Evaluation of the immunological status in clinically healthy and subclinically infected bovine mammary glands

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Summary

Mastitis is a major disease affecting dairy cattle worldwide. Especially the subclinical form of the disease causes high economic losses. The evaluation of udder health is based on somatic cell count (SCC) and bacteriological examinations. A SCC threshold of 100,000 cells/mL in quarter foremilk samples is used to differentiate between healthy and diseased mammary glands. However, the overall goal of this thesis was the analysis of the immunological status of clinically healthy and subclinically infected bovine mammary glands to contribute to a more detailed understanding of subclinical mastitis.

Initially, the results of 615,187 quarter foremilk samples taken between 2000 and 2008 in Hesse, Germany, were analyzed. The data indicated SCC >100,000 cells/mL in 38% and ≤100,000 cells/mL in 62% of all samples; 31% revealed SCC ≤25,000 cells/mL. Mastitis pathogens were detected in 83% of samples with SCC >100,000 cells/mL and in 8.5% of samples in the SCC range from 1,000 to 100,000 cells/mL. Based on these results, inflammatory processes were already suspected in udder quarters with SCC ≤100,000 cells/mL. We argued that such inflammation can be detected by examination of the relationship of immune cells in milk. Hence, in a second and a third study 20 cows, respectively, were selected and differential cell count (DCC) patterns of apparently healthy (SCC ≤100,000 cells/mL) and diseased quarters (>100,000 cells/mL) were compared. While in the second study 100 milk cells of each quarter were differentiated into lymphocytes, macrophages and polymorphonuclear neutrophilic leukocytes (PMNL) using light microscopy, a flow cytometric method was applied for differentiation of 5,000 cells in the third study. In both studies almost all DCC patterns of quarter foremilk samples taken from apparently healthy mammary glands were dominated by lymphocytes. Interestingly, microscopic DCC patterns of three milk samples with 43,000 to 45,000 cells/mL and flow cytometric DCC patterns of six samples with SCC values from 9,000 to 46,000 cells/mL indicated already inflammatory reactions based on the predominance of PMNL. Both studies revealed PMNL as dominant cell population with proportions of ≥49% in milk samples drawn from diseased quarters. In both studies, almost all samples tested revealed macrophages as second predominant cell population in

relationship to lymphocytes and PMNL. Further analysis of the flow cytometric data demonstrated significant differences of the cellular components between quarters infected by major or minor pathogens and culture-negative quarters. In the second and third study, inflammatory reactions in milk of udder quarters classified as healthy according to SCC ≤100,000 cells/mL were detected based on DCC.

The aim of a fourth study was to identify cytological parameters for differentiation between healthy and diseased udder quarters. Quarter foremilk samples were collected from 48 cows and bacteriological status, SCC, and microscopic DCC were determined. Mean lymphocyte percentage was significantly higher in group N (normal secretion, n = 96 samples) than in the three groups of diseased quarters (LM, latent mastitis, n = 15 samples; UM, unspecific mastitis, n = 47 samples; M, mastitis, n = 30 samples). Mean PMNL percentage was significantly lower in group N than in groups UM and M, but not LM. Macrophages did not vary significantly between by the four groups. The mean value of phagocytes (macrophages and PMNL) was significantly lower in group N than in the other groups. Both logarithmic (log) PMNL:Lym and log phagocyte:lymphocyte ratios were significantly lower in group N than in groups LM, UM, and M. However, calculating Fisher values for all variables, the log PMNL:lymphocyte ratio revealed the highest value. A subsequent study concentrated on establishment and verification of cutoff values differentiating between healthy and diseased quarters applying the log PMNL:lymphocyte ratio. Initially, quarter milk and blood samples were taken from eight cows for five consecutive days to investigate short-term repeatability of DCC in milk of healthy mammary glands. While SCC and bacteriological status were determined only for milk samples, DCC was analyzed in all blood and milk samples using flow cytometry. Neither milk nor blood DCC patterns were significantly influenced by sampling day, parity, lactation stage, or quarter position suggesting that DCC can be reliably applied to evaluate udder health. A cutoff value of 0.495 for log PMNL:lymphocyte was established. For the second part of the study 16 animals were randomly selected in a different herd and quarter milk samples were taken on three consecutive days. When the cutoff value was applied to the data along with SCC, high sensitivity and good specificity of 97.3% and 92.3%, respectively, were found under field conditions.

Since all DCC studies revealed lymphocytes as predominant cell population in milk of healthy udder quarters, in a sixth study quantitative relationships of $CD2^+$ T and $CD21^+$ B lymphocytes were investigated using flow cytometry. Percentages of $CD2^+$ T cells were significantly higher in apparently healthy quarters (SCC ≤100,000 cells/mL, n = 65) than in diseased quarters (n = 15), whereas the behaviour of $CD21^+$ B cells was the contrary. As a result of this antidromic effect, a new variable – the CD2/CD21 index – was defined. While diseased quarters indicated values <10, the CD2/CD21 index was >10 in apparently healthy quarters. To test whether CD2/CD21 indices <10 are primarily related to major pathogens, in a second part of this study quarters with SCC ≤100,000 cells/mL (n = 31) and >100,000 cells/mL (n = 32) – either culture-negative or containing minor or major pathogens – were selectively examined. Interestingly, CD2/CD21 indices <10 were found in quarters showing SCC ≤100,000 cells/mL and minor or major pathogens at the time of the current or previous sampling. The results of our examinations indicated a clear connection between the CD2/CD21 index and the bacteriological status. A CD2/CD21 index of 10 may be suitable to aid differentiation between unsuspicious and suspicious or diseased udder quarters.

The aim of a seventh study was to explore DCC patterns of host-microbial interactions for improvement of disease diagnosis. Data collected from six bovine, two human, and one avian study with viral, parasite, or bacterial agents were analyzed. In all studies, no classic data structure (e.g., percentage of an individual cell population) discriminated disease-positive (D+) from disease-negative (D–) samples without overlapping. In contrast, advanced data analysis, like the 3D approach, distinguished three (steady, positive, and negative) feedback phases, in which D– data characterized the steady phase, and D+ data were found in the positive and negative phases. Due to the advanced data analysis methods, in the seventh study host-microbial interactions could be assessed, which might be helpful for earlier diagnosis, differentiation of D+ classes, and lower rates of false-negatives.

In conclusion, this thesis indicated immunological processes and inflammatory reactions appearing already in the SCC range of apparently healthy mammary glands. In addition, new concepts for data analysis and potential new tools for the diagnosis of subclinical mastitis were described.

1st Chapter

General Introduction

Definition of mastitis

Mastitis is an inflammation of the bovine mammary gland, resulting from injury or more commonly from bacterial infection. The term mastitis is from the Greek word 'mastos', for breast, and 'itis', from inflammation. Mastitis is a multifactorial disease with subclinical or clinical manifestations. Subclinical mastitis is characterized by decreased milk production, altered composition of the secretion, and presence of bacteria, but, in contrast to clinical mastitis without any visible changes (e.g., flecks or swelling) in milk and udder (Harmon, 1994).

Costs of mastitis

Mastitis is the most costly disease in milk production worldwide, and approximately 70 to 80% of the financial losses are caused by subclinical mastitis (Reneau and Packard, 1991; Seegers et al., 2003). Financial losses are due to milk production losses, drugs, discarded milk, veterinarian services, labor, milk quality, culling, and occurrence of other diseases. Considering all these financial penalties, average total economic losses for one clinical mastitis case of €210 (Huijps et al., 2008) and €623 (Heikkilä et al., 2012) were calculated. Depending on the month of lactation, the costs can vary between €112 and €946 (Huijps et al., 2008; Heikkilä et al., 2012). The economic losses caused by subclinical mastitis per cow ranged between €53 and €120 depending on the bulk tank somatic cell count (SCC) (Huijps et al., 2008). The total production loss per cow due to subclinical mastitis was amounted to $110 and $295 in herds with bulk tank SCC of ≥200,000 to <400,000 cells/mL and ≥400,000 cells/mL, respectively (Ott, 1999). Moreover, mastitis is one of the major problems that adversely affect dairy cow welfare (Menzies et al., 1995).

Causes of mastitis

Mastitis is considered to be a multifactorial disease, closely related to the production system and environment that the cows are kept in. Almost always, mastitis is caused by pathogenic microorganisms. While predominantly bacteria

induce mastitis, yeasts and fungi, mycobacteria, as well as mycoplasmas can also be infectious agents (Watts, 1988). Bacteria can be categorized into two groups: contagious and environmental. Contagious bacteria live and multiply on and in the infected mammary gland of a cow and are spread from animal to animal as well as from quarter to quarter mainly during unhygienic milking procedures (Dego et al., 2002). The most important contagious agents are *Staphylococcus aureus* and *Streptococcus agalactiae*. Environmental agents exist in the environment of the cows and their transmission to the udder occurs through contamination of the teat end. *Escherichia coli* and *Streptococcus uberis* are the most common environmental pathogens.

Diagnosis of mastitis

Traditional and well-established methods of mastitis diagnosis include estimation of SCC and identification of the causative microorganisms, which involves culturing on agar plates (Viguier et al., 2009). Several SCC cutoffs distinguishing between healthy and diseased mammary glands have been evaluated and discussed in the literature (Schepers et al., 1997; Schukken et al., 2003). In Germany, udder quarters with SCC ≤100,000 cells/mL in foremilk samples are considered to be in the physiological range (DVG, 2002). However, SCC vary with status of lactation, age, stress of the animals, time and frequency of milking, and season, but primarily in response to udder infection (Dohoo and Meek, 1982, Harmon, 1994). Culturing of mastitis pathogens needs a long incubation time of 48 h and requires experienced personnel. In addition, despite bacteria reside in the mammary gland culturing could give false negative results. Negative bacteriological results could depend on intermittent pathogen shedding (Sears et al., 1990), presence of antimicrobials, or other inhibitors in milk (Reiter, 1978). At the time of examination pathogens could also be ingested by phagocytes or survive intracellularly in the host (Newbould and Neave, 1965; Hill et al., 1978). Shedding of too low amounts of pathogens or ceased growth may be further reasons for negative bacteriological results (Sears et al., 1990). Recently, polymerase chain reaction has been proposed as an alternative to bacteriology as a rapid test (Koskinen et al., 2009) but it is expensive and sensitivity was found to be lower than at standard bacterial

culture (Paradis et al., 2012). Hence, there is a major need for new biomarkers that are specific for mastitis and easy to detect at an early stage of the disease (Viguier et al., 2009).

Differential cell counts

Somatic cell count is a robust quantitative measurement, but does not divide the cells present in milk into different cell types (Kehrli and Shuster, 1994; Rivas et al., 2001). The SCC measures all types of cells in milk, including epithelial cells, lymphocytes, macrophages, and polymorphonuclear neutrophilic leukocytes (PMNL) (Kehrli and Shuster, 1994).

In the mammary gland, number and distribution of leukocytes are important for the success of udder defenses against invading pathogens (Leitner et al., 2003). Lymphocytes, macrophages, and PMNL play an important role in inflammatory responses within the mammary gland (Paape et al., 1979; Sordillo and Nickerson, 1988). Lymphocytes are able to recognize antigens through membrane receptors that are specific for invading pathogens (Sordillo et al., 1997). They regulate the induction and suppression of immune responses (Nickerson, 1989). T and B lymphocytes are the two distinct subsets of lymphocytes, which differ in function. T lymphocytes can be further subdivided into $\alpha\beta$ T lymphocytes including CD4$^+$ (T-helper) and CD8$^+$ (T-cytotoxic or T-suppressor) lymphocytes, and $\gamma\delta$ T cells (Sordillo and Streicher, 2002). T-helper cells produce cytokines in response to recognition of antigen-major histocompatibility complex (MHC) class II complexes on antigen-presenting cells, such as B lymphocytes or macrophages. Since they have the ability to secrete certain cytokines, they play an important role in the activation of B lymphocytes, T lymphocytes, and macrophages (Sordillo et al., 1997; Sordillo and Streicher, 2002). Cytotoxic T lymphocytes recognize and eliminate host cells expressing foreign antigens in association with MHC class I molecules. Suppressor T lymphocytes control or modulate the immune response during bacterial infection. Activated during bacterial infections they can suppress important host immune responses (Sordillo et al., 1997; Oviedo-Boyso et al., 2007). $\gamma\delta$ T cells can be cytotoxic and may provide a unique line of defense against bacterial infections (Sordillo and Streicher, 2002). Further, this

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lymphocyte subpopulation is tightly associated with the epithelial surface and destroys damaged epithelial cells (Oviedo-Boyso et al., 2007). B lymphocytes can serve as antigen-presenting cells, secrete cytokines, and differentiate into plasma cells (Sordillo and Streicher, 2002). Their main function is to produce antibodies against invading pathogens (Oviedo-Boyso et al., 2007). Macrophages are active phagocytic cells in the mammary gland and capable of ingesting bacteria, cellular debris, and accumulated milk components (Sordillo and Nickerson, 1988). Milk or tissue macrophages recognize invading pathogens and initiate an immune response by the release of chemoattractants, inducing the rapid recruitment of PMNL into the mammary gland (Paape et al., 2002; Oviedo-Boyso et al., 2007). The main task of PMNL is to defend against invading bacteria at the beginning of an acute inflammatory process (Paape et al., 1979; Oviedo-Boyso et al., 2007). Not only does the number of PMNL increase enormously but their defensive responses (e.g., phagocytic activity) also increase (Targowski, 1983; Paape et al., 2003).

Due to the specific functions of the individual cell populations, the distribution of leukocytes differs between normal milk without any symptoms of mastitis and mastitis milk. Specifically, in normal milk lymphocyte proportions between 14 and 80%, macrophage proportions between 12 and 46%, and those of PMNL between 6 and 50% were described recently (Rivas et al., 2001; Merle et al., 2007; Koess and Hamann, 2008). In milk from cows with mastitis, the proportions of PMNL can reach 95% (Paape et al., 1979; Kehrli and Shuster, 1994). During various phases of inflammation SCC differs in total numbers, whereas differential cell count (DCC) varies in composition of the cell populations involved (Nickerson, 1989). Hence, in addition to SCC, determination of immune cells in milk is beneficial for describing the udder health status (Pillai et al., 2001; Rivas et al., 2001). So far, however, little knowledge exists on DCC and the qualitative role of milk leukocytes in udder quarters classified as healthy because DCC in low-SCC milk are difficult to perform (Dosogne et al., 2003).

Scope of this thesis

The major scope of this thesis was to investigate the immunological status of clinically healthy and subclinically infected bovine mammary glands by cell differentiation methods. Initially, the udder health situation in a representative part of the dairy cow population in Hesse, Germany, was evaluated in chapter 2, analyzing the distribution of SCC, prevalence of mastitis pathogens, and prevalence of mastitis pathogens in dependence of SCC. Subsequently, in chapter 3 immune cells in milk of udder quarters classified as healthy based on SCC values of ≤100,000 cells/mL were differentiated into lymphocytes, macrophages, and PMNL using microscopy. Chapter 4 addresses the differentiation of immune cells in milk of udder quarters classified as healthy based on SCC values of ≤100,000 cells/mL using a flow cytometric method. In chapter 5 combinations of cell populations were evaluated to increase the power of differentiation between healthy and diseased udder quarters based on DCC data. Subsequently, the consistency of DCC results on subsequent days was analyzed and effective cutoff values for the diagnosis of mastitis were established in chapter 6. In a further study (chapter 7) the quantitative relationship of $CD2^+$ T and $CD21^+$ B lymphocytes in quarter foremilk samples was investigated to check early changes of the immunological status of the mammary gland. Finally, in chapter 8 DCC data patterns of host microbial-interactions were explored for improvement of disease diagnosis.

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2nd Chapter

Somatic cell counts and bacteriological status in quarter foremilk samples of cows in Hesse, Germany – A longitudinal study

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Abstract

Somatic cell counts (SCC) are generally used as an indicator of udder health. Currently in Germany, 100,000 cells/mL is the threshold differentiating infected and non-infected mammary glands. The aim of our study was the detailed analysis of udder health in a representative part of the dairy cow population in Hesse, Germany. Between 2000 and 2008, 615,187 quarter foremilk samples were analyzed. In addition to evaluation of distribution of SCC and prevalence of mastitis pathogens, pathogen prevalence was also calculated depending on SCC. The data indicated that 38% of all samples had SCC >100,000 cells/mL and 62% showed SCC ≤100,000 cells/mL; 31% of all samples revealed SCC ≤25,000 cells/mL. Coagulase-negative staphylococci were the dominant pathogens in the Hessian quarter foremilk samples (17.17% of all samples) followed by *Corynebacterium* species (13.56%), *Streptococcus uberis* (8.7%), and *Staphylococcus aureus* (5.01%). Mastitis pathogens were detected in 83% of all samples with SCC >100,000 cells/mL. However, the prevalence of mastitis pathogens in the SCC range from 1,000 to ≤100,000 cells/mL was 8.5% (5.51% minor pathogens, 2.01% major pathogens, and 0.98% other pathogens). For farms producing high quality milk an exceptional hygienic management is compulsory. One of the farms randomly selected showed clearly different results from the Hessian survey. Fifteen percent more samples lay in the SCC range ≤100,000 cells/mL with a lower prevalence of mastitis pathogens of 1.91% (1.03% minor pathogens, 0.83% major pathogens, and 0.05% other pathogens). Based on these results, inflammatory processes can obviously be detected in mammary glands of udder quarters healthy according to the current definitions. However, we argue that such inflammation can be detected by examination of the relationship of immune cells in milk.

Introduction

Mastitis is the most costly disease in milk production worldwide, and approximately 70 to 80% of financial losses are caused by subclinical mastitis (Reneau and Packard, 1991; Seegers et al., 2003). Subclinical mastitis is characterized by decreased milk production, altered composition of the secretion, and presence of bacteria, but without any visible changes in milk and udder (Harmon, 1994).

For mastitis diagnosis, traditional and well-established tests including SCC and microbial culture-based methods are standard (Viguier et al., 2009). Several SCC cutoffs distinguishing between infected and noninfected mammary glands and indicating IMI have been evaluated and discussed in the literature (Schepers et al., 1997; Schukken et al., 2003). According to current definitions of udder health in Germany, quarter foremilk samples with SCC ≤100,000 cells/mL are in the physiological range (DVG, 2002). It is well-known that the crossover of normal cellular defense in the mammary gland into an inflammatory reaction starts from a level of >100,000 cells/mL (Harmon, 1994; DVG, 2002). However, SCC vary with the status of lactation, age, stress of the animals, time and frequency of milking, season, and, mainly, udder infection status (Dohoo and Meek, 1982; Harmon, 1994).

The implementation of classical mastitis prevention programs (Neave et al., 1969) in combination with the introduction of penalty limits for bulk milk SCC have led to substantial progress in controlling subclinical mastitis worldwide. The geometric bulk milk SCC in Hesse, Germany, was 255,000 cells/mL in 1993 and decreased to 200,000 cells/mL in 2008 (HVL, 1994, 2009). A decrease in the geometric bulk milk SCC from 600,000 cells/mL in 1971 to 200,000 cells/mL in 2002 was observed in the Netherlands (Sampimon et al., 2005). In Finland, the geometric bulk milk SCC decreased from 325,000 cells/mL to 132,000 cells/mL between 1988 and 2002 (Myllys et al., 1998; Pitkälä et al., 2004). A moderate decrease of the geometric bulk milk SCC from 356,000 cells/mL in 1998 to 316,000 cells/mL in 2005 was described evaluating the data of one large US milk cooperative localized in New York state (Nightingale et al., 2008). In addition to mastitis control programs, the selection for animals showing low SCC in animal breeding programs might have

had a strong influence on the development of mastitis pathogens. In particular, the prevalence of *Staphylococcus aureus* decreased. Data for *Staphylococcus aureus* are also available for the German federal state of Hesse, Finland, the Netherlands, and the state of Wisconsin (United States). In Hesse, *Staph. aureus* showed a prevalence of 13.0% in 1995 and 2.9% in 2008 (LHL, 2009). In Finland, the prevalence of the bacteria was reduced from 5.1% in 1988 (Myllys et al., 1998) to 3.4% in 2001 (Pitkälä et al., 2004). The prevalence of *Staph. aureus* in the Netherlands decreased from 6.2% in 1973 to 1.8% in 2003 (Sampimon et al., 2009). In Wisconsin, the proportion of *Staph. aureus* in all culture-positive samples decreased from 17.7% in 1994 to a value of 9.7% in 2001 (Makovec and Ruegg, 2003). In contrast, CNS are currently the most isolated pathogens from milk samples in many countries (Pitkälä et al., 2004; Piepers et al., 2007; Sampimon et al., 2009).

The objective of our research was a detailed evaluation of the udder health situation in a representative part of the dairy cow population in Hesse, Germany, analyzing the distribution of SCC, prevalence of mastitis pathogens, and prevalence of mastitis pathogens in dependence of SCC. Additionally, for a more detailed evaluation of udder health in cows with low SCC and comparison with the total Hessian average, a single farm practicing exceptional hygienic management was analyzed. However, because mastitis prevention programs have been implemented in Hesse, Germany, for many years, a high level of udder health could be expected.

Materials and Methods

Animals and Farms

From 2000 to 2008, 615,187 quarter foremilk samples were taken from dairy cows in the German federal state of Hesse and analyzed by the Animal Health Services (Hesse, Germany). This random test represented approximately 12.5% of all Hessian dairy cows. Samples were collected from farms producing high quality milk (n = 98,430; 16% of all samples), from conventional producing farms (n = 412,175; 67% of all samples), and from farms with severe udder health problems (n = 104,852; 17% of all samples). Almost all lactating cows of the farms were tested.

In addition, one farm (A) with exceptionally hygiene management for the production of high quality milk was randomly selected to evaluate its cytobacteriological status; 12,660 quarter foremilk samples were taken on that farm from 1995 to 2003. According to the legislative requirements in Germany, farms producing high quality milk have to perform monthly SCC and bacteriological examinations of quarter foremilk samples from all lactating cows.

On farm A, 60 Holstein-Friesian cows were housed in a pen barn and were milked twice a day in a milking parlor. The average herd milk yield averaged 9,000 kg/yr.

Milk Sampling

Quarter foremilk samples were obtained according to DVG (2000) standards. Before milking, teat ends were scrubbed with 70% ethanol and the first 2 squirts of milk were discarded. A volume of 10 mL of milk per udder quarter was collected in a sterile 14-mL plastic sample tube (Greiner bio-one, Frickenhausen, Germany).

Laboratory Analysis

Somatic cells were determined using a *Fossomatic 5000* (Foss Electric, Hillerød, Denmark) and used as guideline for the selection of udder quarters for bacteriological analysis. Quarter foremilk samples showing SCC >100,000 cells/mL were cultured categorically. All samples of a herd with SCC ≤100,000 cells/mL were only examined bacteriologically when highly contagious mastitis pathogens such as *Staph. aureus, Streptococcus agalactiae*, or group G streptococci were present. Furthermore, all samples from farms producing high quality milk were analyzed bacteriologically independent from SCC.

Culture and isolate identification were performed according to IDF (1981) standards. Promptly after collecting the quarter foremilk samples and cooled transportation to the laboratory, about 10 µL of milk was streaked onto a quadrant of a 7% bovine blood agar plate containing 0.05% esculin (Merck, Darmstadt, Germany) and incubated for 48 h at 37°C; the plates were examined after 24 and 48 h of incubation. Isolates were classified into (1) major pathogens: *Staph. aureus, Streptococcus (Strep.) agalactiae, Strep. dysgalactiae, Strep. uberis, Escherichia coli*, coliforms (e.g., *Klebsiella* spp.,

Serratia spp., *Enterobacter* spp., other *Enterobacteriaceae*); (2) minor pathogens: CNS, *Corynebacterium* spp.; (3) other pathogens: *Arcanobacterium pyogenes, Proteus, Prototheca zopfii, Pseudomonas* spp., group G streptococci (predominantly *Strep. canis*), yeasts, and fungi. Contaminated samples were defined as a mixture of at least 2 environmental type organisms without isolation of a major mastitis pathogen.

Statistical Analysis

The data sets of the results of the SCC examinations in the federal state Hesse and on farm A were divided into 15 different SCC ranges from ≤1,560 to >12,800,000 cells/mL. Their limits were calculated using a logarithmic partition to the base 2 of the SCC scale according to the SCS standard (SCS = $\log_2$ (SCC/100,000) + 3) (Ali and Shook, 1980). All records with a missing SCC value or a value <1,000 cells/mL were excluded from the data sets (Hessian survey: n = 27,281; farm A: n = 122). These records were not included in the total numbers (Hessian survey: n = 615,187; farm A: n = 12,660). For a more practical interpretation, a wider scale range was determined according to the SCS standard defining 4 SCC groups: group I: ≤6,250 cells/mL, group II: >6,250 to ≤25,000 cells/mL, group III: >25,000 to ≤100,000 cells/mL, and group IV: >100,000 cells/mL.

Distributions of SCC in Hesse and on farm A were tested for Gaussian distribution using the Shapiro and Wilk test as well as the Chi-squared test (BMDP Statistical Solutions Ltd., Cork, Ireland). Because of technical reasons, random tests of 2,000 records of each data set were used for the calculation of the Shapiro and Wilk test, whereas the whole data sets of both Hesse and dairy farm A were used for the Chi-squared test.

Additionally, a subset comprising data of Hesse from 2003 (n = 79,204) was analyzed to identify the effect of fixed and random effects on SCC by applying a linear mixed model. Selection of data for verification of results, as presented in Figure 1, was done because of computational limitations. The SCC were log-transformed according to Ali and Shook (1980) to obtain better statistical properties. The data subset was analyzed using the MIXED procedure of SAS 9.1 (SAS Institute, Cary, USA) and the following statistical model:

$$Y_{ijk} = \mu + H_i + Q_j + e_{ijk},$$

where Y_{ijk} = observed value for SCS in herd i and udder quarter j of cow k; μ = overall mean; H_i = random effect of herd i (i = 1 to 1,338); Q_j = fixed effect of quarter j (j = 1 to 4); and e_{ijk} = random error term.

In addition to the SCC data (n = 235,556), the results of the bacteriological examinations of 145,965 quarter foremilk samples analyzed in Hesse were available for the period 2000 to 2003. On farm A, the bacteriological data could be evaluated for the period 1995 to 2003 (n = 12,660). For statistical calculation, the classification of the bacteriological results into major, minor and other pathogens was carried out according to Reneau (1986). The classification and calculation of the frequency of each pathogen was performed independently from SCC and depending on the 15 different SCC ranges defined above using SAS 9.1 (SAS Institute, Cary, USA).

The data subset comprising results from 2003 (n = 79,204) was also analyzed to identify the impact of fixed and random effects on specific pathogens by applying a generalized linear mixed model. Because pathogens were classified as binary traits, the residuals cannot be normally distributed. The best function to describe the relationship between the dependent and independent variables is not linear but S-shaped. This is the primary reason why a linear logistic model with mixed effects was used. As described by König et al. (2005) the probability of observing the event of interest (e.g., prevalence of the pathogen) was

- $$\pi_i = \text{Prob}\,(Y_i = 1|\theta)$$

- where θ is a parameter vector including fixed and random effects. The logit of the observation Y_i was

- $$\log\left[\frac{\pi_i}{1-\pi_i}\right] = \eta_i$$

Since π is the probability of Y = 1, it follows that $1 - \pi$ is the probability of Y = 0 and so $\dfrac{\pi}{1-\pi}$ is the ratio of the two probabilities, which, when stated in the form of odds, gives the odds of having Y = 1. Analysis of variance of the

measurements was carried out using logistic models implemented in the SAS glimmix macro (Wolfinger and O'Connell, 1993) that included the fixed effects of the udder quarter and the defined SCC group and the random effect of the herd. The *F*-ratios used in the analysis of variance are identical to the Wald/rank(K) *F*-statistics as defined by Littell et al. (1999). Wald-type tests were also used to identify significant fixed effects on different pathogens (type III tests of fixed effects).

The final generalized linear model used to determine the impact of fixed and random effects on prevalence of pathogens was:

$$\bullet \quad \text{logit}\,(\pi_{rstu}) = \log\left[\frac{\pi_{rst}}{1 - \pi_{rst}}\right] = \eta_{rst} = \varphi + \gamma_r + \lambda_s + \varepsilon_t$$

where π_{rstu} = probability of occurrence of a pathogen in udder quarter s and SCC group r for cow u in herd t; φ = overall mean effect; γ_r = fixed effect of SCC-group; λ_s = fixed effect of udder quarter; and ε_t = random effect of the herd.

Results

Evaluation of Udder Health in Hessian Dairy Farms

For the evaluation of the udder health situation in Hesse, Germany, a data set with the results of 615,187 quarter foremilk samples was used. The samples were derived from farms with different levels of udder health. In general, farms producing high quality milk showed a bulk tank SCC of 180,000 cells/mL. The bulk tank SCC of conventional milk producers averaged 200,000 cells/mL, whereas the bulk tank SCC in farms with massive udder health problems was higher (400,000 cells/mL).

Distribution of Somatic Cell Counts. Somatic cell counts of the quarter foremilk samples analyzed ranged from 1,000 to 30,000,000 cells/mL. The distribution of all quarter foremilk samples in the 15 SCC ranges is illustrated in Figure 1. In total, 38% of all quarter foremilk samples indicated an inflammatory reaction (SCC >100,000 cells/mL) according to the current DVG definitions (2002). A high frequency (62%) of the samples had SCC in a physiological range (≤100,000 cells/mL). Thirty-one percent of all samples had SCC between 25,000 and 100,000 cells/mL, and 31% had SCC ≤25,000 cells/mL.

For a more practical interpretation, 4 SCC groups (I to IV) were defined. Six percent of the quarter foremilk samples analyzed in Hesse belonged to group I (≤6,250 cells/mL), 25% belonged to group II (>6,250 to ≤25,000 cells/mL), 31% belonged to group III (>25,000 to ≤100,000 cells/mL), and 38% belonged to group IV (>100,000 cells/mL).
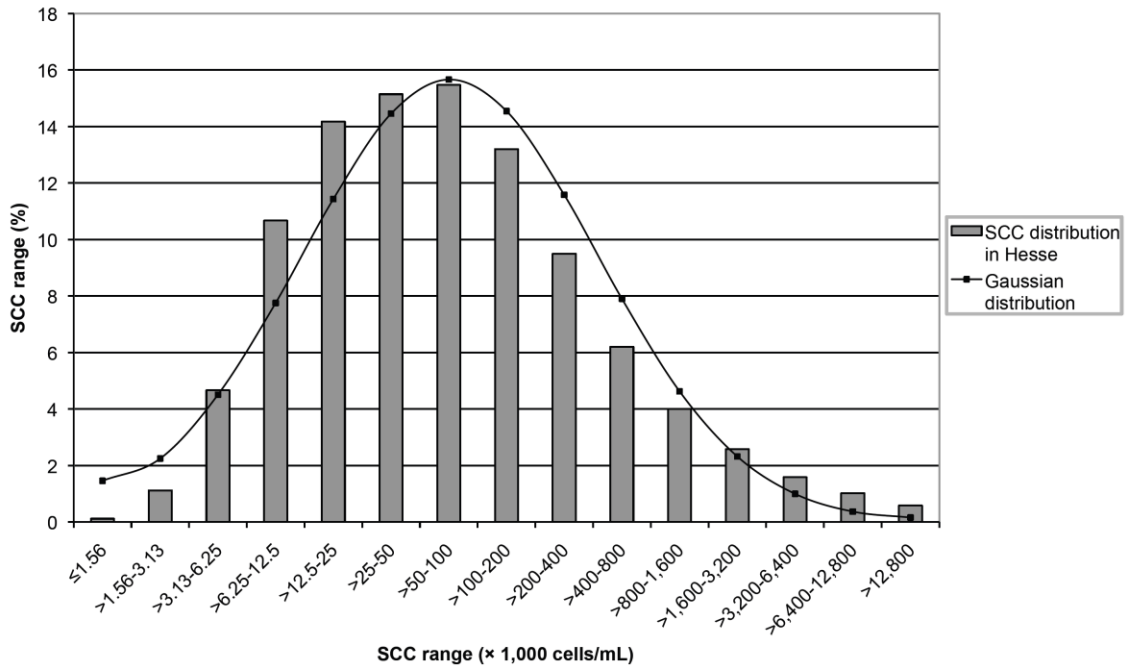
Figure 1. Statistical distribution of SCC of all quarter foremilk samples analyzed in the German federal state of Hesse taken from 2000 to 2008 (n = 615,187) by classification into 15 SCC ranges using a logarithmic partition to the basis 2 of the SCC scale according to the SCS standard. The bar charts show the distribution of SCC observed in Hesse, the line shows the distribution of SCC expected by the exact Gaussian distribution.

The distribution of the logarithm of SCC in Hesse was similar to a Gaussian distribution (Figure 1). However, the goodness-of-fit test for normality according to the Shapiro and Wilk test as well as the Chi-squared test showed a significant deviation ($P < 0.001$) from the exact Gaussian distribution. In particular, in both tails of the distribution deviations from normality could be observed (Figure 1). More samples than expected were observed on the left side of the middle area of the Gaussian distribution. In contrast, fewer samples than expected were observed on the right side of the distribution.

To evaluate the effect of quarter position on SCC, a data set including 79,204 quarter foremilk samples tested in 2003 was analyzed exemplarily using a statistical model. The data from 2003 were representative for the whole data set from 2000 to 2008. For statistical reasons, SCC were transformed to SCS. The analysis indicated a highly significant ($P < 0.001$) effect of quarter position on SCS. The mean SCS value of the front left quarter was significantly ($P < 0.05$) lower than that of the other 3 quarters (Table 1). In contrast, the highest mean SCS value could be calculated for the rear right quarter. In total, the mean SCS of both front quarters of 2.38 (SEM 0.01) was significantly ($P < 0.001$) lower than in both rear quarters (2.49, SEM 0.01).

Table 1. Least-square means and standard error of mean for SCS in different udder quarter positions from 79,204 quarter foremilk samples analyzed in Hesse, Germany, in 2003.

	SCS	
	LSM	SEM
FR	2.45[a]	0.02
RR	2.52[b]	0.02
FL	2.31[c]	0.02
RL	2.45[a]	0.02

[1]Udder quarter position: FR = front right, RR = rear right, FL = front left, RL = rear left.

[a,b,c]Least square means within the same row with different letters differ significantly at $P < 0.05$.

Distribution of Bacteriological Results. A high frequency of the quarter foremilk samples (62%) taken between 2000 and 2008 lay in a physiological range with SCC ≤100,000 cells/mL and offered a proper standard of udder health. Bacteriological results were available for 145,065 of the 235,556 quarter foremilk samples taken from 2000 to 2003. The prevalence of mastitis pathogens in the samples examined was 51.96% (Table 2). The most frequently isolated pathogens were CNS (33.05%), followed by *Corynebacterium* spp. (26.1%), *Strep. uberis* (16.75%), *Staph. aureus* (9.63%), coliforms (2.21%*), Strep. dysgalactiae* (1.61%), *E. coli* (1.17%), and *Strep. agalactiae* (0.55%). Other pathogens were detected in 4.31% of all culture-positive samples. A proportion of 4.62% of all culture-positive samples was contaminated.

Approximately 40% (n = 60,327) of all quarter foremilk samples with SCC ≤100,000 cells/mL were analyzed bacteriologically (Table 3). One fraction (n = 30,759) of these samples was cultured because of the presence of highly contagious mastitis pathogens such as *Staph. aureus, Strep. agalactiae* or group G streptococci in a herd. The remaining fraction (n = 29,568) originated from high quality milk producers whose samples were generally analyzed bacteriologically. Pathogens were found in 8.5% of these quarter foremilk samples. Minor pathogens (*Corynebacterium* spp., CNS) as well as major pathogens (*Staph. aureus, Strep. uberis*) were detected beginning at an SCC level of ≤1,560 cells/mL (Table 3). Within the SCC groups I to III, the frequencies of minor pathogens (5.51%), major pathogens (2.01%), and other pathogens (0.98%) were higher than expected. Concerning the total number of quarter foremilk samples with SCC values ≤100,000 cells/mL analyzed from 2000 to 2003 (n = 150,818), no mastitis pathogens were detected in 94 to 98% of the cases within the individual SCC ranges (Table 3).

Table 2. Prevalence and distribution of mastitis pathogens in quarter foremilk samples analyzed bacteriologically in the German federal state of Hesse between 2000 and 2003 (n = 145,065)

Item	n	All quarters (%)[1]	Culture-positive (%)[2]
Quarters analyzed bacteriologically	145,065		
Culture-negative quarters	69,691	48.04	
Culture-positive quarters	75,374	51.96	
Staphylococcus aureus	7,262	5.01	9.63
Streptococcus agalactiae	414	0.29	0.55
Streptococcus dysgalactiae	1,211	0.83	1.61
Streptococcus uberis	12,623	8.70	16.75
Escherichia coli	882	0.61	1.17
Coliforms[3]	1,664	1.15	2.21
Coagulase-negative staphylococci	24,913	17.17	33.05
Corynebacterium species	19,673	13.56	26.10
Other pathogens[4]	3,246	2.24	4.31
Contamination	3,486	2.40	4.62

[1]Percentage of all quarters analyzed bacteriologically.
[2]Percentage of all culture-positive quarters.
[3]Coliforms = *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., other *Enterobacteriaceae*.
[4]Other pathogens: *Arcanobacterium pyogenes, Proteus, Prototheca zopfii, Pseudomonas* spp., group G streptococci, yeasts, and fungi.

Table 3. Prevalence and distribution of mastitis pathogens (%) isolated from quarter foremilk samples obtained in the German federal state Hesse within 2000 to 2003 (n = 235,556) in 15 different SCC ranges

Group/ SCC range (× 1,000 cells/mL)	n	Major pathogens[1]						Minor pathogens[2]			No pathogens	Contam.[3]
		Staph. aureus	Strep. agalactiae	Strep. dysgalactiae	Strep. uberis	E. coli	Coli-forms	CNS	Coryne-bacterium spp.	Others[3]		
Group I												
≤1.56	415	0.48	0	0	0	0	0	0.48	0.24	0	98.56	0
>1.56-3.13	3,470	0.35	0	0	0	0	0.03	0.58	0.35	0.06	98.40	0.14
>3.13-6.25	12,681	0.54	0	0	0	0	0.04	0.66	0.28	0.13	98.09	0.13
Group II												
>6.25-12.5	26,815	0.42	0.01	0.01	0.14	0	0.04	0.83	0.48	0.14	97.79	0.14
>12.5-25	33,837	0.43	0.02	0.01	0.15	0	0.06	0.78	0.94	0.20	97.19	0.22
Group III												
>25-50	36,246	0.47	0.03	0.02	0.18	0.01	0.06	0.92	1.39	0.13	96.59	0.20
>50-100	37,354	0.56	0.06	0.02	0.41	0.01	0.09	1.79	1.95	0.15	94.65	0.31
Group IV												
>100-200	30,406	3.73	0.21	0.36	10.77	0.47	1.32	30.77	27.21	2.54	18.26	4.36
>200-400	21,421	6.46	0.37	0.92	14.29	0.65	1.51	30.10	22.53	2.64	16.48	4.05
>400-800	13,426	10.49	0.64	1.69	16.13	0.89	1.43	26.95	18.95	3.40	16.19	3.24
>800-1,600	8,513	13.83	0.80	3.01	16.43	1.52	2.69	22.62	15.11	4.26	16.43	3.30
>1,600-3,200	5,270	13.78	0.70	3.29	20.34	2.03	4.22	20.53	11.37	6.09	14.86	2.79
>3,200-6,400	3,011	11.94	0.53	3.19	24.54	2.56	3.26	17.36	9.48	7.25	17.70	2.19
>6,400-12,800	1,943	14.29	0.88	4.23	21.71	5.00	4.18	15.11	6.03	8.10	18.82	1.65
>12,800	748	11.36	0.53	6.55	23.26	8.29	3.48	12.03	3.74	7.49	21.68	1.59

[1]Major pathogens: *Staph. aureus = Staphylococcus aureus*; *Strep. agalactiae = Streptococcus agalactiae*; *Strep. dysgalactiae = Streptococcus dysgalactiae*; *E. coli = Escherichia coli*; Coliforms = *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., other *Enterobacteriaceae*.
[2]Other pathogens: *Arcanobacterium pyogenes*, *Proteus*, *Prototheca zopfii*, *Pseudomonas* spp., group G streptococci, yeasts, and fungi.
[3]Contaminated samples.

Mastitis pathogens were detected in 82.91% of the 84,738 quarter foremilk samples with SCC values >100,000 cells/mL (group IV) analyzed from 2000 to 2003 (Table 3); CNS were the most frequently isolated pathogens. Their prevalence decreased from a maximum of 30.77% (SCC 100,000 to 200,000 cells/mL) to 12.03% (SCC of >12,800,000 cells/mL). *Staphylococcus aureus* were predominantly identified in samples with SCC >400,000 cells/mL (10.49 to 14.29%). *Corynebacterium* spp. were isolated with higher frequencies in samples with SCC between 100,000 and 800,000 cells/mL. *Streptococcus uberis* showed the highest prevalences (20.34 to 24.54%) in samples with SCC >1,600,000 cells/mL. The prevalence of *Strep. agalactiae* was generally very low (<1%). However, *Strep. agalactiae* was more frequently detected (0.53 to 0.80%) in the SCC range from 400,000 to 12,800,000 cells/mL. The major pathogen *Strep. dysgalactiae* was predominantly identified (3.01 to 6.55%) in samples with SCC >800,000 cells/mL. The prevalence of *E. coli* increased from 0.47% at SCC of >100,000 to 200,000 cells/mL to a maximum of 8.29% at SCC of >12,800,000 cells/mL. Coliforms were predominantly isolated in samples

Table 4. Results of ANOVA for the prevalence of the individual pathogens in 79,204 quarter foremilk samples analyzed in Hesse, Germany, in 2003[1]

	Effect	
Pathogen	Udder Quarter[1]	SCC group[1]
Staphylococcus aureus	***	***
Streptococcus agalactiae	*	NC
Streptococcus dysgalactiae	**	NC
Streptococcus uberis	***	***
Escherichia coli	NS	***
Coliforms[2]	***	***
Coagulase-negative staphylococci	***	***
Corynebacterium species	NS	***
Contamination	***	***
Other pathogens[3]	NS	***
No pathogens	***	***

[1]Analyzed factors were quarter positions (front right, rear right, front left, and rear left) and SCC group (I ≤6,250 cells/mL, II >6,250 to ≤25,000 cells/mL, III >25,000 to ≤100,000 cells/mL, IV >100,000 cells/mL); NC = not calculated.
[2]Coliforms = *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., other *Enterobacteriaceae.*
[3]Other pathogens: *Arcanobacterium pyogenes, Proteus, Prototheca zopfii, Pseudomonas* spp., group G streptococci, yeasts, and fungi.
*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS = $P > 0.05$.

showing SCC from 800,000 to >12,800,000 cells/mL. The prevalence of other pathogens in group IV ranged between 2 and 8%. The proportion of contaminated samples decreased at increasing SCC.

Table 5. Least square means for the prevalence of pathogens in different udder quarter positions from 79,204 quarter foremilk samples analyzed in Hesse, Germany, in 2003

Pathogen	Udder Quarter[1]			
	FR	RR	FL	RL
Staphylococcus aureus	1.08[a]	0.77[b]	0.86[b]	0.86[b]
Streptococcus agalactiae	0.30[a,b]	0.22[a]	0.39[b]	0.29[a,b]
Streptococcus dysgalactiae	0.51[a]	0.64[a,b]	0.55[a]	0.82[b]
Streptococcus uberis	1.01[a]	0.82[b]	0.85[b,c]	0.98[a,c]
Escherichia coli	0.38[a]	0.36[a,b]	0.25[b]	0.28[a,b]
Coliforms[2]	0.42[a]	0.60[b]	0.37[a]	0.40[a]
Coagulase-negative staphylococci	4.17[a]	4.46[b]	4.09[a]	4.81[c]
Corynebacterium species	3.75[a,b]	3.73[a,b]	3.87[a]	3.62[b]
Contamination	0.68[a]	0.89[b]	0.76[a]	0.93[b]
Other pathogens[3]	0.07[a]	0.06[a]	0.06[a]	0.08[a]
No pathogens	83.65[a]	83.82[a]	84.79[b]	82.22[c]

[a-c]Least square means within the same row with different letters differ significantly at $P < 0.05$.
[1]Udder quarter position: FR = front right, RR = rear right, FL = front left, RL = rear left.
[2]Coliforms = *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., other *Enterobacteriaceae*.
[3]Other pathogens: *Arcanobacterium pyogenes, Proteus, Prototheca zopfii, Pseudomonas* spp., group G streptococci, yeasts, and fungi.

As an exemplar, a data set including all quarter foremilk samples (n = 79,204) tested cytobacteriologically in 2003 was evaluated applying a generalized linear mixed model for the identification of fixed and random effects on the prevalence of the individual pathogens. The analysis indicated that the position of the udder quarter influenced the prevalence of most pathogens significantly (Table 4). The major pathogen *Staph. aureus* was significantly ($P < 0.001$) more frequently isolated from the front right quarter than from the other 3 quarters (Table 5). The highest prevalences for *Strep. agalactiae* and *Corynebacterium* spp. were found in front quarters, whereas *Strep. dysgalactiae* and CNS were predominantly isolated from rear quarters. *Streptococcus uberis* and other pathogens showed the highest prevalences in

the front right and rear left quarters. Coliforms and *E. coli* were predominantly found in right quarters. Most contaminated samples originated from rear quarters. The proportion of samples without any isolation of pathogens was significantly ($P < 0.001$) higher in the front left quarter and lower in the rear left quarter than in right quarters.

A significant ($P < 0.001$) influence on the prevalence of all pathogens with the exception of *Strep. agalactiae* and *Strep. dysgalactiae* could be calculated for the 4 SCC groups defined (Table 4). The prevalence of most pathogens in groups I and II was significantly lower than in those of groups III and IV (Table 6). The proportion of samples without any isolation of pathogens decreased significantly ($P < 0.001$) with increasing SCC groups. The distribution of *Strep. agalactiae*, *Strep. dysgalactiae*, and *E. coli* depending on the 4 SCC groups could not be evaluated because of their extremely low prevalences in groups I to III.

Table 6. Least square means for the prevalence of pathogens in the 4 SCC groups defined from 79,204 quarter foremilk samples analyzed in Hesse, Germany, in 2003

Pathogen	SCC group[1]			
	I	II	III	IV
Staphylococcus aureus	0.37[a,b]	0.35[a]	0.57[b]	7.71[c]
Streptococcus agalactiae	–	–	–	–
Streptococcus dysgalactiae	–	–	–	–
Streptococcus uberis	0.03[a]	0.03[a]	0.07[a]	1.04[b]
Escherichia coli	–	–	–	–
Coliforms[2]	0.25[a]	0.21[a]	0.31[a]	2.19[b]
Coagulase-negative staphylococci	1.58[a]	1.88[a]	3.55[b]	27.81[c]
Corynebacterium species	0.83[a]	2.30[b]	3.78[c]	22.78[d]
Contamination	0.42[a]	0.49[a]	0.50[a]	4.11[b]
Other pathogens[3]	0.003[a]	0.08[a]	0.07[a]	1.27[b]
No pathogens	95.78[a]	94.00[b]	90.05[c]	17.52[d]

[a-d]Least square means within the same row with different letters differ significantly at $P < 0.05$.

[1]SCC groups: I = ≤6,250 cells/mL; II = >6,250 to ≤25,000 cells/mL; III = >25,000 to ≤100,000 cells/mL; IV = >100,000 cells/mL.

[2]Coliforms = *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., other *Enterobacteriaceae*.

[3]Other pathogens: *Arcanobacterium pyogenes, Proteus, Prototheca zopfii, Pseudomonas* spp., group G streptococci, yeasts, and fungi.

Evaluation of Udder Health in a Dairy Farm Producing High Quality Milk (Farm A)

Farm A was chosen randomly to show the distribution of SCC and bacteriological results in a single farm practicing exceptional hygiene. The evaluation of udder health in this farm was based on the results of 12,660 quarter foremilk samples analyzed.

Distribution of Somatic Cell Counts. Somatic cell counts of all quarter foremilk samples taken on farm A lay in a range between 1,000 and 24,000,000 cells/mL; the distribution of SCC on farm A is presented in Figure 2. A total of 23% of all quarter foremilk samples on farm A indicated an inflammatory reaction (SCC >100,000 cells/mL) according to the current DVG definitions (2002) of udder health, representing 15% fewer samples than in the Hessian survey. The high frequency (77%) of samples with SCC ≤100,000 cells/mL became evident on this farm, too, and was 15% above the Hessian average. Forty percent of all samples revealed SCC between 25,000 and 100,000 cells/mL and 37% revealed SCC ≤25,000 cells/mL, ranging 9% and 6%, respectively, above the Hessian average.

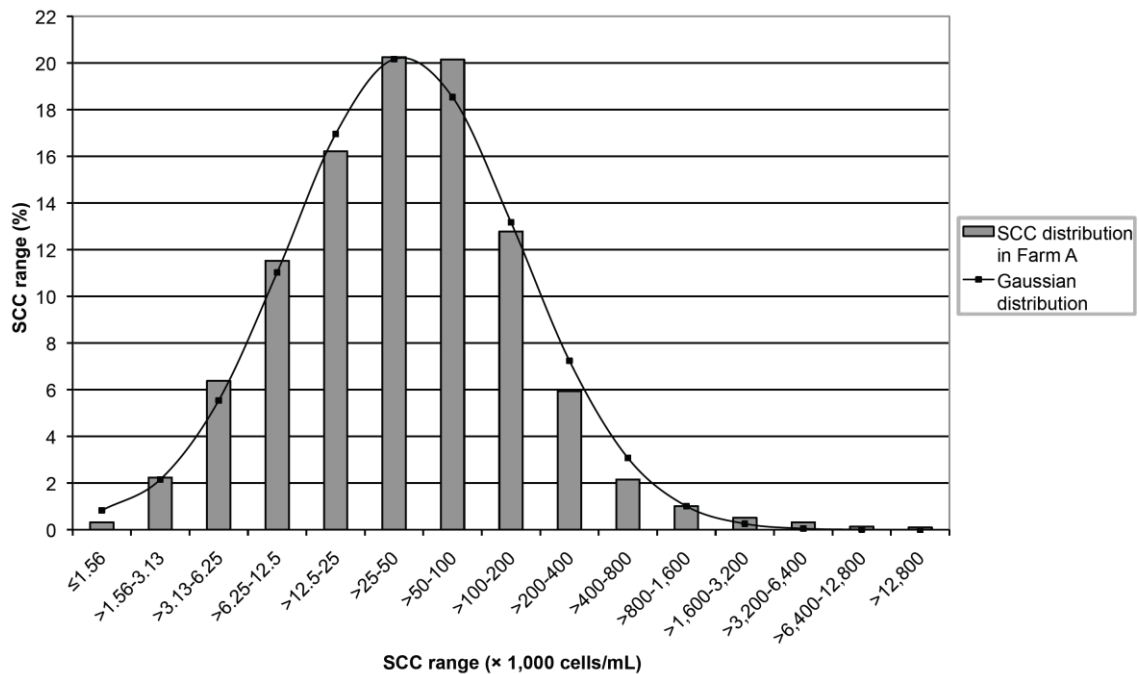**Figure 2.** Statistical distribution of SCC of all quarter foremilk samples analyzed on farm A taken from 1995 to 2003 (n = 12,660) by classification into 15 SCC ranges using a logarithmic partition to the basis 2 of the SCC scale according to the SCS standard. The bar charts show the distribution of SCC observed on farm A, the line shows the distribution of SCC expected by the exact Gaussian distribution.

Dividing SCC frequencies of farm A into the same SCC groups (I to IV) used for the Hessian surveillance, 9% of the quarter foremilk samples belonged to group I, 28% to group II, 40% to group III, and 23% to group IV.

Again, the goodness-of-fit test for normality according to the Shapiro and Wilk test as well as the Chi-squared test applied to the distribution of SCC on farm A (Figure 2) showed a significant deviation ($P < 0.001$) from the exact Gaussian distribution, as already seen for the Hessian samples and for the same reasons. However, the distribution observed was close to the normal distribution (Figure 2).

Distribution of Bacteriological Results. Bacteriological results were available for all of the 12,660 quarter foremilk samples taken from 1995 to 2003. The prevalence of mastitis pathogens was 21.65% (Table 7). Pathogens isolated most frequently were CNS (35.32%), followed by *Corynebacterium* spp. (29.92%), *Strep. uberis* (21.71%), *Staph. aureus* (5.29%), coliforms (1.86*%),* *Strep. dysgalactiae* (0.62%), and *E. coli* (0.51%). Other pathogens were

Table 7. Prevalence and distribution of mastitis pathogens isolated from quarter foremilk samples taken on farm A between 1995 and 2003 (n = 12,660)

Item	n	All quarters (%)[1]	Culture-positive (%)[2]
Quarters analyzed bacteriologically	12,660		
Culture-negative quarters	9,919	78.35	
Culture-positive quarters	2,741	21.65	
Staphylococcus aureus	145	1.15	5.29
Streptococcus agalactiae	0	0	0
Streptococcus dysgalactiae	17	0.13	0.62
Streptococcus uberis	595	4.70	21.71
Escherichia coli	14	0.11	0.51
Coliforms[3]	51	0.40	1.86
Coagulase-negative staphylococci	968	7.65	35.32
Corynebacterium species	820	6.48	29.92
Other pathogens[4]	22	0.17	0.79
Contamination	109	0.86	3.98

[1]Percentage of all quarters analyzed bacteriologically.
[2]Percentage of all culture-positive quarters.
[3]Coliforms = *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., other *Enterobacteriaceae.*
[4]Other pathogens: *Arcanobacterium pyogenes, Proteus, Prototheca zopfii, Pseudomonas* spp., group G streptococci, yeasts, and fungi.

detected in 0.80% of all culture-positive samples. A proportion of 3.98% of all culture-positive samples was contaminated. The major pathogen *Strep. agalactiae* could not be isolated from any sample taken on farm A.

Mastitis pathogens were found in 1.91% of 9,715 quarter foremilk samples showing SCC ≤100,000 cells/mL (Table 8). Minor pathogens (CNS) were detected at a minimum of ≤1,560 cells/mL, whereas major pathogens (*Staph. aureus*) could be isolated beginning at a level of >3,130 to 6,250 cells/mL. The frequencies of minor pathogens (1.03%), major pathogens (0.83%), and other pathogens (0.05%) were clearly lower within the SCC groups I to III compared with the Hessian average. No mastitis pathogens were detected in 96 to 99% of the cases within the individual SCC ranges (Table 8).

Mastitis pathogens were detected in 87.95% of the 2,905 quarter foremilk samples with SCC values >100,000 cells/mL (group IV) analyzed within this period (Table 8); CNS were the most frequently isolated pathogens. Their prevalence decreased from a maximum of 33.62% (SCC 100,000 to 200,000 cells/mL) to 17.64% (SCC of 6,400,000 to >12,800,000 cells/mL) and increased again to a level of 33.33% at SCC >12,800,000 cells/mL. *Staphylococcus aureus* was predominantly isolated in samples with SCC from >200,000 to 6,400,000 cells/mL (3.85 to 10.16%).

The prevalence of *Corynebacterium* spp. decreased constantly from 31.83% at SCC of >100,000 to 200,000 cells/mL to 8.35% at SCC of >12,800,000 cells/mL. *Streptococcus uberis* showed prevalences varying between 14.83% and 35.29% in the SCC range >100,000 cells/mL. The prevalence of *Strep. dysgalactiae* increased from 0.06% at SCC of >100,000 to 200,000 cells/mL to 8.33% at SCC of >12,800,000 cells/mL. *Streptococcus agalactiae* was not detected. The prevalence of *E. coli* in group IV ranged between 0 and 8.33%. The prevalence of coliforms increased from 1.05% at SCC of >100,000 to 200,000 cells/mL to 8.33% at SCC of >12,800,000 cells/mL, except that no coliform bacteria were isolated in samples showing SCC between 6,400,000 and 12,800,000 cells/mL. The prevalence of other pathogens in group IV lay between 0 and 12%. The proportion of contaminated samples ranged between 0 and 5%.

Table 8. Prevalence and distribution of mastitis pathogens (%) isolated from quarter foremilk samples obtained in dairy farm A within the years 1995 to 2003 (n = 12,660) in 15 different SCC ranges

Group/ SCC range (× 1,000 cells/mL)	n	Major pathogens[1]						Minor pathogens[2]			No pathogens	Contam.[3]
		Staph. aureus	Strep. agalactiae	Strep. dysgalactiae	Strep. uberis	E. coli	Coliforms	CNS	Coryne- bacterium spp.	Others[3]		
Group I												
≤1.56	40	0	0	0	0	0	0	2.50	0	0	97.50	0
>1.56-3.13	283	0	0	0	0	0	0	0.71	0.35	0	98.94	0
>3.13-6.25	807	0.37	0	0	0	0	0	0.25	0	0	99.38	0
Group II												
>6.25-12.5	1459	1.24	0	0	0.14	0	0	0.34	0.14	0	98.14	0
>12.5-25	2052	0.34	0	0	0.19	0	0	0.58	0.34	0	98.55	0
Group III												
>25-50	2564	0.55	0	0	0.08	0	0	0.74	0.31	0	98.32	0
>50-100	2550	0.71	0	0	0.47	0	0.04	0.98	0.63	0.08	96.97	0.12
Group IV												
>100-200	1618	1.29	0	0.06	14.83	0.31	1.05	33.62	31.83	0.62	11.77	4.62
>200-400	752	3.85	0	0.80	26.33	0.53	1.60	29.65	23.01	0.53	11.58	2.12
>400-800	273	5.50	0	1.47	28.94	0.73	1.47	27.47	20.15	0	11.71	2.56
>800-1,600	128	10.16	0	1.56	17.19	0	7.03	23.44	20.31	0	15.62	4.69
>1,600-3,200	65	6.16	0	1.54	21.54	1.54	6.15	23.08	13.85	3.08	18.44	4.62
>3,200-6,400	40	7.50	0	2.50	30	2.50	7.50	20	12.50	0	15	2.50
>6,400-12,800	17	0	0	5.88	35.29	0	0	17.64	11.76	11.76	17.67	0
>12,800	12	0	0	8.33	33.33	8.33	8.33	33.33	8.35	0	0	0

[1]Major pathogens: *Staph. aureus* = *Staphylococcus aureus*; *Strep. agalactiae* = *Streptococcus agalactiae*; *Strep. dysgalactiae* = *Streptococcus dysgalactiae*; *E. coli* = *Escherichia coli*; Coliforms = *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., other *Enterobacteriaceae*.
[2]Other pathogens: *Arcanobacterium pyogenes*, *Proteus*, *Prototheca zopfii*, *Pseudomonas* spp., group G streptococci, yeasts, and fungi.
[3]Contaminated samples.

Discussion

Udder health status in the German federal state Hesse was evaluated on the basis of quarter foremilk samples taken from 2000 to 2008. Quarter foremilk samples are accepted for the evaluation of udder health because a high correlation between SCC in foremilk samples and in total representative samples was reported (Dohoo and Meek, 1982). In general, foremilk refers to the first secretions removed from the teat at milking time and represents a portion of cisternal milk (Stockler et al., 2009). It is known that the milk fraction has a substantial affect on SCC because milk secreted during the milking process has lower SCC than milk from pre- or postmilking (Olde Riekering et al., 2007). However, the volume of foremilk varies from 5 to 60 mL (Fernando and Spahr, 1983; Vangroenweghe et al., 2002; Sarikaya and Bruckmaier, 2006). Sarikaya and Bruckmaier (2006) found a significant decrease of SCC comparing the first 10 mL of milk with the following 140 mL in udder quarters secreting >100,000 cells/mL, whereas this decrease was only moderate for udder quarters with a SCC <100,000 cells/mL. Comparing SCC in the first 5 mL of milk with the following 20 mL in infected and uninfected mammary glands, Fernando and Spahr (1983) reported slightly higher values for the first 5 mL of milk. In our study, foremilk was defined as the first 10 mL of milk collected from the teat after the first 2 squirts were discarded. Therefore, we presume that our SCC values are representative for the analysis of the udder health status. Foremilk samples were taken from farms producing high quality milk, from conventional farms, and from farms with massive udder health problems. This random test of 615,187 quarter foremilk samples represented 12.5% of all Hessian cows. The large data set generated indicated that approximately two-thirds of all quarter foremilk samples lay in the physiological range of SCC ≤100,000 cells/mL. This high frequency confirmed a proper level of udder health in the Hessian dairy cow population. The geometric mean of SCC in bulk milk samples in Hesse showed a value of 200,000 cells/mL in 2008 (HVL, 2009). Comparable data are available from the Netherlands, Belgium, and Finland. In the Netherlands, a geometric mean of SCC in bulk milk samples of 200,000 cells/mL was observed in 2002 (Sampimon et al., 2005). The geometric mean of SCC of bulk milk samples in Belgium was 221,000 cells/mL

in 2006 (Piepers et al., 2007). In Finland, the geometric mean of bulk milk SCC was 132,000 cells/mL in 2002 (Pitkälä et al., 2004).

The statistical distribution of the logarithm of SCC of all quarter foremilk samples tested in the years 2000 to 2008 in Hesse did not exactly show a Gaussian distribution. The reasons for the deviation of the empirical distribution curve may be due to the very large sample size and the origin of the samples. The data were collected from dairy farms offering different levels of udder health. Using a logarithmic partition for the SCC scale, the empirical distribution of SCC was still skewed to the right. Fewer samples at the level of ≤3,130 cells/mL and a level of >50,000 cells/mL to 1,600,000 cells/mL were observed than expected by exactly normally distributed values. In contrast, more samples lay in the range from >3,130 cells/mL to 50,000 cells/mL and at SCC >1,600,000 cells/mL than expected by normal distribution.

The distribution of SCC on dairy farm A showed 15% more samples in the SCC range ≤100,000 cells/mL than in the Hessian average. This represented a very high standard of udder health in this high quality milk producing farm and is due to exceptional hygiene management. The distribution of SCC on farm A differed from a Gaussian distribution for the same reasons as determined for the total Hessian random test, but deviation was reduced.

The results of our study indicated that SCC in front quarters were significantly lower than in rear quarters, which is in agreement with the results of Schepers et al. (1997). Because high SCC is usually an indicator of an immune response to IMI with bacterial pathogens (Dohoo et al., 1984), a higher prevalence of pathogens should be expected in rear quarters. However, in our data set the prevalence of bacteria differed between the individual quarters but these differences did not explain the higher SCC in rear quarters. Rear quarters might be more susceptible to infections than front quarters, as published previously (Pearson and Mackie, 1979), because of larger capacity and mass, greater vulnerability to direct trauma (e.g., horning), and greater exposure to environmental effects. In addition, teats of the rear quarters are frequently nearer the floor, especially in older cows, and would thus be contaminated or subjected to injury more readily.

In the Hessian survey in the period 2000 to 2003, the SCC value was used as a guideline for the selection of quarter foremilk samples for

bacteriological analysis. The data showed 51.96% of samples to be culture-positive. Our results are comparable to those obtained from the Netherlands (Sampimon et al., 2009), Belgium (Piepers et al., 2007), and Finland (Pitkälä et al., 2004). Similar to results from Hesse (17.17%), CNS were the most frequently isolated pathogens in the Netherlands, Belgium, and Finland (9.7 to 16.61%). However, it is not clear whether IMI with CNS will always result in inflammation (Sampimon et al., 2009). High frequencies of isolation of *Corynebacterium* spp. have been noted in Hesse (13.56%) and Finland (11.52%), in contrast to the Netherlands and Belgium, where only 2.5% and 0.1% of the samples showed *Corynebacterium* spp., respectively. These differences were probably related to changes in herd management and bacteriological ecology in the herd environment (Pitkälä et al., 2004). The prevalence of *Staph. aureus* in the Netherlands, Belgium, and Finland (1.8 to 3.4%) was lower than in Hesse (5.01%). Implementation of standard mastitis prevention programs (Neave et al., 1969) led to a clear decrease of mastitis caused by *Staph. aureus* in many countries. In contrast to the low prevalence of *Strep. uberis* in the Netherlands and in Finland (1.1% and 0.65%, respectively), the prevalence of this species was higher in Belgium (2.7%) and Hesse (8.7%). These differences may originate from different hygiene and management systems in these countries, because *Strep. uberis* is an environmental pathogen. *Streptococcus agalactiae* showed a low prevalence in Hesse (0.29%), Belgium (0.1%), and Finland (0.02%), but was not detected in any of the samples taken in the Netherlands in 2003. The implementation of mastitis prevention programs led to a clear reduction of the prevalence of *Strep. agalactiae*, too. The prevalence of *Strep. dysgalactiae* in Hesse (0.83%) was similar to that in the Netherlands (1.2%), but differed from Belgium (0.4%) and Finland (0.05%). The epidemiology of this pathogen is intermediate between contagious and environmental. When it is known which factors increase the incidence of IMI caused with these pathogens, the control program for *Strep. dysgalactiae* may need to be adjusted (Sampimon et al., 2009). The low prevalences of coliforms and *E. coli* observed in Hesse (1.15% and 0.61%, respectively), Belgium (0.1% coliforms), and Finland (0.14% coliforms) were expected because these species are generally involved in acute clinical mastitis

of short duration (Todhunter et al., 1991). In the Dutch study, *E. coli* was considered in the group of other bacteria because of only 2 isolations.

In our investigation, major pathogens (*Staph. aureus, Strep. uberis*) as well as minor pathogens (*Corynebacterium* spp., CNS) were detectable starting from an SCC level of 1,000 cells/mL. Similar data were found in a Dutch study (ten Napel et al., 2009). However, *Staph. aureus* is a natural species of mammalian skin and mucous epithelia (Sutra and Poutrel, 1994). *Streptococcus uberis* is considered an environmental pathogen (Radostits et al., 2007). Both major pathogens cause mastitis, which could take subclinical or clinical courses. *Corynebacterium* spp. readily colonize the teat canal of dairy cows (Brooks and Barnum, 1984). Coagulase-negative staphylococci are part of the normal teat skin flora and can colonize the teat canal (Devriese and De Keyser, 1980); some CNS species are detectable in the environment (White et al., 1989). In case of both minor pathogens, it is possible that they can contaminate the milk samples but not cause IMI (Linde et al., 1980). However, it is not clear whether the pathogens that we isolated from quarter foremilk samples with SCC values from 1,000 to 100,000 cells/mL originated from contamination or whether they caused an IMI. In any case, no signs of an IMI were visible based on SCC. Further research is needed for a detailed evaluation of immunological processes in such udder quarters.

The results of the bacteriological examinations on farm A were similar to the Hessian survey, and the low prevalence of mastitis pathogens in the SCC range ≤100,000 cells/mL could be expected because of the exceptional level of hygiene management.

Somatic cell counts >100,000 cells/mL are normally related to inflammatory processes inside the mammary gland. In the Hessian study, bacterial diagnosis was possible in approximately 83% of these cases. Negative bacteriological results in these cases could depend on spontaneous elimination of infection (Eberhart et al., 1979; Smith et al., 1985), intermittent shedding of the pathogens (Sears et al., 1990), or presence of antimicrobials or other inhibitors in milk (Reiter, 1978). At the time of examination, pathogens could also be ingested by phagocytes or survive intracellularly in the host (Newbould and Neave, 1965; Hill et al., 1978). Exudations of too-low masses of the pathogens or ceased growth of the pathogens are further reasons for negative

results (Sears et al., 1990). Negative results following antibiotic therapies should be excluded in our study because of withdrawal period regulations.

An SCC range of ≤100,000 cells/mL is within the physiological range, but can be related to subclinical mastitis in the presence of pathogens. However, SCC values ≤100,000 cells/mL are normally not taken into account to evaluate inflammatory processes. Bacteria detected could also be derived from contamination of the skin, teat canal, or environment. We presume that not all of these bacteria contaminated the milk samples. Therefore, it is obvious that they originated from inside the mammary gland and might cause an IMI even in the SCC range <100,000 cells/mL.

Our data showed that in 62% of all udder quarters SCC ≤100,000 cells/mL were detected, suggesting a high standard of udder health in the Hessian dairy cow population analyzed. In view of the mastitis pathogens, prevalences were clearly lower in samples with ≤100,000 cells/mL than in samples with >100,000 cells/mL. This finding confirmed the 100,000 cells/mL threshold differentiating between infected and noninfected mammary glands. However, our data also indicated that minor and major pathogens were detected even at a minimum of 1,000 cells/mL. This result led us to suspect inflammatory processes in the SCC range ≤100,000 cells/mL. We assert that these inflammatory processes could be elucidated by examination of the relationship of immune cells in milk. Differential cell counts might be a better indicator than SCC for a profound evaluation of inflammation, especially at SCC levels ≤100,000 cells/mL.

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3rd Chapter

Microscopic differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands

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Abstract

Somatic cell count (SCC) is generally regarded as an indicator of udder health. A cutoff value of 100×10^3 cells/ml is currently used in Germany to differentiate between normal and abnormal secretion of quarters. In addition to SCC, differential cell counts (DCC) can be applied for a more detailed analysis of the udder health status. The aim of this study was to differentiate somatic cells in foremilk samples of udder quarters classified as normal secreting by SCC $<100 \times 10^3$ cells/ml. Twenty cows were selected and 72 normal secreting udder quarters were compared with a control group of six diseased quarters (SCC $>100 \times 10^3$ cells/ml). In two severely diseased quarters of the control group (SCC of 967×10^3 cells/ml and $1{,}824 \times 10^3$ cells/ml) *Escherichia coli* and *Staphylococcus aureus* were detected. DCC patterns of milk samples ($n=25$) with very low SCC values of $\leq 6.25 \times 10^3$ cells/ml revealed high lymphocyte proportions of up to 92%. Milk cell populations in samples ($n=41$) with SCC values of $(>6.25$ to $\leq 25) \times 10^3$ cells/ml were also dominated by lymphocytes (mean value 47%), whereas DCC patterns of milk from udder quarters ($n=6$) with SCC values $(>25$ to $\leq 100) \times 10^3$ cells/ml changed. While in samples ($n=3$) with SCC values of $(27\text{-}33) \times 10^3$ cells/ml macrophages were predominant (35-40%), three milk samples with $(43\text{-}45) \times 10^3$ cells/ml indicated already inflammatory reactions based on the predominance of polymorphonuclear leucocytes (PMN) (54-63%). In milk samples of diseased quarters PMN were categorically found as dominant cell population with proportions of $\geq 65\%$. Macrophages were the second predominant cell population in almost all samples tested in relationship to lymphocytes and PMN. To our knowledge, this is the first study evaluating cell populations in low SCC milk in detail. Udder quarters classified as normal secreting by SCC $<100 \times 10^3$ cells/ml revealed already inflammatory processes based on DCC.

Introduction

For mastitis diagnosis, traditional and well-established tests including somatic cell count (SCC) and microbial culture-based methods are standard (Viguier et al. 2009). According to current definitions of udder health in Germany, SCC ≤100 × 10^3 cells/ml in quarter foremilk samples are in the physiological range (DVG, 2002). It is well known that the crossover of normal cellular defence in the mammary gland into an inflammatory reaction starts at a level of >100 × 10^3 cells/ml (Harmon, 1994; DVG, 2002). However, SCC vary with the status of lactation, age, stress of the animals, time and frequency of milking, season, and status of udder infection (Dohoo & Meek, 1982; Harmon, 1994). SCC is a robust quantitative estimate, but it does not divide the cells present in milk into different cell types (Kehrli & Shuster, 1994; Rivas et al. 2001).

In the mammary gland, number and distribution of leucocytes are important for the success of udder defences against invading pathogens (Leitner et al. 2003). Lymphocytes, macrophages, and polymorphonuclear leucocytes (PMN) play an important role in immune reactions within the mammary gland (Paape et al. 1979; Sordillo & Nickerson, 1988). Induction and suppression of immune responses are regulated by lymphocytes (Nickerson, 1989). They recognize antigens through membrane receptors specific for invading pathogens (Sordillo et al. 1997). Macrophages are active phagocytic cells in the mammary gland and capable of ingesting bacteria, cellular debris and accumulated milk components (Sordillo & Nickerson, 1988). Milk or tissue macrophages recognize the invading pathogens and initiate an immune response by the release of chemo-attractants inducing the rapid recruitment of PMN into the mammary gland (Paape et al. 2002; Oviedo-Boyso et al. 2007). The main task of PMN is the defence of invading bacteria at the beginning of an acute inflammatory process (Paape et al. 1979; Oviedo-Boyso et al. 2007). Not only does the number of PMN increase enormously, but also their level of defence activity (Targowski, 1983; Paape et al. 2003).

The distribution of leucocyte types varies in normal milk without any symptoms of mastitis. Some previous studies found lymphocyte proportions between 14 and 80%, macrophage proportions between 12 and 46%, and

those of PMN between 6 and 50% (Rivas et al. 2001; Merle et al. 2007; Koess & Hamann, 2008). In mastitis milk, PMN proportions of up to 95% have been reported (Paape et al. 1979; Kehrli & Shuster, 1994). During various phases of inflammation SCC differs in total numbers, whereas differential cell count (DCC) varies in composition of the cell populations involved (Nickerson, 1989). Therefore, in addition to SCC, determination of different types of immune cells present in milk is beneficial for describing udder health status (Pillai et al. 2001; Rivas et al. 2001). So far, however, there is little knowledge on DCC and the qualitative role of milk leucocytes in healthy udders because DCC in low-SCC milk are difficult to perform (Dosogne et al. 2003).

Data from a previous study (Schwarz et al. 2010) indicated a high standard of udder health in a representative part of the dairy cow population in the German federal state Hesse and confirmed the threshold of 100×10^3 cells/ml differentiating between normal and abnormal secretion of quarters. However, unexpectedly high numbers of mastitis pathogens in the SCC range $\leq 100 \times 10^3$ cells/ml were found. They could already be detected at a threshold of 1×10^3 cells/ml. Based on these data we suspected inflammatory processes even in the SCC range of mammary glands classified as healthy according to current definitions. Therefore, the objective of this study was the detailed evaluation of health status in udder quarters with SCC clearly $<100 \times 10^3$ cells/ml based on a statistical analysis of DCC. Leucocytes were isolated from quarter foremilk samples and differentiated into lymphocytes, macrophages, and PMN using microscopy.

Materials and Methods

Animals and farms

Twenty dairy cows in good condition and without previous history of mastitis were selected from four German dairy farms (A-D) for a detailed analysis of their udder health status based on DCC in quarter foremilk samples. The animals, Holstein-Frisian cows (*n*=18) and German Simmental cows (*n*=2), were in different lactations (1-6) and stages of the lactation. Six cows were in their first, seven in their second, three in their third, one in her fourth, two in their fifth, and one in her sixth lactation. Seven animals were in an early stage

of lactation (28-86 d), eight were in mid lactation (107-177 d) and five in a late stage of lactation (212-289 d). Foremilk samples from 72 udder quarters of the 20 cows classified as normal secreting (SCC $\leq 100 \times 10^3$ cells/ml and no pathogen) were selected for DCC analysis. A further 6 quarter foremilk samples with SCC of $(100-1,824) \times 10^3$ cells/ml were chosen from 6 cows as control group. Clinical mastitis symptoms such as flecks in milk, swelling or redness of the udder quarters could only be observed in quarters with SCC $>100 \times 10^3$ cells/ml.

In Farms A-D, 52-109 dairy cows were housed in pen barns and milked twice a day in milking parlours. Milking operations were similar in all farms. After forestripping into a foremilk cup, the milkers used damp cotton tissues for udder cleaning. Teats were dipped after milking with iodine solution. Feeding comprised a total mixed ration consisting of grass and maize silage, rape grist and cereals. Water was available ad libitum. Farm A produced high quality milk, while farms B-D were conventional milk producers. The average herd annual milk yields of the four farms ranged between 6500 and 9900 kg.

Milk sampling

Quarter foremilk samples were obtained according to DVG (2000) standards. Before milking, teat ends were scrubbed with 70% ethanol and the first two squirts of milk were discarded. Ten millilitres of milk per udder quarter was collected aseptically in a sterile 14-ml plastic sample tube (Greiner Bio-one, Frickenhausen, Germany). Four millilitres was used for SCC and bacteriological examinations, the remaining 6 ml was subjected to DCC analysis.

Quarter foremilk samples were taken in farms B-D during morning milking. Further processing occurred within 4 h. Samples on farm A were collected during evening milking and analysed within 15 h.

Somatic cell counts and bacteriological examinations

SCC was determined using a *Fossomatic 5000* (Foss Electric, Hillerød, Denmark). Cytobacteriological analysis of all quarter foremilk samples was performed according to IDF (1981) standards. Promptly after collecting the quarter foremilk samples and cooled-transportation to the laboratory, 10 µl of milk was streaked onto a quadrant of a 7% bovine blood agar plate containing

0.05% aesculin (Merck, Darmstadt, Germany), incubated for 48 h at 37 °C, and examined for bacterial growth.

Differential cell counts

Six millilitres of each quarter foremilk sample was transferred into a sterile 14-ml plastic tube. Milk samples were then centrifuged at 200 g at 4 °C for 15 min. Cream layers and supernatants were discarded and cells were washed once in PBS by centrifugation at 200 *g* at 4 °C for 15 min. Cell pellets were finally resuspended in 20 µl PBS. To obtain as many cells as possible for DCC analysis on the microscope slide, the whole sediment of the tube was spread over an area of 2 cm². Cell staining was performed according to the method of Pappenheim (1912).

Evaluation of the slides followed using light microscopy and oil immersion (100-fold magnification). One-hundred cells of each slide were counted meander-shaped and differentiated into lymphocytes, macrophages and PMN. Cell identification occurred according to standard methods (Coles, 1974; Lee et al. 1980). Lymphocytes were identified based on their circular form (5-10 µm) and the typical shape of the nucleus that almost fills the cell leaving a very thin rim of cytoplasm. Cells of 8-30 µm in size containing a little nucleus and pale staining were considered as macrophages. The group of PMN was characterized as cells of 10-14 µm in size and segmented nuclei. They were intensely coloured and contained granula in the cytoplasm.

Statistical analyses

Associations between values for individual cell populations and values for SCC were analysed by applying linear mixed models as implemented in the SAS program (version 9.1, SAS Institute, Cary, NC, USA). The statistical model included fixed and random effects as well as a regression on SCC up to the third polynomial degree, in order to fit regression curves. The non-significant regression coefficients of different polynomial structures were removed from the model by using *F*-statistics sum of square type I tests at $P<0.05$ instead of likelihood ratio tests. Based on type I sums of squares at $P<0.05$, a sequential analysis approach is appropriate for polynomial formulated models (Littell et al. 1998). The applied statistical model [1] was defined as follows:

$y_{ijkl} = \mu + herd_i + parity_j + DIM_k + cow_l + \alpha_1 SCC_{ijkl} + \alpha_2 SCC^2_{ijkl} + \alpha_3 SCC^3_{ijkl} + e_{ijkl}$

where y_{ijkl} = observation for the individual cell population (lymphocytes, macrophages, PMN) of the individual udder quarter of cow l; μ = overall mean effect; $herd_i$ = fixed effect of the i-*th* herd of cow l; $parity_j$ = fixed effect of the j-*th* lactation number of cow l [1, 2, ≥3]; DIM_k = fixed effect of the k-*th* stage of lactation of cow l [early, 28-86 d; mid, 107-177 d; late, 212-289 d]; cow_l = random effect of cow l; SCC_{ijkl} = value for SCC of the individual udder quarter of cow l; $\alpha_1, \alpha_2, \alpha_3$ = linear, quadratic, and cubic regression on SCC; and e_{ijkl} = random residual effect.

For verification of results of model [1], and to test DCC data especially for differences in the SCC range ≤100 × 10^3 cells/ml, analysis of variance was additionally done by including a fixed effect of the SCC group and removing the SCC covariates from the statistical model. Therefore, all 78 udder quarters were classified into SCC groups I-IV as defined in a previous study (Schwarz et al. 2010). Group IV represented the control quarters. Twenty-five (31%) of the 78 samples analysed belonged to group I according to their SCC values of ≤6.25 × 10^3 cells/ml. Forty-one samples (53%) showed SCC values of (>6.25 to ≤25) × 10^3 cells/ml (group II). Six samples (8%) with SCC of (>25 to ≤100) × 10^3 cells/ml were categorized into group III. A further 6 samples (8%) with SCC >100 × 10^3 cells/ml were assorted into group IV. The applied statistical model [2] was defined as follows:

$y_{ijklm} = \mu + herd_i + parity_j + DIM_k + cow_l + group_m + e_{ijklm}$

where y_{ijklm} = observation for the individual cell population (lymphocytes, macrophages, PMN) of the individual udder quarter of cow l; μ = overall mean effect; $herd_i$ = fixed effect of the i-*th* herd of cow l; $parity_j$ = fixed effect of the j-*th* lactation number of cow l [1, 2, ≥3]; DIM_k = fixed effect of the k-*th* stage of lactation of cow l [early, 28-86 d; mid, 107-177 d; late, 212-289 d]; cow_l = random effect of cow l; $group_m$ = fixed effect of the m-*th* SCC-group [I, ≤6.25 × 10^3 cells/ml; II, (>6.25 to ≤25) × 10^3 cells/ml; III, (>25 to ≤100) × 10^3 cells/ml; IV, >100 × 10^3 cells/ml]; and e_{ijklm} = random residual effect.

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Results

Somatic cell counts and bacteriological status of quarter foremilk samples

The 78 udder quarters selected showed a geometric mean value for SCC of 11.78×10^3 cells/ml and a median of 10×10^3 cells/ml. The quarter with the lowest SCC contained 1×10^3 cells/ml, the quarter with the highest SCC contained 1824×10^3 cells/ml. Udder pathogenic microorganisms were identified in only two of the 78 quarter foremilk samples. In two of the six control quarters (SCC of 967×10^3 cells/ml and 1824×10^3 cells/ml) *Escherichia coli* and *Staphylococcus aureus* were detected.

Differential cell counts of quarter foremilk samples depending on somatic cell counts

For a more detailed evaluation of the udder health status 100 cells per quarter foremilk sample were differentiated into lymphocytes, macrophages, and PMN. In view of DCC (*n*=78) the proportions of lymphocytes lay between 2 and 92% with a mean of 44.56% and a SD of 21.63% (Table 1). Proportions of macrophages ranged between 8 and 68% with a mean of 34.85% and a SD of 15.20%. PMN proportions varied between 0 and 88% with a mean of 19.94% and a SD of 21.40%.

Table 1. General overview about differential cell counts (DCC) of 78 quarter foremilk samples analysed by microscopy

	DCC in %		
	Lymphocytes	Macrophages	PMN
Mean	44.56	34.85	19.94
SD	21.63	15.20	21.40
Minimum	2	8	0
Maximum	92	68	88

Abbreviation: PMN, polymorphonuclear leucocytes

Because of the wide variations found within the cell populations, particularly in case of lymphocytes and PMN, DCC data were tested for correlation with SCC using the statistical model [1].

Lymphocytes decreased continuously from 92% at SCC of 1 × 10^3 cells/ml to only 5% at an SCC of 1824 × 10^3 cells/ml (Fig. 1A). The statistical analysis (model [1]) indicated a significant ($P<0.001$) negative correlation between percentages of lymphocytes and SCC (Table 2).

Table 2. Results of variance analysis (model [1]) for the percentage of the individual cell populations in 78 quarter foremilk samples analysed by microscopy[†]

		Effect			
Cell population	SCC	Quarter position	Lactation number	Days in milk	Farm
Lymphocytes	0.0001	0.41	0.21	0.14	0.05
Macrophages	0.01	0.68	0.33	0.07	0.05
PMN	0.0001	0.13	0.29	0.77	0.13

[†]Factors analysed were SCC, quarter positions (front right, rear right, front left, and rear left), lactation number (1, 2, ≥3), days in milk (28-86 d, 107-177 d, 212-289 d), and farm (A-D)

Abbreviations: SCC, somatic cell counts; PMN, polymorphonuclear leucocytes

Percentages of PMN (Fig. 1B) and lymphocytes (Fig. 1A) emerged in contrary directions at rising SCC. PMN increased constantly from 0% at SCC of 1 × 10^3 cells/ml to a maximum of 88% at 139 × 10^3 cells/ml. At SCC of 1824 × 10^3 cells/ml the proportion of PMN was 86%. Interestingly, PMN was already the predominant cell population at a SCC level of 43 × 10^3 cells/ml with a proportion of 62%. This event was observed in three udder quarters (SCC (43-45) × 10^3 cells/ml) of three different cows housed in two different farms. The statistical analysis (model [1]) indicated a significant ($P<0.001$) positive correlation between percentages of PMN and SCC (Table 2).

Within the SCC range of (3-100) × 10^3 cells/ml macrophage proportions lay between 8 and 68% (Fig. 1C). At SCC <3 × 10^3 cells/ml and in samples with >100 × 10^3 cells/ml proportions of macrophages were <30%. The statistical analysis (model [1]) revealed a significant ($P<0.01$) negative correlation between macrophage percentage and SCC (Table 2). In addition, the position of the udder quarter, the lactation number and the stage of lactation had no significant impact on the individual cell populations (Table 2). However, percentages of lymphocytes and macrophages were significantly influenced by the farm (Table 2).
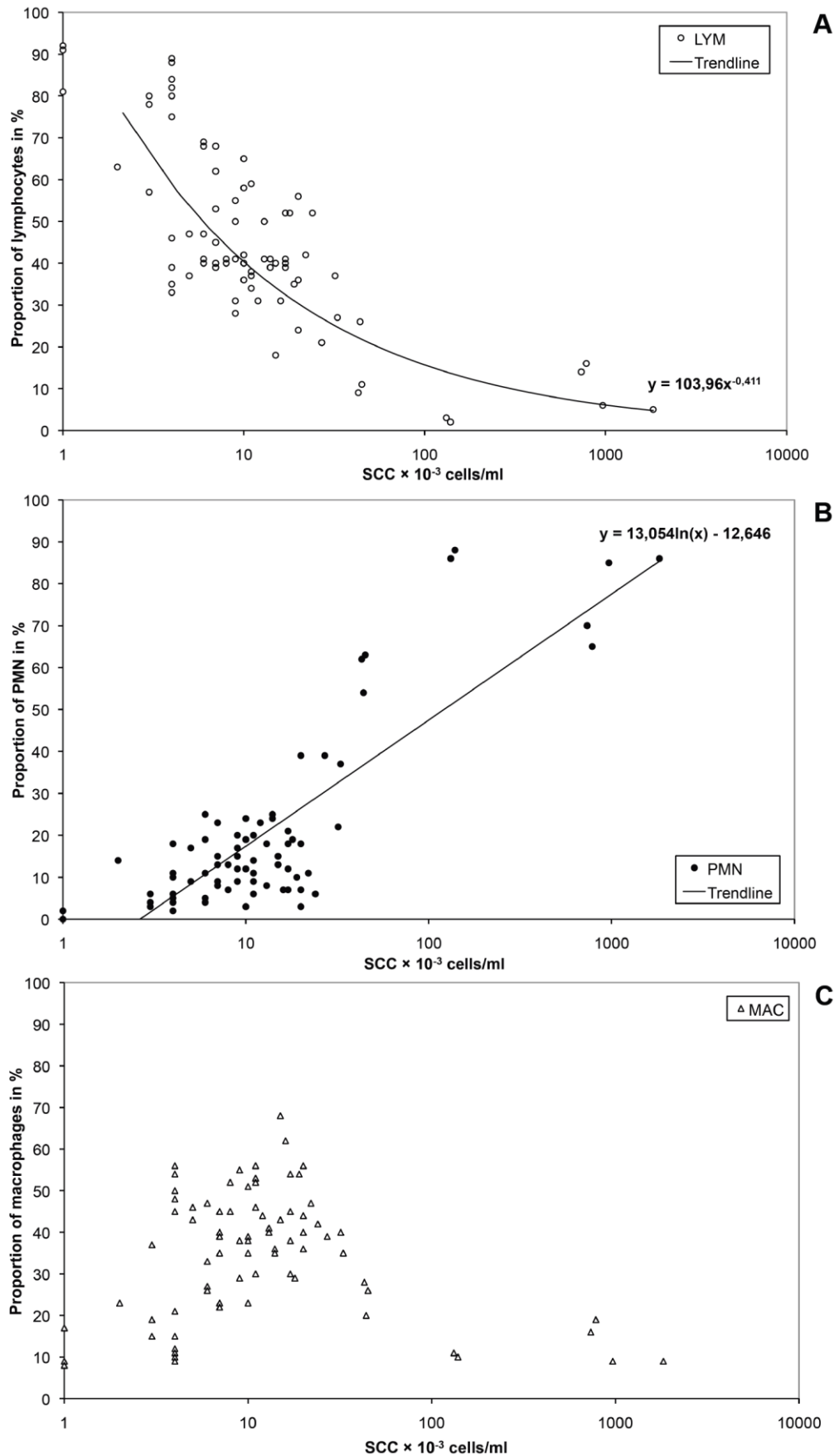
Fig. 1. Differential cell counts (DCC) depending on somatic cell counts (SCC): **A,** Proportions of lymphocytes (○ LYM) pictured in combination with a calculated potential trendline; **B,** Proportions of polymorphonuclear leucocytes (● PMN) pictured in combination with a calculated logarithmic trendline. **C,** Proportions of macrophages (△ MAC); each symbol represents the result of one udder quarter analysed, but overlapping is possible.

Statistical analysis (model [1]) revealed a significant negative correlation between the percentages of lymphocytes and SCC, a significant negative correlation between macrophages and SCC, as well as a significant positive correlation between PMN and SCC. To test DCC data especially for differences in the SCC range $\leq 100 \times 10^3$ cells/ml, a second statistical analysis (model [2]) was performed (Fig. 2).
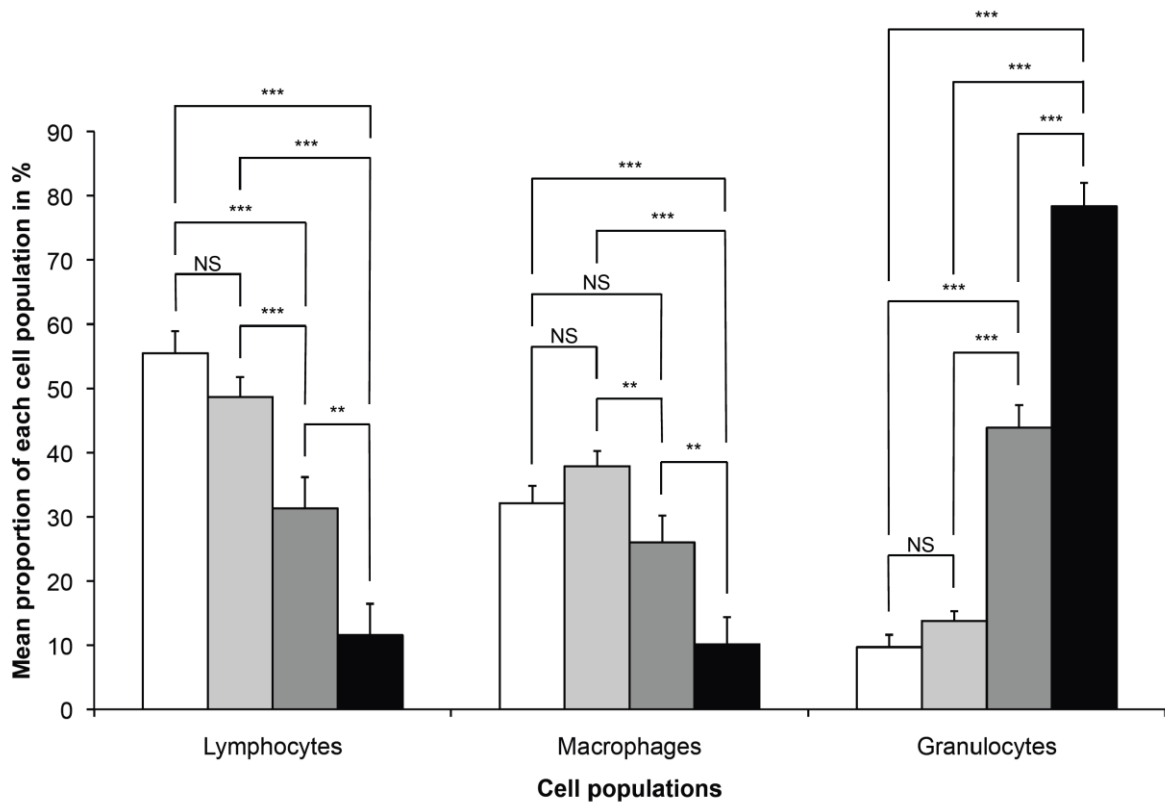

Fig. 2. Comparison of differential cell counts (DCC) within the somatic cell count (SCC) range of healthy mammary glands ($\leq 100 \times 10^3$ cells/ml) using statistical model [2]. All 78 udder quarters analysed were classified into SCC groups I-IV (group I, empty bars, SCC $\leq 6.25 \times 10^3$ cells/ml, n=25; group II, light gray bars, SCC (>6.25-≤ 25) $\times 10^3$ cells/ml, n=41; group III, dark gray bars, SCC (>25-≤ 100) $\times 10^3$ cells/ml, n=6; group IV, black bars, SCC $>100 \times 10^3$ cells/ml, n=6). Group IV represents the control quarters. Data are expressed as mean $\pm$ SEM for percentages of the individual cell populations in the four SCC groups defined.
Significance level: ***$P<0.001$; **$P<0.01$; *$P<0.05$; NS, not significant
Abbrevation: PMN, polymorphonuclear leucocytes

Lymphocytes indicated significantly ($P<0.01$) higher mean percentages in groups I-III (31.31-55.49%) than in group IV (11.56%). Interestingly, mean percentages in groups I and II were significantly ($P<0.001$) higher than those in group III.

Mean percentages of macrophages indicated significant ($P<0.01$) differences between groups I-III (26.02-37.88%) and IV (10.14%). In addition, mean percentages were significantly ($P<0.01$) lower in group III than those in group II.

Mean percentages of PMN differed significantly ($P<0.001$) between groups I-III (9.69-43.87%) and IV (78.37%). In addition, mean percentages of PMN were significantly ($P<0.001$) lower in groups I and II than those in group III.

Discussion

Together with SCC, determination of DCC in milk is an important tool characterizing udder health (Pillai et al. 2001). There are clear SCC cutoffs to differentiate between normal and abnormal secretion of quarters. But even in healthy udders, inflammations can be suspected under special circumstances (Schwarz et al. 2010). The immunological status of mammary glands classified by DCC is poorly investigated. Reviewing the literature Medzhitov (2007) reported that there might be a lack of knowledge on host defence in asymptomatic infections because almost all studies performed so far concentrated on symptomatic infections. In the present study, we differentiated leucocytes purified from quarter foremilk samples to improve knowledge of the immunological status of clinically healthy and subclinically infected bovine mammary glands. While SCC of $>100 \times 10^3$ cells/ml is normally related to inflammatory processes inside the mammary gland, a SCC range of $\leq100 \times 10^3$ cells/ml is in a physiological band (Harmon, 1994; DVG, 2002), but can also be related to latent mastitis in the presence of pathogens (Schwarz et al. 2010). Here, we predominantly analysed milk samples with SCC $<50 \times 10^3$ cells/ml to have a high informative value about DCC in low-SCC milk. Our control group (SCC $>100 \times 10^3$ cells/ml) included only six quarters. However, the SCC range $>100 \times 10^3$ cells/ml has been studied extensively before and PMN has been reported generally to be the dominant cell population in mastitic milk (e.g., Leitner et al. 2000; Merle et al. 2005; Koess & Hamann, 2008).

It is known that the milk fraction collected has an impact on both SCC and DCC (Sarikaya et al. 2005; Sarikaya & Bruckmaier, 2006; Olde Riekerink et

al. 2007). SCC values in foremilk samples of quarters with a total quarter milk SCC >100 $\times$ 10^3 cells/ml were significantly higher than in cisternal milk (Sarikaya & Bruckmaier, 2006). In samples with SCC <100 $\times$ 10^3 cells/ml differences of SCC between foremilk and cisternal milk were only minor. While no changes of DCC during milking could be observed in milk with SCC <200 $\times$ 10^3 cells/ml, proportions of PMN were higher and proportions of macrophages were lower in milk (SCC >200 $\times$ 10^3 cells/ml) collected post milking compared to milk collected premilking or during the milking process (Olde Riekerink et al. 2007). Sarikaya et al. (2005) also reported that the proportion of macrophages decreased while that of PMN increased during milking. Since we concentrated predominantly on the analysis of low-SCC milk, we presume that the foremilk samples taken in our study are representative for the analysis of the udder health status.

Our results indicate that lymphocytes were the predominant cell population in healthy mammary glands. Milk samples with an extremly low SCC value of ≤6.25 $\times$ 10^3 cells/ml revealed high lymphocyte proportions of up to 92% (mean value: 55%). In a SCC range of (>6.25 to ≤25) $\times$ 10^3 cells/ml a high mean proportion of lymphocytes (49%) was determined too. Information on DCC in milk samples with such low SCC from other field studies is rare. Only Koess & Hamann (2008) reported a mean value of 25% for the proportion of lymphocytes in the SCC range of (0-50) $\times$ 10^3 cells/ml. Merle et al. (2007) measured a mean proportion of 25% of lymphocytes in milk samples with SCC <100 $\times$ 10^3 cells/ml. In an experimental study (Rivas et al. 2001) lymphocyte proportions between 54 and 80% were measured pre-inoculation in udder quarters with SCC <200 $\times$ 10^3 cells/ml. Data from our study showed higher proportions of lymphocytes in milk with SCC <100 $\times$ 10^3 cells/ml than reported before. This difference resulted from the analysis of milk with very low SCC, because lymphocytes were the dominant cell population in these samples. The proportions of 2-16% of lymphocytes in milk secreted by diseased udder quarters (SCC >100 $\times$ 10^3 cells/ml) were clearly lower than those in healthy quarters. Similar observations were described before (Rivas et al. 2001; Merle et al. 2007; Koess & Hamann, 2008).

In udder quarters classified as normal secreting (SCC ≤100 ×
10^3 cells/ml) PMN proportions ranged from 0 to 63%. Our data showed that
PMN, particularly in milk samples with SCC values ≤6.25 × 10^3 cells/ml, were
rare (mean PMN proportion: 10%). At a SCC level of (>6.25 to ≤25) ×
10^3 cells/ml the mean proportion of PMN of 14% was also low. Comparable
data for such low SCC values are not available from the literature. Only a mean
PMN proportion of 30% in udder quarters with SCC of (0-50) × 10^3 cells/ml was
reported previously (Koess & Hamann, 2008). However, because of an
increased transfer of PMN from blood into the mammary gland at the beginning
of an inflammation (Kehrli & Shuster, 1994; Paape et al. 2002; Paape et al.
2003), a high percentage of PMN in milk is an important indicator of
inflammatory reactions (Pillai et al. 2001; Paape et al. 2002). PMN have been
reported previously as predominant cell population in secretions of diseased
mammary glands (Paape et al. 1979; Kehrli & Shuster, 1994). We made the
unexpected observation that in milk of udder quarters classified as normal
secreting PMN dominated already at SCC ≥43 × 10^3 cells/ml. This finding
suggested that inflammatory processes appear already in a SCC range that is
clearly below the cutoff value of 100 × 10^3 cells/ml. Factors that might have
triggered the elevated proportion of PMN might be manifold. A dairy cow is
under constant pressure from udder pathogenic microorganisms in the
environment. The elevated PMN proportion could be evidence for the initial
phase of an inflammation. In this regard it is also possible that PMN are able to
defend against pathogens successfully and prevent mastitis. However, although
we could not isolate any pathogens in such quarters they might be not healthy
anyhow. Negative bacteriological results could depend on intermittent pathogen
shedding (Sears et al. 1990), presence of antimicrobials or other inhibitors in
milk (Reiter, 1978). At the time of examination pathogens could also be
ingested by phagocytes or survive intracellularly in the host (Newbould &
Neave, 1965; Hill et al. 1978). Shedding of too low amounts of pathogens or
ceased growth may be further reasons for negative bacteriological results
(Sears et al. 1990).

The interdependence of infections, inflammatory processes, and immune
responses in individual udder quarters is discussed controversially in the

literature. Some authors suggested that individual udder quarters within a cow can be influenced by infections of neighbouring quarters (Merle et al. 2007), whereas others did not find any evidence for an interdependence of udder quarters (Wever & Emanuelson, 1989) because they did not find DCC to be affected by the bacteriological status of adjacent quarters. Our data indicated no immunological interdependence between the four udder quarters at low and high SCC levels. In the three udder quarters of three different cows with SCC of (43-45) $\times$ 10^3 cells/ml in foremilk samples, elevated PMN proportions between 54 and 63% were determined. In the remaining nine quarters of these cows clearly lower SCC values of (4-19) $\times$ 10^3 cells/ml, lower PMN proportions of 6-18%, and no bacterial infection were detected. Furthermore, no interactions between the quarters were observed in the six cows of the control group with high SCC values >100 $\times$ 10^3 cells/ml and PMN proportions of 65-92% in one udder quarter. In these animals SCC <100 $\times$ 10^3 cells/ml and PMN proportions of 4-39%, respectively, were detected in the other three quarters.

Another factor that might have triggered the elevated percentage of PMN is stress (Davis et al. 2008). Although we did not measure parameters considering stress, such as corticosterone in plasma, the influence of stress in our study might be minimal because the animals analysed were kept under optimal conditions and according to national guidelines. No obvious symptoms of stress (i.e. kicking during pre-milking preparation of the udder or during taking the quarter foremilk samples) were observed.

The antidromic trend of lymphocyte and PMN percentages at increasing SCC is caused by the composition of milk leucocytes. Since they primarily consist of lymphocytes, macrophages, and PMN (Sordillo & Nickerson, 1988), the increase of the percentage of one cell population implies the decrease of at least one of the other cell populations. However, our statistical analysis indicated a significant impact of the farms on percentages of both macrophages and lymphocytes. This impact might be due to a non-randomized selection of cows within the farms and different numbers of cows selected per farm. While cows with healthy mammary glands were predominantly selected from farms A, B and D, samples from cows with diseased quarters were predominantly collected from farm C.

Beside PMN, macrophages also possess phagocytic functions. Milk samples with extremly low SCC values of $\leq 6.25 \times 10^3$ cells/ml showed a mean proportion of 32% of macrophages. In the SCC range of $(>6.25$ to $\leq 25) \times 10^3$ cells/ml the mean macrophage proportion was 38%. In the literature a mean value of 43% of macrophages was reported for the SCC range of $(0-50) \times 10^3$ cells/ml (Koess & Hamann, 2008). In the case of diseased udder quarters (SCC $>100 \times 10^3$ cells/ml) we measured macrophage proportions of 9-28%. These results lay also within a wide range of 4-48% as mentioned in other studies (Rivas et al. 2001; Merle et al. 2007; Koess & Hamann, 2008). While we found that macrophages were the second dominant cell population in almost all samples tested in relationship to lymphocytes and PMN, they had been reported to be the predominant cell population in milk of healthy mammary glands (Lee et al. 1980). This difference might be explainable by different definitions of healthy mammary glands. In our study we focused on the analysis of immune cells in milk with very low SCC values. However, Lee et al. (1980) defined mammary glands as healthy based on negative bacteriological examinations and did not present any SCC values.

Beside leucocytes, epithelial cells can also be found in milk. In the literature (Lee et al. 1980; Koess & Hamann, 2008) low proportions of epithelial cells of 1-3%, which were similar to our examinations (data not shown), were described. Other researchers reported epithelial cell proportions of 10-19% (Miller et al. 1991) or even 44% (Leitner et al. 2000). However, proportions of ≥ 10% should be discussed critically. Miller et al. (1991) analysed milk of primiparous cows during the first 75 days of lactation, whereas Leitner et al. (2000) measured epithelial cells by flow cytometry based on a non-specific identification procedure.

Variations in the distribution of leucocytes in milk from non-infected mammary glands as shown in other studies, were probably dependent on differences in methods, sampling, investigators (Schröder & Hamann, 2005), breed (Leitner et al. 2003), stage of lactation (Vangroenweghe et al. 2001; Dosogne et al. 2003) and variable SCC. Contrary to other authors (Schröder & Hamann, 2005), we did not observe influences of the composition of the sample

tubes (glass or plastic) on differences of phagocytic cell percentages (data not shown).

In our study bacteriological examinations revealed udder pathogenic microorganisms in only 2 of the 78 udder quarters analysed (2 of the 6 control quarters). *Esch. coli*, however, was found in a quarter with high SCC of 967 $\times$ 10^3 cells/ml (DCC: lymphocytes 6%, PMN 85%, macrophages 9%), whereas *Staph. aureus* was detected in a quarter with SCC of 1824 $\times$ 10^3 cells/ml (DCC: lymphocytes 5%, PMN 86%, macrophages 9%). These few bacteriological findings did not allow any assessment. However, other studies (Piccinini et al. 1999) also found PMN to be the dominant cell population in milk of udder quarters infected with major pathogens.

Conclusion

SCC is an undisputed and well-established criterion for the evaluation of udder health and milk quality. However, in addition to SCC, DCC can be used for a more detailed analysis of the udder health status. Analyzing DCC of mammary glands classified as normal secreting by SCC <100 $\times$ 10^3 cells/ml, inflammatory reactions were already detectable at a SCC level of ≥43 $\times$ 10^3 cells/ml due to predominating PMN proportions in foremilk samples of the corresponding udder quarters. This is the first study indicating inflammatory reactions in udder quarters with SCC that were clearly below the current threshold of 100 $\times$ 10^3 cells/ml.

Acknowledgements

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4th Chapter

Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands

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Abstract

Somatic cell counts (SCC) are generally used as indicator of udder health. In Germany, a cutoff value of 100,000 cells/mL is currently used to differentiate between healthy and diseased mammary glands. In addition to SCC, differential cell counts (DCC) can be applied for a more detailed evaluation of the udder health status. The aim of this study was to differentiate immune cells in milk of udder quarters classified as healthy based on SCC values of <100,000 cells/mL. Twenty cows were selected and 65 healthy udder quarters were compared with a control group of 15 diseased udder quarters (SCC >100,000 cells/mL). Cells were isolated from milk of all quarters to measure simultaneously percentages of lymphocytes, macrophages, and polymorphonuclear neutrophilic leukocytes (PMNL) by flow cytometric analysis. The bacteriological status of all 80 quarters was also determined. Differential cell count patterns of milk samples (n = 15) with extreme low SCC values of ≤6,250 cells/mL revealed high lymphocyte proportions of up to 88%. Milk cell populations in samples (n = 42) with SCC values from >6,250 to ≤25,000 cells/mL were also dominated by lymphocytes, whereas DCC patterns of 6 out of 41 milk samples with SCC values from ≥9,000 to ≤46,000 cells/mL indicated already inflammatory reactions based on the predominance of PMNL (56-75%). In 13 of 15 milk samples of the diseased udder quarters (SCC >100,000 cells/mL), PMNL were categorically found as dominant cell population with proportions of ≥49%. Macrophages were the second predominant cell population in almost all samples tested in relation to lymphocytes and PMNL. Further analysis of the data demonstrated significant differences of the cellular components between udder quarters infected by major pathogens (e.g., *Staphylococcus aureus*; n = 5) and culture-negative udder quarters (n = 56). Even the percentages of immune cells in milk from quarters infected by minor pathogens (e.g., coagulase-negative staphylococci; n = 19) differed significantly from those in milk of culture-negative quarters. Our flow cytometric analysis of immune cells in milk of udder quarters classified as healthy by SCC <100,000 cells/mL revealed inflammatory reactions based on DCC.

Introduction

For mastitis diagnosis, traditional and well-established tests including SCC and microbial culture-based methods are standard (Viguier et al., 2009). According to current definitions of udder health in Germany, SCC ≤100,000 cells/mL in quarter foremilk samples are in the physiological range (DVG, 2002). It is accepted that the crossover of normal cellular defense in the mammary gland into an inflammatory reaction starts at a level of >100,000 cells/mL (Harmon, 1994; DVG, 2002). However, SCC vary with the status of lactation, age, stress of the animals, time and frequency of milking, season, but primarily in response to udder infection (Dohoo and Meek, 1982; Harmon, 1994). Somatic cell count is a robust quantitative measurement, but does not divide the cells present in milk into different cell types (Kehrli and Shuster, 1994; Rivas et al., 2001a).

In the mammary gland, number and distribution of leukocytes are important for the success of udder defenses against invading pathogens (Leitner et al., 2003). Lymphocytes, macrophages, and polymorphonuclear neutrophilic leukocytes (PMNL) play an important role in inflammatory responses within the mammary gland (Paape et al., 1979; Sordillo and Nickerson, 1988). Induction and suppression of immune responses are regulated by lymphocytes (Nickerson, 1989). They recognize antigens through membrane receptors specific for invading pathogens (Sordillo et al., 1997). Macrophages are active phagocytic cells in the mammary gland and capable of ingesting bacteria, cellular debris, and accumulated milk components (Sordillo and Nickerson, 1988). Milk or tissue macrophages recognize the invading pathogens and initiate an immune response by the release of chemoattractants inducing the rapid recruitment of PMNL into the mammary gland (Paape et al., 2002; Oviedo-Boyso et al., 2007). The main task of PMNL is to defend against invading bacteria at the beginning of an acute inflammatory process (Paape et al., 1979; Oviedo-Boyso et al., 2007). Not only does the number of PMNL increase enormously but their defensive responses also increase (Targowski, 1983; Paape et al., 2003).

The distribution of leukocyte types varies in normal milk without any symptoms of mastitis. Some recent studies found lymphocyte proportions between 14 and 80%, macrophage proportions between 12 and 46%, and those of PMNL between 6 and 50% (Rivas et al., 2001a; Merle et al., 2007; Koess and Hamann, 2008). In milk from cows with mastitis, the proportions of PMNL can reach 95% (Paape et al., 1979; Kehrli and Shuster, 1994). During various phases of inflammation both overall SCC and differential cell count (DCC) change (Nickerson, 1989). Therefore, in addition to SCC, determination of different types of immune cells present in milk is beneficial for describing the udder health status (Pillai et al., 2001; Rivas et al., 2001a). So far, however, there is little knowledge on DCC and the qualitative role of milk leukocytes in udders classified as healthy because DCC in low-SCC milk are difficult to perform (Dosogne et al., 2003).

Data of a previous study (Schwarz et al., 2010) indicated a high standard of udder health in a representative part of the dairy cow population in the German federal state Hesse and confirmed the threshold of 100,000 cells/mL differentiating between healthy and diseased mammary glands. However, unexpectedly high numbers of mastitis pathogens were found in the SCC range ≤100,000 cells/mL. In some cases, pathogens were detected in milk samples from some cows with an SCC of 1,000 cells/mL. Based on these data, we suspected inflammatory processes even in the SCC range of mammary glands classified as healthy according to the current definitions. Therefore, the objective of this study was a detailed evaluation of the health status in udder quarters with SCC clearly below 100,000 cells/mL based on a statistical analysis of DCC. Leukocytes were isolated from quarter foremilk samples and differentiated simultaneously into lymphocytes, macrophages, and PMNL using flow cytometry.

Materials and Methods

Animals and Farms

Twenty Holstein-Frisian cows in good health status were selected from 3 German dairy farms (A-C) for detailed analysis of their udder health status based on DCC in quarter foremilk samples. The animals were in different

lactations (1 to 6) and stages of the lactation. In total, 80 foremilk samples were selected for DCC analysis. Sixty-five samples were obtained from udder quarters of 18 cows classified as healthy (SCC ≤100,000 cells/mL). Fifteen foremilk samples were chosen as the control group. Seven of those samples with SCC from 100,000 to 624,000 cells/mL were derived from 7 individual cows. A further 8 quarter foremilk samples with SCC ranging from 100,000 to 1,394,000 cells/mL were collected from 2 cows that were clinically diagnosed with mastitis. Symptoms included flecks in the milk, swelling, or redness of the involved quarters.

In Farms A to C, 50 to 160 dairy cows were housed in pen barns and milked twice per day in milking parlors. The milking operations were similar in all farms. After forestripping into a foremilk cup, the milkers used damp cotton tissues for udder cleaning. Teats were dipped after milking with iodine solution. In all of the dairy farms, animals were fed with a TMR consisting of grass and maize silage, rape grist, and cereals. Water was available ad libitum. All farms were conventional milk producers and the average herd milk yields ranged between 8,000 and 10,000 kg/yr (305-d milk yield: 7,393 to 9,385 kg/yr).

Milk Sampling

Quarter foremilk samples were obtained according to German Veterinary Society (DVG, 2000) standards. Before milking, teat ends were scrubbed with 70% ethanol and the first 2 squirts of milk were discarded. Aliquots of 110 mL of milk per udder quarter were collected aseptically in sterile 14-mL plastic sample tubes and 2 sterile 50-mL plastic tubes (SARSTEDT, Nümbrecht, Germany). Ten milliliters were saved for SCC and bacterial measurements and 100 mL for DCC analysis. Quarter foremilk samples were taken in all farms during morning milking. Further processing occurred within 7 h.

SCC and Bacteriological Examinations

Somatic cell counts were determined using a *Fossomatic 5000* (Foss Electric, Hillerød, Denmark). The cytobacteriological analysis of all quarter foremilk samples was performed according to the IDF (1981) standards. Promptly after collection, quarter foremilk samples were cooled in a cold box and transported to the laboratory. Ten microliters milk were streaked onto a

quadrant of a 7% bovine blood agar plate containing 0.05% esculin (Merck, Darmstadt, Germany), incubated for 48 h at 37°C, and examined.

Differential Cell Counts

Isolation of Milk Cells. Milk samples were centrifuged for 15 min at 200 × g and 4°C before cream layers and supernatants were discarded. To wash the cells, cell pellets were resuspended in PBS and then centrifuged again for 15 min at 200 × g and 4°C. Washed pellets were resuspended at a final dilution of 1 × 10^6 cells/100 µL based on predetermined SCC values for each milk sample.

Antibody Staining of Milk Cells. For DCC analysis, 100 µL of the cell suspension was transferred into a 5-mL BD Falcon™ tube (Becton, Dickinson and Co., Heidelberg, Germany). For identification of PMNL and macrophages, nonconjugated monoclonal antibodies against CD11b- and CD14-molecules were used (Table 1). Volumes of 5 µL of anti-CD11b (IgG2b) and 10 µL of anti-CD14 (IgG1), both diluted 1:10 in PBS, were added and the tubes incubated on ice for 30 min. After adding 2 mL of FACS Lysing Solution (Becton, Dickinson and Co.) a further incubation step of 15 min followed. Cells were centrifuged (5 min at 250 × g) then resuspended in 3 mL of PBS and allowed to stand for 5 min. After a second wash step, cells were resuspended in 50 µL of PBS and then incubated with secondary antibodies. Specifically, 10 µL of rat-anti-mouse-IgG2b diluted 1:10 in PBS and 5 µL nondiluted rat-anti-mouse-IgG1 were applied (Table 1). After incubation for 30 min, cells were washed twice again and finally resuspended in 500 µL of PBS. Cells were kept on ice during all procedures, centrifuged at 4°C, and incubated in the dark.

Flow Cytometry Analysis. Stained samples were analyzed with a FACSCalibur™ flow cytometer (Becton, Dickinson and Co.) with standard optical equipment using an argon ion laser and a red diode laser with excitation wavelengths of 488 and 635 nm, respectively. The software CellQuest Pro (Becton, Dickinson and Co.) was used for data collection and analysis. Five thousand cells from each sample were differentiated into lymphocytes, macrophages, and PMNL. Lymphocytes were identified by size (forward light scatter, FSC-H) and granularity (side light scatter, SSC-H). The PMNL were measured as CD11b$^+$ cells, whereas macrophages were defined as both

CD11b$^+$ and CD14$^+$ cells. Gates enclosing the antibody-positive cells were placed outside the upper limit of background fluorescence. Cells with no antibody labeling served as a negative control and were regarded as a measure for background fluorescence. In addition, isotype control antibodies (rat-IgG1, κ isotype control, 554686 and rat-IgG2a, κ isotype control, 554688, Becton, Dickinson and Co.) were used to allow measurement of background staining.

Table 1. Monoclonal antibodies used for differentiation and labeling of bovine milk cells applying flow cytometry analysis

Type	Description	Name	Isotype	Specificity	Host	Company
α-CD11b	PMNL, macrophages, and monocytes	MM10A	IgG2b	Bovine	Mouse	VMRD[1]
α-CD14	Macrophages and monocytes	MM61A	IgG1	Bovine	Mouse	VMRD
α-IgG1	APC[2] marker	550874	IgG1, κ	Murine	Rat	BD[3]
α-IgG2b	FITC[4] marker	553395	IgG2a, κ	Murine	Rat	BD

[1]VMRD Inc., Pullman, WA.
[2]Allophycocyanin
[3]Becton, Dickinson and Co., Heidelberg, Germany.
[4]Fluorescein isothiocyanate.

Statistical Analysis

Associations between the individual cell populations and SCC, as well as between the individual cell populations and the bacteriological status of the udder quarters were analyzed by applying linear mixed models with the SAS program (version 9.1, SAS Institute, Cary, NC). The statistical model included the fixed effects of herd, lactation number, position of the udder quarter, as well as a regression on SCC up to the third polynomial degree, to fit regression curves. The nonsignificant regression coefficients of different polynomial structures were removed from the model by using F-statistics sum of square type I tests at $P < 0.05$ instead of likelihood ratio tests. Based on type I sums of squares at $P < 0.05$, a sequential analysis approach is appropriate for polynomial formulated models (Littell et al., 1998). Variance analysis of SCC groups was done by modeling a fixed SCC group effect and removing SCC covariates from the statistical model.

Results

SCC and Bacteriological Status of Quarter Foremilk Samples

Quarter foremilk samples were taken from 20 cows housed in 3 German dairy farms (A to C) to determine the bacteriological status and SCC. The 80 udder quarters selected showed an SCC mean value of 85,780 cells/mL with a standard deviation of 233,040 cells/mL (Table 2). Bacteria were identified in 24 of the 80 quarter foremilk samples. Thirteen of the 15 control quarters (SCC >100,000 cells/mL) were culture-positive. In one quarter with an SCC value of 454,000 cells/mL *Staphylococcus aureus* was isolated. In 3 quarters (SCC 181,000 to 1,394,000 cells/mL) *Streptococcus (Strep.) uberis* was detected. A double infection with *Strep. uberis* and *Strep. dysgalactiae* was diagnosed in an udder quarter with SCC of 139,000 cells/mL. In 5 further quarters (SCC 104,000 to 624,000 cells/mL), CNS were identified. *Corynebacterium* spp. could be isolated from 3 quarters with SCC from 116,000 to 587,000 cells/mL. In 11 of the 65 udder quarters with SCC <100,000 cells/mL, bacteria were found, which indicated a latent mastitis in these quarters according to DVG (2002) definitions. Interestingly, all of those bacteria were CNS and detected in the SCC range from 7,000 to 59,000 cells/mL.

Table 2. General overview about SCC and differential cell counts (DCC) of the 80 quarter foremilk samples analyzed using flow cytometry

Item	SCC ($\times$ 1,000 cells/mL)	DCC (%)		
		Lymphocytes	Macrophages	PMNL
Mean	85.78	48.50	21.74	29.76
SD	233.04	23.39	12.07	22.44
Minimum	2	2.37	2.37	4.48
Maximum	1,394	87.75	55.71	85.27

DCC of Quarter Foremilk Samples Depending on SCC

For a more detailed evaluation of the udder health status, 5,000 cells per quarter foremilk sample were differentiated into lymphocytes, macrophages, and PMNL by flow cytometry. Over all samples (n = 80), the proportions of lymphocytes ranged between 2.37 and 87.75%, with a mean of 48.50% and a standard deviation of 23.39% (Table 2). Macrophages ranged between 2.37

and 55.71%, with a mean of 21.74% and a standard deviation of 12.07%. Polymorphonuclear neutrophilic leukocytes proportions varied between 4.48 and 85.27%, with a mean of 29.76% and a standard deviation of 22.44%.
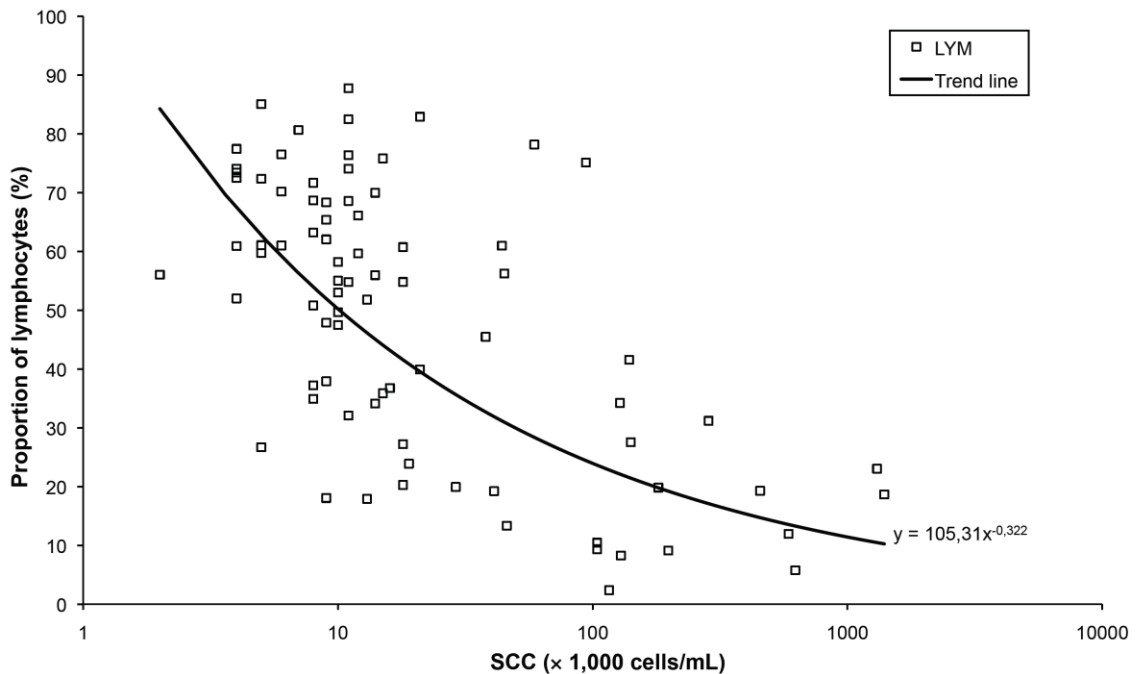

Figure 1. Differential cell counts depending on SCC: proportions of lymphocytes (□ LYM), pictured in combination with a calculated potential trendline; each symbol represents the result of 1 udder quarter analyzed, but overlapping is possible.

Because of the wide variations found within the cell populations, particularly in the case of lymphocytes and PMNL, DCC data were tested statistically for correlation with SCC. The proportion of lymphocytes decreased from >60% at SCC values <10,000 cells/mL to 18.67% at an amount of 1,394,000 cells/mL (Figure 1). However, lymphocytes were the predominant cell population in 75% of the 65 healthy mammary glands (SCC ≤100,000 cells/mL), as clearly demonstrated for a single udder quarter (Figure 2A and B). The milk leukocytes secreted from this healthy quarter (SCC of 10,000 cells/mL) consisted of 58.20% lymphocytes, 23.06% macrophages, and 18.74% PMNL (Figure 2A and B). The statistical analysis indicated a significant ($P < 0.001$) negative correlation between percentages of lymphocytes and SCC (Table 3).

As indicated by the calculated trend lines, percentages of PMNL (Figure 3) and lymphocytes (Figure 1) emerged in contrary directions as SCC increased.
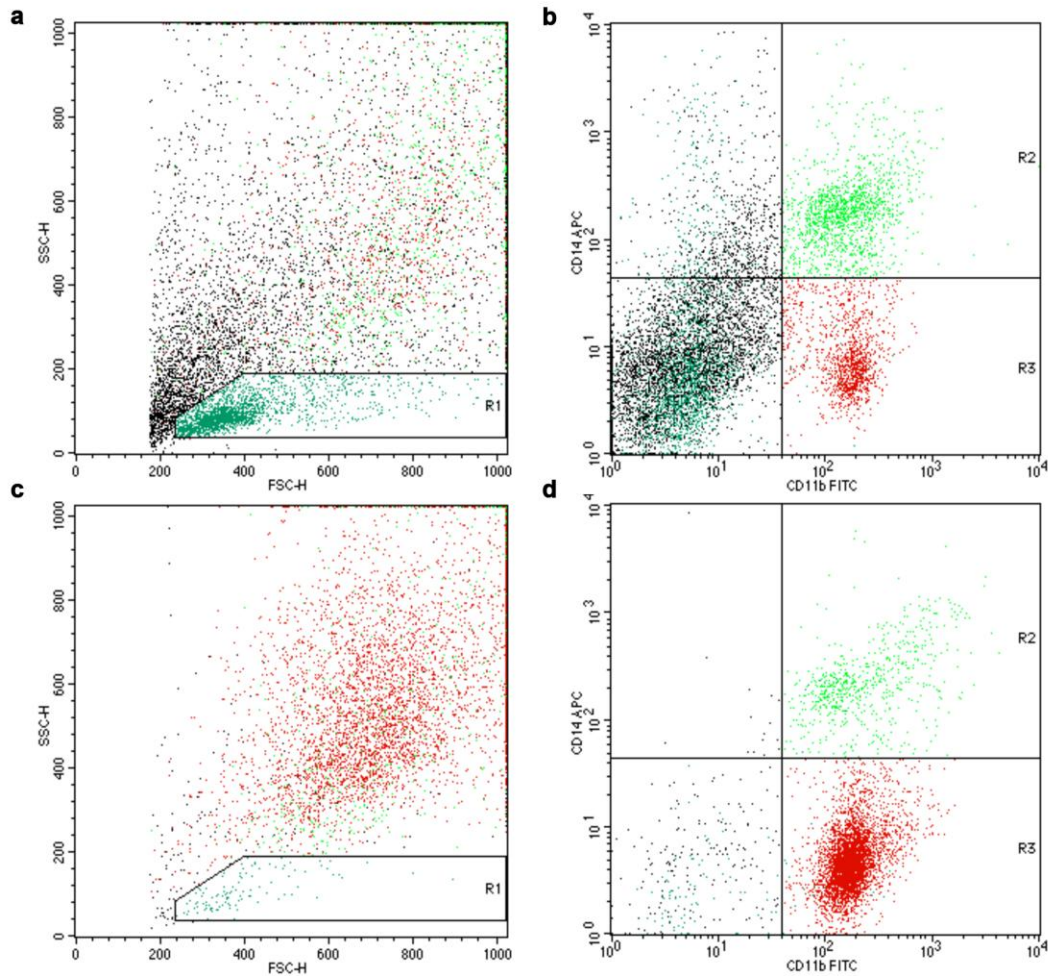
Figure 2. Dot plots of the flow cytometric analysis of milk leukocytes: forward scatter (FSC-H) versus side scatter (SSC-H) plots (a and c) show the size (FSC-H) and granularity (SSC-H) of the leukocytes. The CD11b FITC versus CD14 APC plots (b and d) show the results of the fluorescence analysis of the leukocytes. Lymphocytes (R1) were recognized based on physical parameters. Macrophages (R2) were identified as CD11b^{+} and CD14^{+} cells, whereas PMNL (R3) were defined as CD11b^{+} cells. Data show representative FACS profiles of a healthy mammary gland with SCC of 10,000 cells/mL (a and b) and a diseased mammary gland with SCC of 116,000 cells/mL and detection of *Corynebacterium* spp. (c and d).

Polymorphonuclear neutrophilic leukocytes increased from <30% within the SCC range of <10,000 cells/mL to 63.65% at SCC of 1,394,000 cells/mL. Apparent inflammatory reactions (e.g., increased PMNL proportion of 74.43%) could be detected in samples with SCC values ≥9,000 cells/mL. This relationship was observed in 6 udder quarters (SCC from 9,000 to 46,000 cells/mL) of 2 different cows housed in farm C. However, PMNL were

Table 3. Results of variance analysis for the percentage of the individual cell populations in 80 quarter foremilk samples analyzed using flow cytometry[1]

		Effect		
Cell population	SCC	Quarter position	Lactation number	Farm
Lymphocytes	***	NS	*	*
Macrophages	NS	NS	*	NS
PMNL	***	NS	NS	*

[1]Analyzed factors were SCC, quarter positions (front right, rear right, front left, and rear left), lactation number (1, 2, and ≥ 3) and farm (A to C).
*** $P < 0.001$; * $P < 0.05$; NS = $P > 0.05$.

the predominant cell population in 86% of the 15 diseased mammary glands (SCC >100,000 cells/mL), as demonstrated for a single udder quarter (Figure 2C and D). Although 85.27% of milk leukocytes of this quarter were PMNL, percentages of macrophages and lymphocytes of 12.35% and 2.38%, respectively, were low (Figure 2C and D). This quarter showed an SCC value of 116,000 cells/mL and the bacteriological examination revealed *Corynebacterium* spp. The statistical analysis indicated a significant ($P < 0.001$) positive correlation between percentages of PMNL and SCC (Table 3).
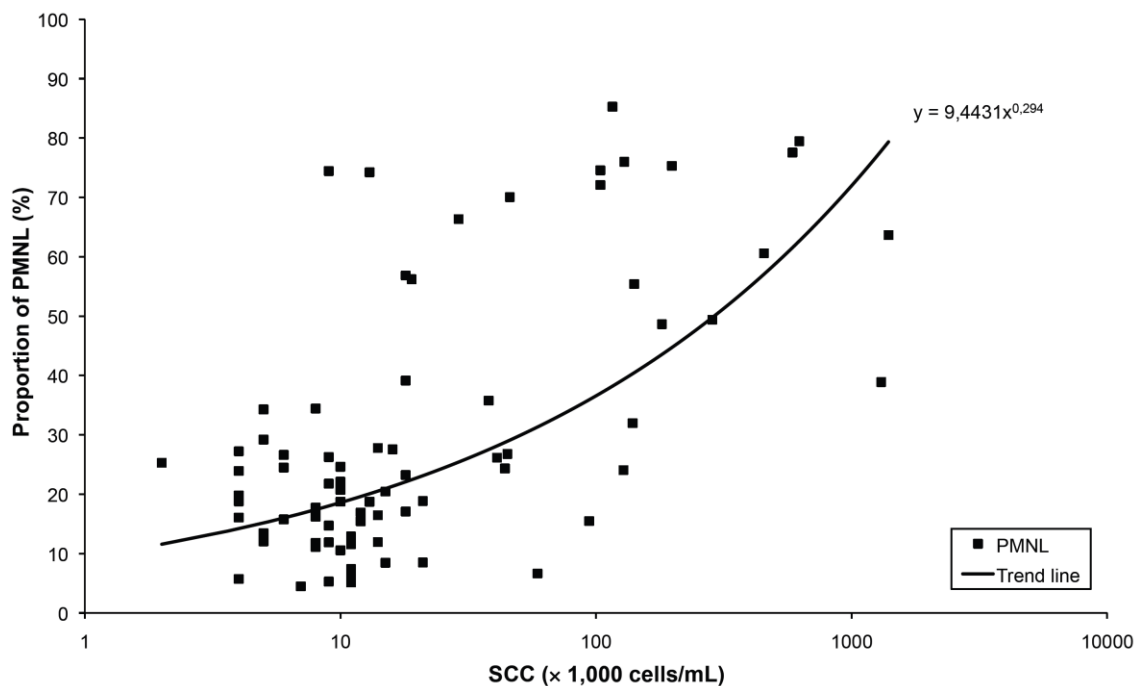

Figure 3. Differential cell counts depending on SCC: proportions of PMNL (■), pictured in combination with a calculated logarithmic trendline; each symbol represents the result of 1 udder quarter analyzed, but overlapping is possible.

Within an SCC range of 8,000 to 100,000 cells/mL, the proportions of macrophages ranged between 7.51 and 55.71% (Figure 4). At SCC <8,000 cells/mL and in samples with >100,000 cells/mL, the proportions of macrophages were <40%. The statistical analysis revealed no significant correlation between percentages of macrophages and SCC (Table 3). In addition, the percentages of the individual cell populations were significantly influenced by lactation number and the farm but not by the position of the udder quarter (Table 3).
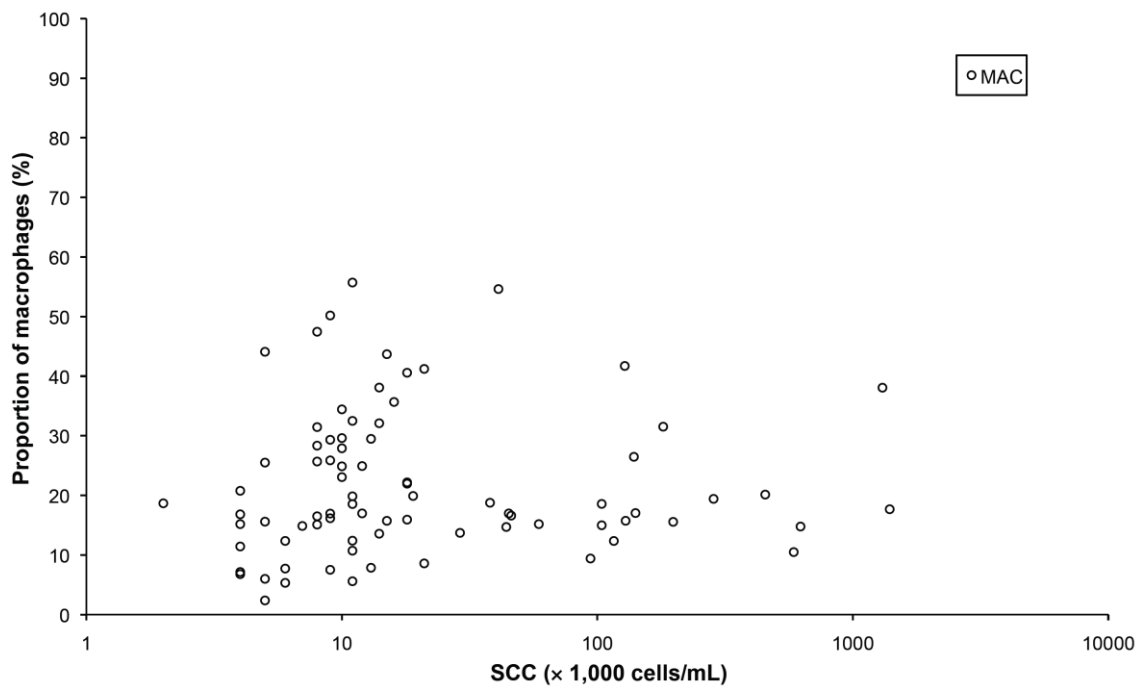

Figure 4. Differential cell counts depending on SCC: proportions of macrophages (O MAC); each symbol represents the result of 1 udder quarter analyzed, but overlapping is possible.

Statistical analysis revealed a significant negative correlation between lymphocyte percentages and SCC as well as a significant positive correlation between PMNL percentages and SCC. However, to test whether the immunological status differed statistically within the SCC range ≤100,000 cells/mL, all udder quarters analyzed were classified into SCC groups I to IV, as defined in a previous study (Schwarz et al., 2010). Group IV represented the control quarters. Fifteen (18.75%) of the 80 samples analyzed belonged to group I according to their SCC values ≤6,250 cells/mL. Forty-two samples (52.5%) showed SCC values between >6,250 and ≤25,000 cells/mL (group II). Eight samples (10%) with SCC from >25,000 cells/mL to

≤100,000 cells/mL were categorized into group III. Fifteen other samples (18.75%) with SCC >100,000 cells/mL were assorted into group IV.

The mean percentages of lymphocytes were significantly ($P < 0.001$) higher in groups I to III (50.61-62.99%) than in group IV (19.17%; Figure 5). In addition, mean percentages in group I were significantly ($P < 0.05$) higher than those in group III (Figure 5). Significant differences were not detected between the mean percentages of macrophages within the 4 SCC groups (21.15-27.13%; Figure 5). Mean percentages of PMNL differed significantly ($P < 0.001$) between groups I to III (15.35-22.26%) and IV (59.68%; Figure 5). However, significant differences could not be calculated between the mean percentages of PMNL within groups I to III.
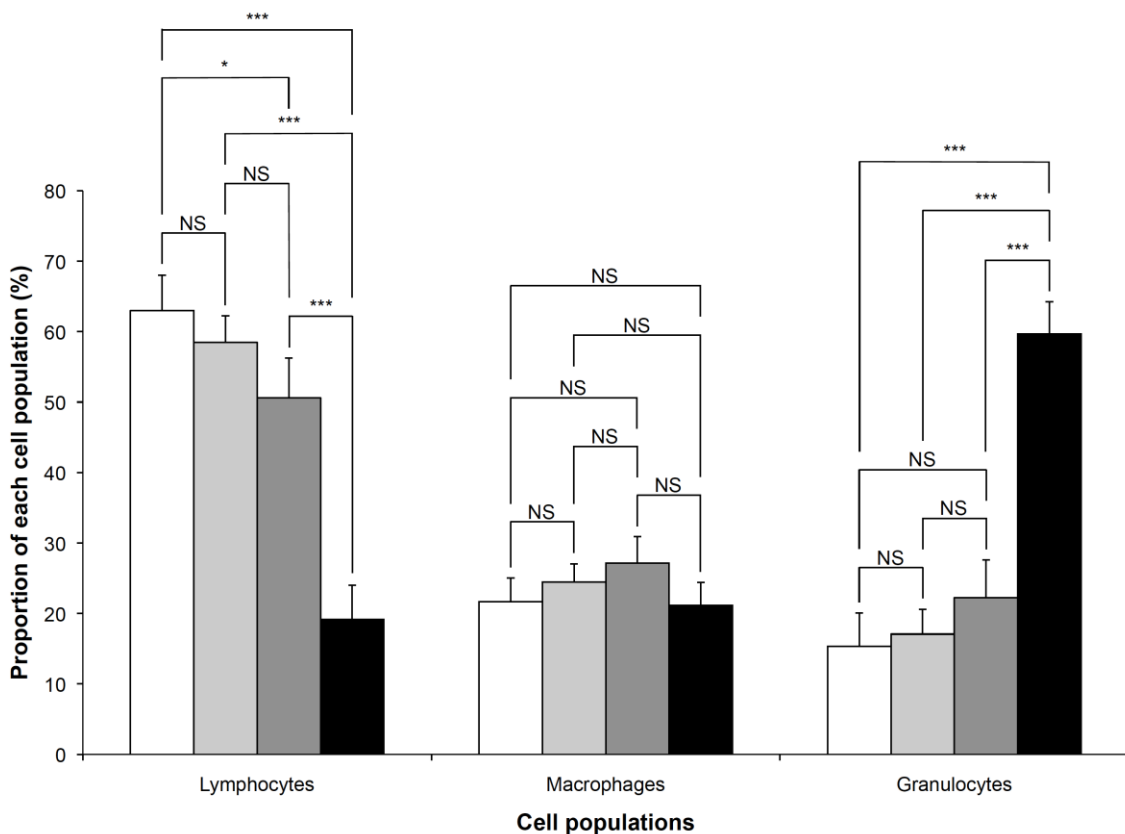

Figure 5. Comparison of differential cell counts within the SCC range of healthy mammary glands (≤100,000 cells/mL). All 80 udder quarters analyzed were classified into SCC groups I to IV (group I, empty bars, SCC ≤6,250 cells/mL, n = 15; group II, light gray bars, SCC >6,250 to ≤25,000 cells/mL, n = 42; group III, dark gray bars, SCC >25,000 to ≤100,000 cells/mL, n = 8; group IV, black bars, SCC >100,000 cells/mL, n = 15). Group IV represents the control quarters. Data are expressed as mean ± standard error of the means for percentages of the individual cell populations in the 4 SCC groups defined. *** $P < 0.001$; * $P < 0.05$; NS = $P > 0.05$.

DCC of Quarter Foremilk Samples Depending on the Bacteriological Status

Significant correlations were identified between the individual cell populations, in particular with respect to lymphocytes or PMNL, and SCC. To analyze and compare DCC data depending on the mastitis pathogens detected, all 80 mammary glands analyzed were classified into three groups (no, minor, and major pathogens) according to Reneau (1986). In 56 (70%) of the 80 quarters, no pathogens could be detected. Minor pathogens were detectable in 19 samples (23.75%), whereas major pathogens were isolated in 5 samples (6.25%).
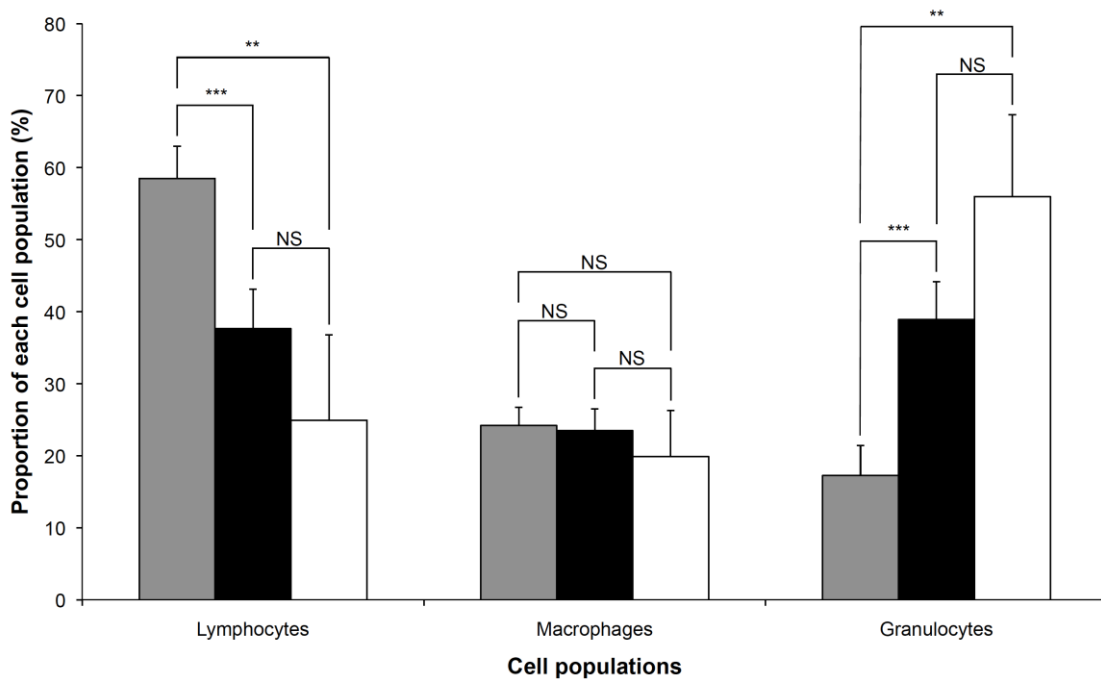

Figure 6. Differential cell counts depending on the bacteriological status of the mammary glands. The 80 udder quarters analyzed were classified into no pathogens (dark grey bars, n = 56), minor pathogens (e.g., CNS, black bars, n = 19), or major pathogens (e.g., *Staphylococcus aureus*, empty bars, n = 5). Data are expressed as mean $\pm$ standard error of the means for percentages of the individual cell populations in the 3 groups defined. *** $P < 0.001$; ** $P < 0.01$; NS = $P > 0.05$.

The mean lymphocyte percentage in milk of culture-negative udder quarters (58.48%) was significantly ($P < 0.01$) higher than in milk of culture-positive quarters (24.91-37.63%; Figure 6). In case of macrophages the mean percentages (19.87-24.18%) indicated no significant differences depending on the bacteriological status (Figure 6). Polymorphonuclear neutrophilic leukocytes

showed significantly ($P < 0.01$) higher mean percentages in culture-positive udder quarters (38.90-55.96%) than in culture-negative quarters (17.23%; Figure 6).

Discussion

Together with SCC, determination of DCC in milk is an important tool characterizing udder health (Pillai et al., 2001). Clear SCC cutoffs exist defining an udder as healthy or not. But even in healthy udders, inflammations can be suspected under special circumstances (Schwarz et al., 2010). The immunological status of mammary glands classified as healthy based on DCC is poorly investigated. Reviewing the literature, Medzhitov (2007) reported that a lack of knowledge on host defense in asymptomatic infections might exist because almost all studies performed so far concentrated on symptomatic infections. In this study, we differentiated leukocytes purified from quarter foremilk samples to improve knowledge of the immunological status of clinically healthy and subclinically infected bovine mammary glands. Although SCC of >100,000 cells/mL are normally related to inflammatory processes inside the mammary gland, a SCC of ≤100,000 cells/mL is generally accepted as normal or physiological (Harmon, 1994; DVG, 2002), but can also be related to latent mastitis in the presence of pathogens (Schwarz et al., 2010).

Our results indicated that lymphocytes were the predominant cell population in healthy mammary glands. Milk samples with an extreme low SCC value of ≤6,250 cells/mL revealed high lymphocyte proportions of up to 88% (mean value: 63%). In an SCC range from >6,250 to ≤25,000 cells/mL, a high mean proportion of lymphocytes (58%) was determined, too. To our knowledge, information about DCC in milk samples with that low SCC is not available in the literature. Flow cytometry studies measuring lymphocytes based on a combination of physical parameters and nucleic acid staining revealed mean proportions of 5% for udder quarters with SCC from 25,000 to 100,000 cells/mL (Östensson et al., 1988) and 57% for udder quarters with SCC <200,000 cells/mL (Dosogne et al., 2003). The clearly higher proportions of lymphocytes measured in our study are explainable by the analysis of samples with clearly lower SCC and the predominance of lymphocytes in those samples.

The proportions of 2 to 42% of lymphocytes in milk of diseased udder quarters were clearly lower compared to those in healthy udder quarters. Similar observations were described before (Östensson, 1993; Rivas et al., 2001a). In view of the flow cytometric method, considering and identifying preferably all lymphocytes, we measured them based on physical characteristics according to previous studies (Riollet et al., 2001; Rivas et al., 2001b). Because lymphocytes isolated from blood were almost indistinguishable from those isolated from milk (Redelman et al., 1988), the definition of our lymphocyte gate resulted from the analysis of blood and milk cells. However, other recent studies (Rivas et al., 2001a; Koess and Hamann, 2008) used an antibody against CD3-molecules for the identification of lymphocytes and detected CD3^{+} lymphocytes with wide variations from 11 to 88% in non-mastitic milk.

In mammary glands classified as healthy (SCC ≤100,000 cells/mL), the proportion of PMNL ranged from 4 to 74%. Our data showed that PMNL in milk samples with SCC values ≤6,250 cells/mL were rare (mean proportion: 15%). At an SCC level of >6,250 to ≤25,000 cells/mL, the mean proportion of PMNL of 17% was also low. Comparable data for such low SCC values are not available from the literature. However, some authors (Koess and Hamann, 2008) measured PMNL with a mean proportion of 42% in milk of healthy udder quarters with SCC <100,000 cells/mL applying an antibody against CD11b-molecules. In an experimental study, CD11b^{+} PMNL were measured preinoculation with proportions between 11 and 36% in milk of quarters with SCC <200,000 cells/mL (Rivas et al., 2001a). However, inflammation is generally defined as an increase of leukocytes, especially PMNL, in tissues or body fluids infected by pathogens. An increased transfer of PMNL from blood into the mammary gland at the beginning of an inflammation could be detected (Burvenich et al., 1994; Kehrli and Shuster, 1994; Paape et al., 2002, 2003). Therefore, a high PMNL percentage in milk is accepted as an important indicator of inflammatory reactions (Pillai et al., 2001; Paape et al., 2002). Polymorphonuclear neutrophilic leukocytes have previously been reported as predominant cell population in secretions of diseased mammary glands (Paape et al., 1979; Kehrli and Shuster, 1994). We made the unexpected observation, that in milk of mammary glands classified as healthy, PMNL dominated at SCC ≥9,000 cells/mL. This finding suggests that inflammatory processes can appear

within an SCC range that is clearly below the cutoff value of 100,000 cells/mL. Factors that might have triggered the elevated proportion of PMNL might be manifold. A dairy cow is under constant pressure from udder pathogenic microorganisms of the environment. The elevated proportion of PMNL could be evidence for the initial phase of an inflammation. In this regard, it is also possible that PMNL are able to defend pathogens successfully and prevent mastitis. However, although we could not isolate any pathogens in such quarters, they might be infected nonetheless. Negative bacteriological results could depend on intermittent pathogen shedding (Sears et al., 1990), presence of antimicrobials, or other inhibitors in milk (Reiter, 1978). At the time of examination pathogens could also be ingested by phagocytes or survive intracellularly in the host (Newbould and Neave, 1965; Hill et al., 1978). Shedding of too low amounts of pathogens or ceased growth may be further reasons for negative bacteriological results (Sears et al., 1990).

The interdependence of infections, inflammatory processes, and immune responses in individual udder quarters is discussed controversially in the literature. Some authors suggested that individual udder quarters within a cow can be influenced by infections of neighboring quarters (Merle et al., 2007), whereas others did not find any evidence for an interdependence of udder quarters (Wever and Emanuelson, 1989) because they did not find DCC to be affected by the bacteriological status of adjacent quarters. Our data indicated immunological interdependence as well as independence between the 4 udder quarters at low and high SCC levels. In the 6 udder quarters of 2 different cows with SCC from 9,000 to 46,000 cells/mL in foremilk samples, elevated PMNL proportions between 56.21 and 74.43% were determined. In the remaining 2 quarters of these cows (control group), clearly higher SCC values of 104,000 and 587,000 cells/mL, PMNL proportions of 74.56 and 77.56%, and bacterial infections with CNS or *Corynebacterium* spp. were detected. However, no interactions between the individual quarters were observed in 5 of the 7 cows of the control group with 3 healthy and 1 diseased udder quarter. Each of these cows showed SCC values >100,000 cells/mL, PMNL proportions of 72.08 to 85.27%, and an infection with CNS or *Corynebacterium* spp. in 1 udder quarter. In the remaining 3 quarters, SCC values <100,000 cells/mL and PMNL proportions between 5.12 and 27.79%, respectively, were detected.

Another factor that might have triggered the elevated proportion of PMNL is stress (Davis et al., 2008). Although we did not measure parameters considering stress, such as corticosterone in plasma, the influence of stress in our study might be minimal because the animals analyzed were kept under optimal conditions and according to national guidelines. No obvious symptoms of stress (i.e., kicking during pre-milking preparation of the udder or during taking the quarter foremilk samples) could be observed.

The antidromic trend of lymphocyte and granulocyte percentages at increasing SCC is caused by the composition of milk leukocytes. Because they primarily consist of lymphocytes, macrophages, and PMNL (Sordillo and Nickerson, 1988), the increase of the percentage of one cell population implies the decrease of at least one of the other cell populations. However, our statistical analysis indicated significant effects of the farms on percentages of both PMNL and lymphocytes. These effects might be due to a non-randomized selection of the cows within the farms and the different numbers of cows selected per farm. Whereas cows with healthy mammary glands were predominantly selected from farms A and C, samples from cows with diseased quarters were predominantly collected in farm B. In addition, a significant effect of the lactation number on percentages of lymphocytes and macrophages was found. This phenomenon might be explainable due to the exposure of the mammary glands during the lactation. The milking process by milking machines and the constant contact to the environment could be responsible for both increasing SCC values (Harmon, 1994) and increasing percentages of PMNL (Vangroenweghe et al., 2001) detected in the course of the lactation period and in milk of cows with higher lactation numbers. Vice versa, the mammary glands of cows in their first lactation period, in particular at the beginning of the lactation, were only influenced slightly by milking machines and the environment.

Beside PMNL, macrophages posses phagocytic functions, too. We detected proportions of macrophages in healthy mammary glands (SCC ≤100,000 cells/mL) between 2 and 56%. Milk samples with extreme low SCC values of ≤6,250 cells/mL showed a mean proportion of macrophages of 22%. Within the SCC range from >6,250 to ≤25,000 cells/mL, the mean macrophage proportion was 24%. Due to the expression of CD14 (Berthon and Hopkins,

1996; Sopp et al., 1996) and CD11b (Splitter and Morrison, 1991; Howard and Naessens, 1993) on monocytes and macrophages, we defined macrophages as both CD11b$^+$ and CD14$^+$ cells. To our knowledge, this is the first study measuring DCC in milk with such a definition of macrophages. A mean value of 34% of macrophages identified with an antibody against CD14-molecules was reported for the SCC range <100,000 cells/mL (Koess and Hamann, 2008). Rivas et al. (2001a) measured macrophages with an antibody against CD11b-molecules and detected proportions between 1 and 20% in quarters with SCC of <200,000 cells/mL. In case of diseased udder quarters (SCC >100,000 cells/mL), we measured macrophages with proportions of 9 to 42% (mean proportion: 21%). These results were similar to the mean proportion of 16% (Koess and Hamann, 2008) and proportions of macrophages between 21 and 48% (Rivas et al., 2001a). Although we found macrophage percentages with wide variations in both healthy and diseased mammary glands, they had been reported to be the predominant cell population in milk of healthy mammary glands (Lee et al., 1980; Östensson et al., 1988). This difference might be explainable by different definitions of healthy mammary glands. In our study, we focused on the analysis of immune cells in milk with very low SCC values. However, Lee et al. (1980) defined mammary glands as healthy based on negative bacteriological examinations, but did not present any SCC values. Östensson et al. (1988) analyzed foremilk samples of bacteriologically negative quarters with an SCC mean value of 89,000 cells/mL.

Beside leukocytes, epithelial cells can also be found in milk. Although some researchers (Lee et al., 1980; Koess and Hamann, 2008) described low proportions of 1 to 3%, others reported proportions of epithelial cells of 10 to 19% (Miller et al., 1991) or even 44% (Leitner et al., 2000). However, proportions of ≥10% should be discussed critically. Miller et al. (1991) analyzed milk of primiparous cows during the first 75 d of lactation, whereas Leitner et al. (2000) measured epithelial cells based on a nonspecific identification using flow cytometry. Because cows selected in our study were in different lactations and at least 70 d in lactation, we presume that percentages of epithelial cells in their milk were low. Therefore, and because their immunological properties are not well understood, we did not consider epithelial cells. They were completely

disregarded in other flow cytometric DCC studies (Rivas et al., 2001a; Koess and Hamann, 2008), too.

Variations in the distribution of leukocytes in milk from non-infected mammary glands, as shown in other studies, were probably dependent on differences in methods, sampling, investigators (Schröder and Hamann, 2005), breeds (Leitner et al., 2003), stages of lactation (Vangroenweghe et al., 2001; Dosogne et al., 2003), and variable SCC. In contrast to other authors (Schröder and Hamann, 2005), we did not observe influences of the composition of the sample tubes (glass or plastic) used on differences of phagocytic cell percentages (data not shown). However, due to the rapid characterization of a large number of cells as well as the definite identification of individual cell populations, using specific antibody flow cytometric analysis gives more accurate results compared with microscopic analysis (Loken and Stall, 1982; Rivas et al., 2001a; Dosogne et al., 2003; Koess and Hamann, 2008).

In our study, bacteriological examinations revealed udder pathogenic microorganisms in 24 of the 80 udder quarters analyzed (11 of the 65 healthy quarters with SCC <100,000 cells/mL and 13 of the 15 control quarters with SCC >100,000 cells/mL). Statistical analysis of DCC data revealed significant differences of cellular components in milk between culture-positive and culture-negative udder quarters. Interestingly, these results also indicated that, even in case of the presence of minor pathogens, the percentages of lymphocytes and PMNL differed significantly from those in culture-negative quarters. However, DCC patterns of udder quarters with SCC ≤10,000 cells/mL and culture positive for minor pathogens did not indicate inflammatory reactions. Therefore, it can be speculated that the pathogens detected in these samples originated from contaminations of the skin, teat canal, or environment. Nevertheless, in milk samples with >10,000 to 25,000 cells/mL containing minor pathogens, the proportions of PMNL were already twice as high compared to bacteriological negative quarters and reached 25%. This observation suggested that the pathogens originated from mammary gland tissue and that, even at such low cell numbers, PMNL were recruited from blood into milk to phagocyte bacteria. Differential cell count results of quarters infected with major pathogens indicated an IMI and were in agreement with previous observations (Piccinini et al., 1999).

Conclusions

Somatic cell count is an undisputed and well-established criterion for the evaluation of udder health and milk quality. However, in addition to SCC, DCC can be used for a more detailed analysis of the udder health status. Analyzing DCC of udder quarters classified as healthy by having SCC <100,000 cells/mL, inflammatory reactions were detectable at an SCC level of ≥9,000 cells/mL due to predominating PMNL proportions in foremilk samples of the corresponding quarters. Our study indicated inflammatory reactions in udder quarters with SCC that were clearly below the current threshold of 100,000 cells/mL. Further research in this field should concentrate on longitudinal examinations of immune cells in milk of udder quarters classified as healthy that reveal high percentages of PMNL.

Acknowledgements

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5th Chapter

Microscopic differential cell counting to identify inflammatory reactions in dairy cow quarter milk samples

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Abstract

The diagnosis of intramammary infections is mostly based on somatic cell count (SCC) and bacteriological analysis. As an alternative, differential cell counting (DCC) could be a useful method, because it identifies changes in the relative cell populations before the rising of total cell number. The aim of the study was to identify cytological parameters that could be used in the field to classify mammary quarters as healthy or diseased, comparing cyto-bacteriological results with DCC. Overall, 48 cows were randomly selected from 3 herds in Lombardy region of Italy. Herd A was characterized by the absence of contagious microorganisms; in herd B and C, the prevalence of *Staphylococcus aureus* was 20% and 50%, respectively. Foremilk samples were aseptically collected from 188 quarters and submitted to bacteriological analysis, SCC, and DCC. For the statistical analysis, the samples were clustered into 4 health groups, and DCC results were compared in each group. Ninety-six samples were classified as normal secretions (N), 30 as mastitis (M), 15 as latent mastitis (LM), and 47 as unspecific mastitis (UM) based on SCC and bacteriological results. Single percentages of lymphocytes, polymorphonuclear neutrophilic leukocytes (PMNL), or macrophages were firstly evaluated to established variables capable of identifying healthy and inflamed quarters. Then, combinations of cell populations were tested to increase the discrimination power of DCC: phagocytes, logarithmic (log) PMNL:lymphocyte ratio, and log phagocyte:lymphocyte ratio. The mean percentage of lymphocytes was significantly higher in group N than in groups LM, UM, and M. The mean percentage of PMNL was significantly lower in group N than in groups UM and M, but not LM. Mean percentages of macrophages were not significantly influenced by the 4 groups. The mean value of phagocytes was significantly lower in group N than in the other groups. Both the log PMNL:Lym and the log phagocyte:lymphocyte ratios were significantly lower in group N than in groups LM, UM, and M. Fisher (*F*-) values were calculated, and the highest *F*-value was that of log PMNL:lymphocyte ratio (48.23). The explanation for this could be that log PMNL:Lym is the only variable that involved both cell populations statistically influenced by health

groups but excluded macrophages. Microscopic DCC has potential as a tool to identify cows affected by any inflammatory process of the mammary gland, with the best results being achieved using log PMNL:Lym as variable.

Introduction

Bovine mastitis is a major health problem in dairy cattle. Economic losses are mostly associated with decreased production and milk quality due to subclinical infections. Such infections are not evident and can persist in the mammary tissue throughout lactation. In the mammary gland, the number and distribution of leukocytes are important for the successful defense against invading pathogens (Leitner et al., 2003). Lymphocytes, macrophages, and PMNL play an important role in the inflammatory response within the mammary gland (Paape et al., 1979; Sordillo and Nickerson, 1988). Lymphocytes regulate the induction and suppression of immune responses (Nickerson, 1989), recognizing antigens through membrane receptors specific for invading pathogens (Sordillo et al., 1997). Macrophages are active phagocytic cells, capable of ingesting bacteria, cellular debris, and milk components (Sordillo and Nickerson, 1988). Milk or tissue macrophages recognize the invading pathogen and initiate an immune response by releasing chemoattractants that induce rapid recruitment of PMNL (Paape et al., 2002; Oviedo-Boyso et al., 2007). The main task of PMNL is to defend against invading bacteria at the beginning of acute inflammatory process (Paape et al., 1979; Oviedo-Boyso et al., 2007), when both number and cellular activity of PMNL increase enormously (Targowski, 1983; Paape et al., 2003).

The presence of subclinical mastitis can be indicated by SCC and diagnosed by bacteriological analysis or PCR. Somatic cells consist of many cell types, including leukocytes and epithelial cells. Therefore, SCC is routinely used as a measure of inflammation based on the total number of cells in the milk sample. Bacteriological analysis gives the precise etiology of infection, but is time-consuming and requires experienced personnel. Polymerase chain reaction has been proposed as an alternative to bacteriology as a rapid test (Koskinen et al., 2009) but it is expensive. At present, the diagnosis of bovine mastitis is mostly based on cyto-bacteriological analysis of milk samples

(Vangroenweghe et al., 2002). The International Dairy Federation recommends the use of both SCC and bacteriological analysis as criteria for the determination of udder health (Hogan et al., 1999). The German Veterinary Society (DVG, 2002) suggests a threshold of ≤100,000 cells/mL to define a quarter as normal.

Staphylococcus aureus is a contagious pathogen and one of the most widely distributed causative agents of subclinical mastitis (International Dairy Federation, 2006). Environmental pathogens, mainly *Streptococcus uberis* and coliform bacteria, may enter the mammary gland, typically causing clinical mastitis. In the case of contagious pathogens, the prompt identification of infected animals is crucial to implement measures to avoid spread of the infection. Nevertheless, the detection of *Staph. aureus* can be difficult due to the intermittent shedding of the pathogen in milk (Sears et al., 1990) at levels frequently below the detection limit of the bacteriological method (Zecconi et al., 1997), and to the presence of persistent infections without an increase in SCC. Consequently, cyto-bacteriological analysis has only a partial reliability, particularly when performed on a single sample (Schröder and Hamann, 2005). Identification of chronic mastitis caused by environmental pathogens also plays an important role in herd management, but it is often difficult due to the low numbers of bacteria shed in the milk (Hogan and Smith, 2003).

In healthy milk, the percentage of each cell type is widely variable; according to some authors, macrophages are the predominant cell type (Riollet et al., 2001; Lindmark-Mansson et al., 2006), whereas others have shown that lymphocytes are a major population (Park et al., 1992; Leitner et al., 2000a, Schwarz et al., 2011a, b). Moreover, cell percentages can vary dependening on the milk fraction sampled, because cisternal milk shows lower percentage of PMNL compared with alveolar milk (Sarikaya et al., 2005). Different cell patterns have been documented in the presence of different pathogens and in the course of infection (Leitner et al., 2000b). In addition to the etiological agent, the effect of lactation stage and parity number should be taken into account (Dosogne et al., 2003). In the presence of acute mastitis, PMNL are the predominant cell type, accounting for up to 90% of the total mammary leukocyte population (Sordillo and Streicher, 2002). In contrast, in chronic mastitis caused by *Staph. aureus* or CNS, PMNL percentage can be as low as that in uninfected

quarters, whereas the percentage of macrophages is higher (Leitner et al., 2000b).

The changes in relative cell proportions can be considered a valid tool for the identification of inflammatory processes despite low SCC, thus differentiating healthy milk from that with early or late inflammation (Rivas et al., 2001). Recent studies (Schwarz et al., 2011a, b) have shown that differential cell counting (DCC) can reveal inflammatory processes even in milk with 9,000 cells/mL. Therefore, the goals of the present study were (1) to compare, in the field, cyto-bacteriological results with DCC results, and (2) to identify cytological parameters that could be used to classify mammary quarters as healthy or diseased.

Materials and Methods

Animals and Milk Sampling

Three herds in Lombardy region of Italy were enrolled in the study because they were participating in a voluntary control program for contagious or environmental pathogens. Herd A was characterized by the absence of contagious microorganisms; infections caused by *Staph. aureus* were present in the other 2 herds with lower prevalence in herd B (20%) and higher in herd C (approximately 50%). All the 3 herds housed Holstein-Frisian cows (120 in herd A, 180 in herds B and C) in freestalls, and cows were milked twice daily in a milking parlor.

Overall, 48 cows were randomly selected, of which 23 cows were primiparous, 11 were in the second or third lactation, and 14 had calved 4 or more times. Eleven animals were in early lactation (8 to 86 d), 20 were in mid lactation (96 to 210 d), and 17 in late lactation (247 to 531 d). All cows were free of clinical signs of mastitis at sampling.

After cleaning and disinfection of the teat, the first 2 squirts of milk were discarded, and 10 mL foremilk was aseptically collected from 188 quarters in sterile plastic tubes (Bioster, Italy). Samples were kept under refrigeration until arrival at laboratory facilities.

SCC and Bacteriological Analysis

All samples were submitted to bacteriological analysis, which was performed as previously described (Oliver et al., 2004). Briefly, a 10-µL aliquot of each sample was spread onto blood agar plates (5% bovine blood, Oxoid, Basingstoke, UK) and incubated at 37°C. Plates were evaluated after 24 and 48 h, and colonies of growth were isolated. The large and hemolytic colonies that were catalase- and coagulase-positive were identified as *Staph. aureus* and thereafter confirmed by API ID32 Staph (bioMèrieux, Marcy l'Etoile, France). All the other colonies were identified by biochemical tests following Hogan et al. (1999). Somatic cells were counted on a Bentley Somacount 150 (Bentley Instruments, Chaska MN).

DCC

Slides for DCC were prepared following Dulin et al. (1982), with modified centrifugation conditions. Briefly, for each sample, an aliquot of 4 mL was diluted with 10 mL of PBS with 0.5% EDTA (PBS-EDTA). Samples were then centrifuged at 125 × g for 15 minutes, and cell pellets were resuspended in PBS-EDTA. Cell suspensions were centrifuged on a cytocentrifuge (Shandon Cytospin, Thermo Scientific, Waltham, MA) at 20 × g for 5 min. Slides were air-dried and stained with May Grünwald-Giemsa stain. Each slide was evaluated by light microscopy, and 100 to 200 cells were differentiated into lymphocytes, macrophages, and PMNL, according to standard methods (Coles, 1974; Lee et al., 1980). Epithelial cells could not always be distinguished from macrophages and therefore were counted as macrophages.

Statistical Analysis

For the statistical analysis, the 188 milk samples were clustered into 4 health groups according to Bansal et al. (2005). The first group included quarters considered as normal secreting (**N**), with SCC ≤100,000 cells/mL and no pathogens. The second group included quarters with latent mastitis (**LM**), characterized by SCC ≤100,000 cells/mL and a positive bacteriological culture. The third group was classified as unspecific mastitis (**UM**), including quarters with SCC >100,000 cells/mL and culture-negative results. The fourth group was

considered as affected by mastitis (**M**), including culture-positive quarters with SCC >100,000 cells/mL.

The impact of the 4 health groups on individual cell populations was analyzed by applying linear mixed models and using the SAS program (version 9.1, SAS Institute Inc., Cary, NC). The statistical model (model [1]) was defined as follows:

$$y_{ijklmn} = \mu + herd_i + parity_j + DIM_k + cow_l + group_m + quarter_n + e_{ijklmn} \qquad [1]$$

where y_{ijklmn} = observation for the individual cell population of cow l; μ = overall mean effect; $herd_i$ = fixed effect of the ith herd of *cow l*; $parity_j$ = fixed effect of the jth parity of *cow l*; DIM_k = fixed effect of the kth class of days in milk; cow_l = random effect of *cow l*; $group_m$ = fixed effect of the health group; $quarter_n$ = fixed effect of the position of the udder quarter; and e_{ijklmn} = random residual effect.

For a second analysis, udder quarters were classified into 2 categories: healthy and diseased. Healthy udder quarters were assigned a score of 0 and consisted the group N, whereas diseased udder quarters (group **D**) were assigned a score of 1 and included groups LM, UM, and M. Because the defined disease was treated as a binary trait, a logistic model was applied to assess the effect of individual cell populations on the occurrence of the disease. Analysis of variance was carried out using logistic models as implemented in the SAS Glimmix macro (Wolfinger and O'Connell, 1993). Significance of regression coefficients was determined by using results from sum of square type I tests (Wald-type tests) and F-statistics. The final generalized linear model (model [2]) used to determine the impact fixed effects and covariates on the incidence of the disease was:

$$\text{logit } (\pi_{rstu}) \quad = \log\left[\frac{\pi_{rstu}}{1 - \pi_{rstu}}\right] = \eta_{rst} \qquad [2]$$

$$= \varphi + \gamma_r + \lambda_s + \tau_t + \upsilon_u + \varphi_v + b_1 Y_{rstu}$$

where π_{rst} = probability of occurrence of the disease; φ = overall mean effect; γ_r = fixed effect of parity; λ_s = fixed herd effect; τ_t = fixed effect of the position of the udder quarter; υ_u = fixed effect for classes of days in milk; φ_v = random effect of the cow; Y_{rstu} = value for the individual cell population; and b_1 = linear regression of the disease on the value of the individual cell population.

Results

SCC and Bacteriological Analysis

Foremilk samples were taken from 188 quarters of 48 cows in 3 Italian dairy farms. Overall, 92 samples (48.4%) were classified as diseased. In herd A, out of 70 samples tested, 59 (84.3%) were bacteriologically negative, and only 1 showed a major pathogen, *Escherichia coli*. In addition, CNS were detected in 5 samples, *Enterococcus faecalis* in 4, and *Streptococcus dysgalactiae* in 1 sample. In herd B, 57 out of 78 quarters tested (73.1%) were bacteriologically negative, whereas 13 (16.6%) were positive for *Staph. aureus*. Four samples showed the presence of CNS, and a further 4 samples were considered contaminated. Finally, in herd C, 25 out of 40 quarters tested (62.5%) were bacteriologically negative, whereas 10 (25%) were positive for *Staph. aureus*; CNS were detected in 3 samples, and *Ent. faecalis* in 1 sample.

The clustering of milk samples into the 4 health groups mentioned above, is summarized in Table 1. Following group definitions (Bansal et al., 2005), 96 quarters belonged to group N (normal secretion), and 30 samples were categorized into group M (mastitis). Out of them, 18 samples showed *Staph. aureus* and 6 samples CNS; *Strep. dysgalactiae* was detected in 1 sample, and *Ent. faecalis* in 5 samples. A further 15 samples were classified in group LM (latent mastitis): CNS were detected in 7 samples, *Staph. aureus* in 5 samples, *E. coli* in 1 sample and, contamination in 2 samples. Group UM (unspecific mastitis) included 47 samples.

DCC

The DCC of all quarter foremilk samples analyzed was determined by microscopic differentiation of 100 to 200 cells into lymphocytes, macrophages, and PMNL. In addition to the percentages of individual cell populations, the following variables were considered: phagocytes (**Phag**, combining the percentages of macrophages and PMNL); logarithmic PMNL:lymphocyte ratio (**log PMNL:Lym**), and logarithmic phagocyte:lymphocyte ratio (**log Phag:Lym**), which involved all 3 cell populations.

Table 1. Clustering of milk samples into the 4 health groups according to Bansal et al. (2005), with bacteriological results and SCC

Group[1]	No. of quarters	Bacteriologic result	Bacteria isolated	No. of quarters	SCC, × 1,000 cells/mL	SCC, × 1,000 cells/mL (mean ± SEM)
N	96	No growth				19.62 ± 2.50
LM	15	Positive	CNS	7	1-85	47.86 ± 10.77
			Staphylococcus aureus	5	9-93	56.20 ± 19.16
			Escherichia coli	1	42	42 ± 0
			Contaminated	2	72-83	77.50 ± 5.50
UM	47	No growth				611.67 ± 122.82
M	30	Positive	CNS	6	132-2,783	763.50 ± 412.73
			Staphylococcus aureus	18	122-1,625	585.11 ± 114.06
			Streptococcus dysgalactiae	1	679	679 ± 0
			Enterococcus faecalis	5	350-4,725	1,367.80 ± 840.58

[1]N = normal secretion; LM = latent mastitis; UM = unspecific mastitis; M = mastitis.

Overall (n = 188 samples), the proportion of lymphocytes ranged between 1 and 97%, with a mean of 25% (SD 23.2%), that of macrophages between 0 and 80% with a mean of 19.2% (SD 16.9%), and proportions of PMNL ranged between 0 and 96% with a mean of 55.8% (SD 25.8%).

Table 2. Mean values and standard deviation for the percentage of individual cell populations and combinations of cell populations in farms A, B, and C

Cell population or variable[1]	A		B		C	
	Mean	SD	Mean	SD	Mean	SD
PMNL, %	58.68	23.10	67.03	21.11	31.79	21.05
Lymphocytes, %	28.97	23.56	20.05	22.09	25.56	22.34
Macrophages, %	12.35	9.06	12.92	8.38	42.65	18.06
Phag, %	71.03	23.56	79.95	22.09	74.44	22.34
Log PMNL:Lym	0.40	0.63	0.73	0.65	0.22	0.65
Log Phag:Lym	0.50	0.61	0.81	0.62	0.59	0.52

[1]Phag = phagocytes (macrophages and PMNL); log PMNL:Lym = logarithmic PMNL:lymphocyte ratio; log Phag:Lym = logarithmic phagocyte:lymphocyte ratio.

Mean values and standard deviation for each variable considered in each farm are summarized in Table 2. Mean percentages of PMNL were 58.7, 67.0, and 31.8% in herds A, B, and C, respectively. Mean percentages of lymphocytes showed similar values in the 3 herds (29% in A, 20% in B, and 25.6% in C). Mean percentages of macrophages were almost identical in herds A and B (12.3 and 12.9%, respectively), whereas herd C showed a much higher mean value (42.6%). Mean percentages of phagocytes were similar in all herds (71% in A, 79.9% in B, and 74.4% in C), whereas the mean values of both log PMNL:Lym and log Phag:Lym were higher in herd B compared with those in herds A and C (0.40 and 0.50 in herd A, 0.73 and 0.81 in herd B, 0.22 and 0.59 in herd C, respectively).

The variance analysis indicated that percentages of all individual cell populations were significantly ($P < 0.01$) influenced by farm, and macrophages were further significantly ($P < 0.01$) influenced by DIM. None of the individual cell populations was influenced by quarter position or lactation number (Table 3). The variables Phag, log Phag:Lym, and log PMNL:Lym were

significantly ($P < 0.01$) influenced by the farm but but not by quarter position, lactation number, or DIM (Table 2). Overall, a significant ($P < 0.0001$) effect of the 4 health groups was demonstrated on percentages of lymphocytes and PMNL, but not on macrophages (Table 3).
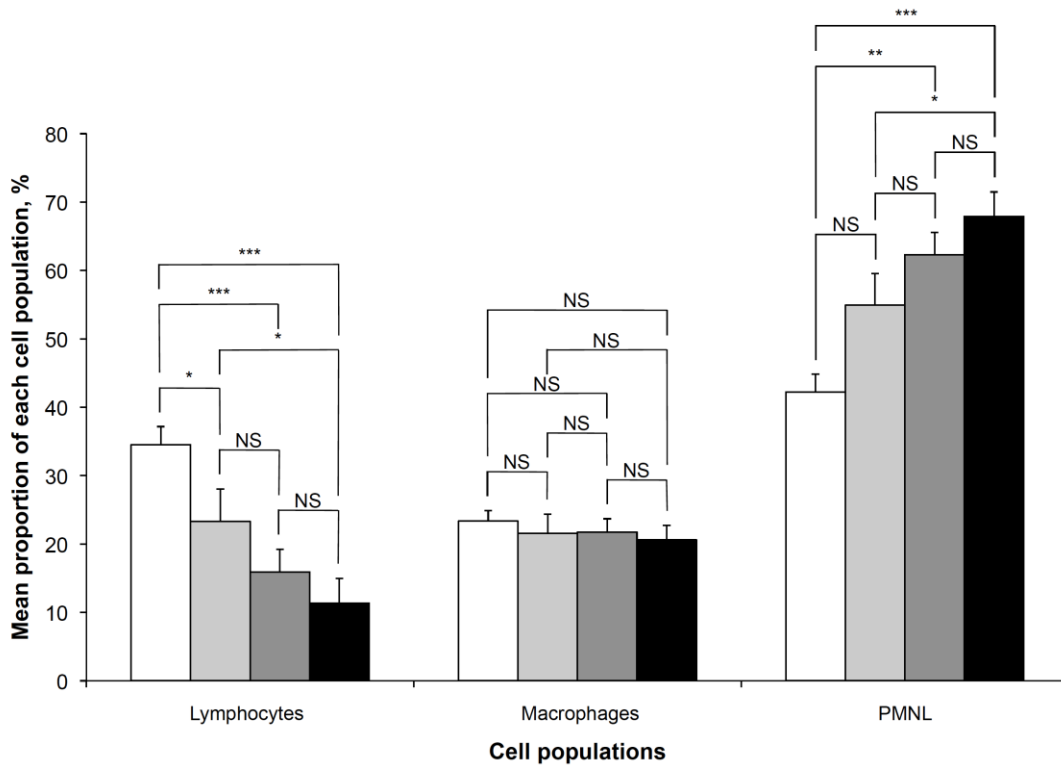

Figure 1. Comparison of mean percentages of lymphocytes, macrophages, and PMNL in milk of udder quarters with different health status. All 188 udder quarters analyzed were classified into groups N, LM, UM, and M (group N = empty bars, SCC ≤100,000 cells/mL and culture-negative, n = 96; group LM = light gray bars, SCC ≤100,000 cells/mL and culture-positive, n = 15; group UM = dark gray bars, SCC >100,000 cells/mL and culture-negative, n = 47; group M = black bars, SCC >100,000 cells/mL and culture-positive, n = 30). N = normal secretion; LM = latent mastitis; UM = unspecific mastitis; M = mastitis. Data are expressed as mean ± SEM for percentages of the individual cell populations in the 4 SCC groups defined; *** P < 0.001; ** P < 0.01; * P < 0.05; NS = P > 0.05.

The mean percentage of lymphocytes in group N (34.5%) was significantly ($P < 0.05$) higher than in groups LM, UM, and M (23.3%, 15.9%, and 11.3%, respectively; Figure 1), and the value in group LM was significantly ($P < 0.05$) higher than that in group M. Mean percentage of PMNL was significantly ($P < 0.01$) lower in group N (42.2%) than in groups UM and M (62.3% and 67.9%, respectively; Figure 1); in addition, group LM (54.9%) value

was significantly ($P < 0.05$) lower than that in group M. Finally, mean percentages of macrophages were not significantly related to the 4 groups (N: 23.3%; LM: 21.5%; UM: 21.7%; M: 20.6%; Figure 1).

All variables combining different cell populations were significantly ($P < 0.001$) influenced by health group (Table 3). The mean value of Phag was significantly lower ($P < 0.05$) in group N (65.5) than in groups LM, UM, and M (76.7, 84.1, and 88.6, respectively; Figure 2). In addition, the value in group LM was significantly ($P < 0.05$) lower than that in group M.
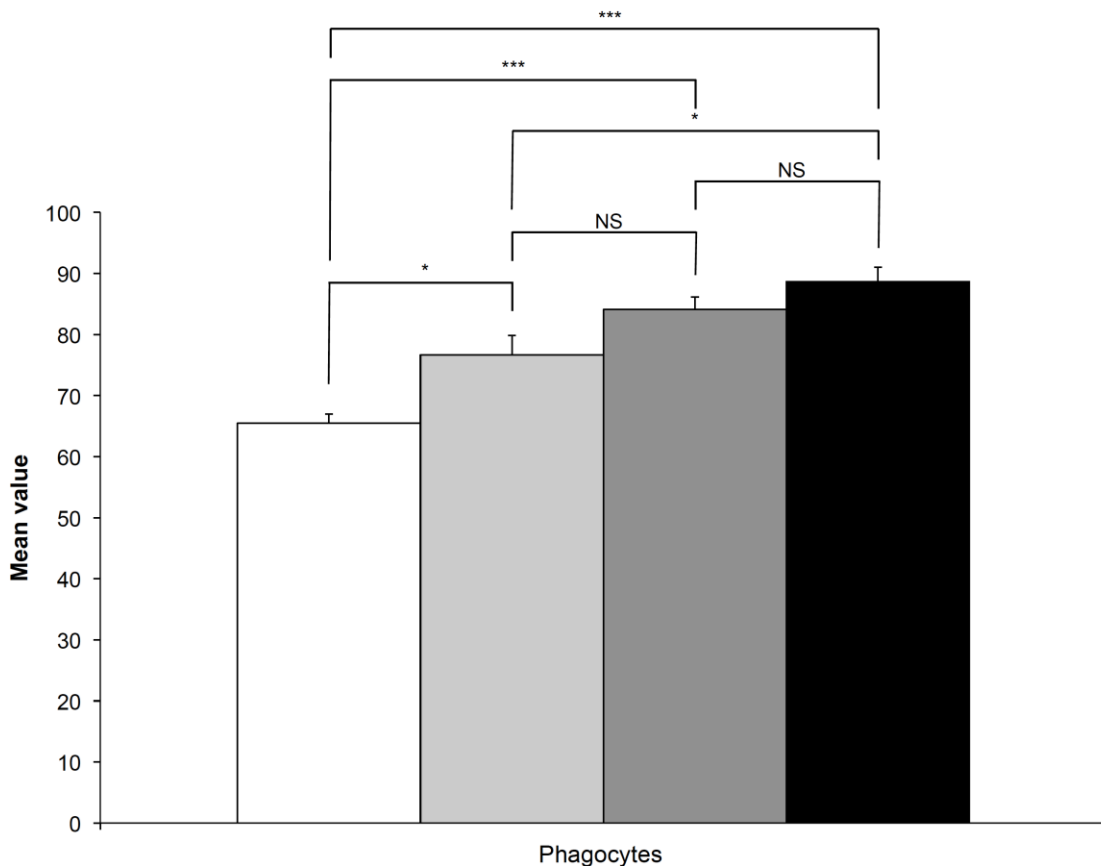
Figure 2. Comparison of mean values of phagocytes (macrophages and PMNL) in milk of udder quarters with different health status. All 188 udder quarters analyzed were classified into groups N, LM, UM, and M (group N = empty bars, SCC ≤100,000 cells/mL and culture-negative, n = 96; group LM = light gray bars, SCC ≤100,000 cells/mL and culture-positive, n = 15; group UM = dark gray bars, SCC >100,000 cells/mL and culture-negative, n = 47; group M = black bars, SCC >100,000 cells/mL and culture-positive, n = 30). N = normal secretion; LM = latent mastitis; UM = unspecific mastitis; M = mastitis. Data are expressed as mean ± SEM; *** $P < 0.001$; * $P < 0.05$; NS = $P > 0.05$.

Table 3. Results of variance analysis for the percentage of individual cell populations as well as combinations of cell populations in 188 quarter foremilk samples analyzed by light microscopy[1]

Cell population or variable[2]	Group	Quarter position	Lactation number	DIM	Farm
PMNL	0.0001	0.07	0.16	0.27	0.0001
Lymphocytes	0.0001	0.22	0.09	0.07	0.005
Macrophages	0.66	0.52	0.75	0.009	0.0001
Phag	0.0001	0.22	0.09	0.07	0.005
Log PMNL:Lym	0.0001	0.08	0.13	0.52	0.0001
Log Phag:Lym	0.0001	0.09	0.11	0.36	0.0007

[1]Analyzed factors were group (normal secretion, latent mastitis, unspecific mastitis, mastitis), quarter positions (front right, rear right, front left, and rear left), lactation number (1, 2&3, ≥4), days in milk (8-86 d, 96-210 d, 247-531 d), and farm (A to C).
[2]Phag = phagocytes (macrophages and PMNL); log PMNL:Lym = logarithmic PMNL:lymphocyte ratio; log Phag:Lym = logarithmic phagocyte:lymphocyte ratio.

The log PMNL:Lym mean value in group N (0.11) was significantly ($P < 0.001$) lower than in groups LM, UM, and M (0.57, 0.73, and 0.94, respectively; Figure 3), and the value in group LM was significantly ($P < 0.05$) lower than that in group M (Figure 3).

Finally, log Phag:Lym showed a significantly ($P < 0.01$) lower value in group N (0.35) than in groups LM, UM, and M (0.73, 0.91, and 1.06, respectively; Figure 4). Group M also demonstrated a significantly ($P < 0.05$) higher mean value than group LM (Figure 4).

The possibility of differentiating between healthy and diseased udder quarters was further evaluated using individual cell populations as well as the variables that combined different cell populations. For this purpose, quarters were split into 2 groups, and F-values were calculated. The first group (N) included all healthy quarters (n = 96), whereas the second groups (D, n = 92) included diseased mammary glands (groups LM, UM, and M). Out of the 3 individual cell populations, lymphocytes were the best one to differentiate between groups N and D (Table 4). Although macrophages showed an F-value of 1.65 and did not differ significantly between group N and D, the F-value of PMNL (15.54) was higher and significantly ($P < 0.001$) different (Table 4). Nevertheless, lymphocytes showed the highest F-value (32.64) and their percentage differed significantly between group N and D ($P < 0.0001$).
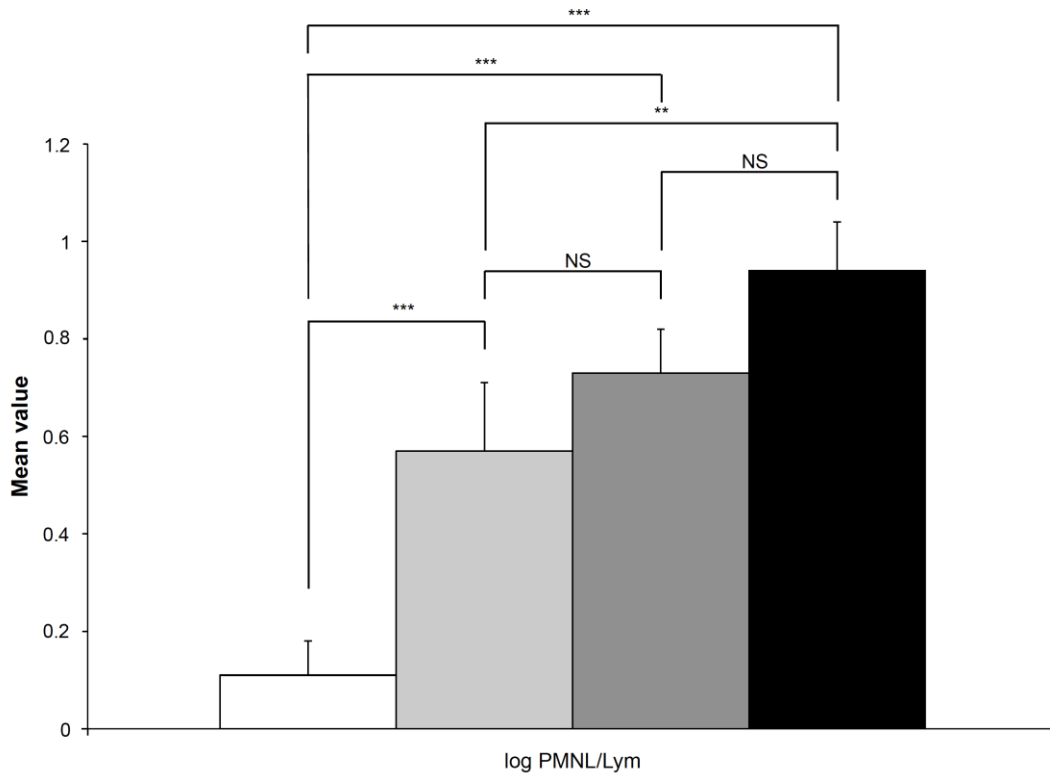
Figure 3. Comparison of mean values of the variable log PMNL/Lym (logarithmic PMNL:lymphocyte ratio) in milk of udder quarters with different health status. All 188 udder quarters analyzed were classified into groups N, LM, UM, and M (group N = empty bars, SCC ≤100,000 cells/mL and culture-negative, n = 96; group LM = light gray bars, SCC ≤100,000 cells/mL and culture-positive, n = 15; group UM = dark gray bars, SCC >100,000 cells/mL and culture-negative, n = 47; group M = black bars, SCC >100,000 cells/mL and culture-positive, n = 30). N = normal secretion; LM = latent mastitis; UM = unspecific mastitis; M = mastitis. Data are expressed as mean ± SEM; *** $P < 0.001$; ** $P < 0.01$; NS = $P > 0.05$.

Table 4. Fisher (*F*)-values and associated probabilities of different markers to discriminate between healthy (group N; n = 96) and diseased (group D, including groups LM, UM, M; n = 92) udder quarters[1]

Marker[2]	*F*-value	*P*-value
PMNL, %	15.54	0.0001
Lymphocytes, %	32.64	<0.0001
Macrophages, %	1.65	0.2007
Phag, %	32.64	<0.0001
Log PMNL:Lym	48.23	<0.0001
Log Phag:Lym	45.90	<0.0001

[1]N = normal secretion; LM = latent mastitis; UM = unspecific mastitis; M = mastitis.

[2]Phag = phagocytes (macrophages and PMNL); log PMNL:Lym = logarithmic PMNL:lymphocyte ratio; log Phag:Lym = logarithmic phagocyte:lymphocyte ratio.
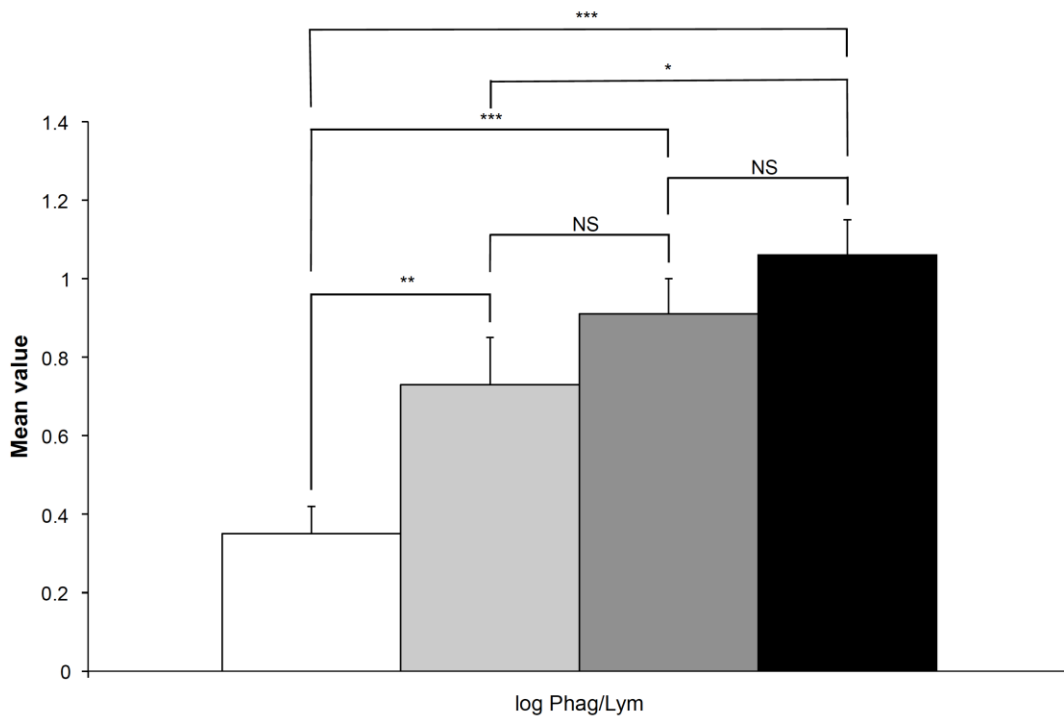
Figure 4. Comparison of mean values of the variable log Phag/Lym (logarithmic phagocyte:lymphocyte ratio) in milk of udder quarters with different health status. All 188 udder quarters analyzed were classified into groups N, LM, UM, and M (group N = empty bars, SCC ≤100,000 cells/mL and culture-negative, n = 96; group LM = light gray bars, SCC ≤100,000 cells/mL and culture-positive, n = 15; group UM = dark gray bars, SCC >100,000 cells/mL and culture-negative, n = 47; group M = black bars, SCC >100,000 cells/mL and culture-positive, n = 30). N = normal secretion; LM = latent mastitis; UM = unspecific mastitis; M = mastitis. Data are expressed as mean ± SEM; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS = $P > 0.05$.

All the cell combinations allowed us to differentiate significantly ($P < 0.001$) between groups N and D. Nevertheless, the highest F-value was shown by log PMNL:Lym (48.23), whereas Phag and log Phag:Lym showed F-values of 32.64 and 45.90, respectively (Table 4).

Discussion

The diagnosis of IMI is mostly based on SCC and bacteriological analysis. Milk samples with SCC < 100,000 cells/mL are currently considered healthy or within normal physiological limits, but inflammatory reactions can be detected in such samples (Schwarz et al, 2011a, b). Indeed, SCC is low in the initial stage of inflammatory reaction, until the invading pathogen is recognized

by immune and epithelial cells that release chemoattractants, thus stimulating migration of PMNL (Paape et al., 2002; Oviedo-Boyso et al., 2007, Koess and Hamann, 2008). Differential cell count could be a useful method, because it identifies changes in the relative cell populations before the increase in total cell number occurs in the course of the inflammatory process. Therefore, DCC could be regarded as the standard technique to determine the presence or absence of inflammation in mammary quarters (Rivas et al., 2001).

The aim of the present study was to detect one or more parameters that could easily identify diseased mammary quarters independently of the prevalent pathogen in the herd using light microscopy DCC. Differential cell count can be performed with either flow cytometry or light microscopy. Cytometric analysis is a very accurate method, but expensive and time-consuming, because it is based on the use of several marker antibodies. Leitner et al. (2000b) reported a high correlation between flow cytometry and light microscopy for PMNL and lymphocytes, but a lower correlation for macrophages and epithelial cells. This lower correlation could be due to the difficult differentiation between macrophages and epithelial cells by light microscopy. Epithelial cells are not always present in the milk (Lee et al., 1980) or they make up only 1 to 3% of cells (Sarikaya et al., 2004, Schwarz et al., 2011a). Different results were reported by Miller et al. (1991) and Leitner et al. (2000b), who recorded higher values (10 to 19% or 44%, respectively). Miller et al. (1991) sampled only primiparous cows in early lactation, whereas Leitner et al. (2000b) evaluated epithelial cell percentages using a nonspecific identification procedure. However, according to Schwarz et al. (2011a), percentages >10% should be critically discussed. Therefore, the potential misclassification of epithelial cells likely represents a minor error that probably does not affect the result. For these reasons, light microscopy DCC is a suitable method applicable to routine analysis. It is cost effective and could be automatized by using a slide scanner and computer imaging software.

Three dairy herds were selected with different prevalences of IMI, which were caused by different etiological agents. The influence of breed was not explored because all 3 herds housed Holstein-Friesian cows. The causative agents of mastitis were environmental pathogens in herd A but contagious bacteria in the other 2 herds. Moreover, *Staph. aureus* isolates from herds B

and C demonstrated low and high diffusiveness, respectively. The results of DCC showed statistically significant differences among the 3 dairy herds considered, as expected and in accordance with previous reports (Schwarz et al., 2011b). The DCC results in herds A and B showed relative higher values of PMNL followed by lymphocytes and macrophages, whereas macrophages were the main population in herd C, followed by PMNL and lymphocytes. These data suggest that most infections in herds A and B were acute infections, with high increases of PMNL, whereas herd C probably had a considerable prevalence of chronic infections, which led to an increase of macrophage percentage, as also shown by Sladek and Rysanek (2009).

No correlation was found between cell populations and either quarter position or lactation number, in accordance with findings described by Schwarz et al. (2011a, b). Dosogne et al. (2003) reported the effect of DIM on DCC in the milk, showing that lymphocytes decreased while PMNL and macrophages increased in the course of lactation. In contrast, our data indicated that only macrophages were influenced by DIM. This discrepancy could be related to the different methods used, because the analyses of Dosogne et al. (2003) were performed by flow cytometer. Despite this, the results of the present study could not bias quarter classification by DCC, because macrophages were the only population not significantly influenced by health group. Even though 2 recent publications (Schwarz et al., 2011a, b) indicated that inflammatory profiles can be found in quarters with SCC <100,000 cells/mL, we decided to follow current recommendations of DVG (2002) to enable comparison between cyto-bacteriological and DCC results. In fact, Harmon (1994) showed that losses in production occur starting from 100,000 cells/mL, and Pyörälä (2003) stated that milk components differ significantly from the physiological norm above this level. Interestingly, 15 milk samples in group N showed marked inflammatory profiles, with PMNL percentages >80%; in particular, one quarter showed an SCC value of 1,000 cells/mL and 91.3% PMNL. Many factors could explain the high percentage of PMNL in these quarters, including chemical and mechanical factors or physical injury. Furthermore, any of these quarters could show false negative results on bacteriological analysis, for different reasons. Negative bacteriological results could depend on intermittent shedding of pathogens or shedding in amounts lower than the detection limit of the method applied or on

the presence of antimicrobials in milk (Sears et al., 1990; Zecconi et al., 1997). It is also possible that the inflammatory response was so effective in those quarters that most bacteria were phagocytosed and killed, or survived only intracellularly by active invasion of cells or survival inside phagosomes (Newbould and Neave, 1965; Hill et al., 1978). Furthermore, in the UM quarters, inflammatory processes were present, even in the absence of bacteria, as indicated by increased SCC and high PMNL percentages. Because cellular profiles were similar in groups UM and M, it could be hypothesized that some UM samples were false negatives.

Of the 3 cell populations, PMNL are known to strongly increase during the course of infection and have been consistently reported as the dominant cell population in mastitic milk (Kehrli and Shuster, 1994; Sordillo et al., 1997; Pillai et al., 2001). Therefore, PMNL would be an obvious choice to differentiate between healthy and infected quarters with low SCC. Accordingly, PMNL were statistically lower in group N than in groups UM and M, but no difference was demonstrated between groups N and LM. The macrophage percentage was very similar in the 4 groups, because macrophages are associated with the late phase of infection (Leitner et al., 2000b; Sladek and Rysanek, 2009) and are expected to increase in chronic infections, which were presumably at low levels in the cows of this study. Lymphocytes were the only individual cell population showing statistically different percentages between the healthy group and all diseased groups. Mammary lymphocytes play an important role in the initiation of immune response of the gland. They are mainly T cells, whose function is to remove old and damaged secretory cells, thus decreasing the susceptibility of the mammary gland to infections (Sordillo et al., 1997).

Combinations of cell populations were evaluated to increase the discrimination power of DCC. Indeed, combining populations increased the F-values, indicating that a larger percentage of quarters would be correctly classified when that parameter was considered. Combining PMNL and macrophages into phagocytes (Phag) increased F-values from 1.65 and 15.54, respectively, to 32.64. Combining PMNL and lymphocytes into the log PMNL:Lym ratio increased F-values from 15.54 and 32.64 to 48.23, and the combination of Phag and Lym into the log Phag:Lym ratio, which includes all 3 cell populations, led to an F-value of 45.90. All combinations of individual cell

populations showed statistically significant differences between groups N and D, but the best *F*-value was related to ratio log PMNL:Lym, presumably because log PMNL:Lym was the only variable that involved both cell populations statistically influenced by health groups but excluded macrophages.

Conclusions

Differential cell counting can detect changes in the relative cell populations in milk without an increase in total cell numbers, thus identifying inflammatory processes in quarters otherwise considered healthy. This information could be particularly useful when control programs for contagious milk pathogens are being applied. In the present study, we considered mammary quarters with or without natural occurring infections to established single or combined variables capable to identify healthy and inflamed quarters. Microscopic DCC was shown to be a potentially useful tool to identify cows affected by any inflammatory process of the mammary gland, with the best results being achieved using log PMNL:Lym as variable. Further studies are needed to determine and validate the cutoff values to be applied in the dairy herd.

Acknowledgements

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6th Chapter

Differential cell count as a new method to diagnose dairy cow mastitis

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Abstract

Changes in relative cell proportions occurring in diseased mammary glands of dairy cows can be determined using diffrential cell count (DCC). The present study was carried out in 2 consecutive trials, with 2 goals: (a) to test the consistency of DCC results on subsequent days, and (b) to establish an effective cutoff value for the diagnosis of mastitis. In the first trial, quarter milk and blood samples were taken from 8 healthy cows for 5 consecutive days. Milk samples were tested by somatic cell count (SCC) and bacteriological analysis, and DCC was performed on blood and milk samples by flow cytometer. In the second trial, 16 animals were randomly selected from a different herd and quarter milk samples taken on 3 consecutive milkings. All samples were cyto-bacteriologically analyzed and DCC was performed on the second sampling. In the first trial, mean SCC was 77,770 cells/mL and 4 samples were bacteriologically positive. No fixed or random effect had a significant influence on percentages of individual cell populations or ratios in blood or in milk. A cutoff value of 0.495 for logarithmic polymorphonuclear neutrophilic leukocytes (PMNL):lymphocyte was established. Mean SCC of milk samples collected in the second trial was 543,230 cells/mL and infection was detected in 53.1% of quarters, mostly caused by *Staphylococcus aureus*. When the cutoff value was applied to the data along with SCC, sensitivity and specificity of the diagnostic method were 97.3% and 92.3%, respectively.

Introduction

Subclinical mastitis is a major health problem in dairy cattle. Economic losses are mostly associated with decreased production and milk quality. Such infections are not evident and can persist in the mammary tissue throughout lactation. *Staphylococcus aureus* is a contagious pathogen and a major agent of subclinical mastitis (IDF, 2006), but the infection can also be caused by a wide range of environmental and opportunistic pathogens (Bradley, 2002).

Subclinical mastitis can be diagnosed by SCC, bacteriological analysis, or PCR. The International Dairy Federation recommends the use of both SCC and bacteriological analysis as criteria for the determination of udder health (Hogan

et al., 1999). Accordingly, the diagnosis of bovine mastitis is mostly based on cyto-bacteriological analysis of milk samples (Vangroenweghe et al., 2002). Despite that, identification of infected quarters presents difficulties related to the possibility of false negative bacteriological results and infections without a concomitant increase of SCC (Schwarz et al., 2010).

Differential cell count (DCC) shows changes in relative cell proportions, which can be used to differentiate healthy glands from inflamed or infected glands, and DCC has been proposed as a valid tool for the identification of inflammatory processes in cases with low SCC (Rivas et al., 2001). Recent studies (Schwarz et al., 2011a, b; Pilla et al., 2012) have shown that DCC can reveal inflammatory processes, even in milk with SCC of 1,000 cells/mL, well below the current threshold of 100,000 cells/mL (DVG, 2002).

Differential cell count can be performed using different methods. Microscopic DCC is a simple and cost-effective method, but most researchers prefer cytometric analysis because of its higher accuracy. Leitner et al. (2000b) found a high correlation between the 2 methods for PMNL and lymphocytes, but a lower correlation for macrophages and epithelial cells, probably because of the difficulty in differentiating between these cell populations with light microscopy. Different cell patterns have been documented during the course of infection in the presence of different pathogens (Leitner et al., 2000b). In acute mastitis, PMNL are the predominant cell type, often accounting for more than 90% of the total mammary leukocyte population (Sordillo and Streicher, 2002). In contrast, in chronic mastitis caused by *Staph. aureus* and CNS, PMNL percentages can vary from the high values seen in acute mastitis to percentages as low as those recorded in uninfected quarters (Leitner et al., 2000b; Riollet et al., 2001; Leitner et al., 2003). Also, the effect of lactation stage and parity number should be taken into account. In early lactation, lymphocytes and monocytes were reported to be higher than in mid and late lactation, while macrophages and PMNL percentages were considerable lower (Dosogne et al., 2003).

Lymphocytes, macrophages, and PMNL play an important role in the immunity of the mammary gland (Paape et al., 1979; Sordillo and Nickerson, 1988). A successful defense against invading pathogens depends on number and distribution of leukocytes (Leitner et al., 2003). In healthy milk, the

percentage of each cell type is widely variable; according to some authors, macrophages are the predominant cell type (Riollet et al., 2001; Lindmark-Mansson et al., 2006), whereas others have shown that lymphocytes are the major population (Park et al., 1992; Leitner et al., 2000a; Schwarz et al., 2011a, b). Leitner et al. (2000a) demonstrated a high repeatability for samples taken from the same cow in different stages of lactation and suggested that the leukocyte pattern in uninfected mammary glands is genetically controlled. To the best of our knowledge, however, no information on short-term repeatability is available. Because the immune system is dynamic and the mammary gland is subjected to persistent stress during lactation, a basic knowledge of the cellular profile in healthy glands is fundamental. Therefore, the goals of the present study were (a) to investigate DCC in milk from healthy mammary quarters and to test whether the results are consistent on subsequent days; and (b) to establish an effective cutoff value for the diagnosis of mastitis that is applicable under field conditions. The study was carried out in 2 consecutive trials, the first to determine DCC stability and cutoff and the second to test this cutoff value under field conditions.

Materials and Methods

Animals and Milk Sampling

Trial 1. To investigate DCC in healthy quarters and its test-retest reliability, the herd enrolled in the first trial was located in Lombardy region of Italy and was certified free of paratuberculosis, bovine viral diarrhoea, and infectious bovine rhinotracheitis; it also had no history of contagious mastitis pathogens in the last 10 yr. The herd consisted of 50 lactating Holstein-Friesian dairy cows housed in freestalls and milked twice daily in a milking parlor.

Eight cows were selected based on low SCC and 2 negative results of bacteriological analysis in the week before samplings. Of these, 3 cows were primiparous, 4 were in the second or third lactation, and 1 had calved 4 times. Two animals were in early lactation (83 to 111 DIM), 3 were in midlactation (144 to 172 DIM), and 3 in late lactation (233 to 357 DIM).

Blood and quarter milk were sampled for 5 consecutive days at morning milking. All cows were free of clinical signs of mastitis at sampling. After

cleaning and disinfection of the teat, the first squirts of milk were discarded, and 250 mL of milk was aseptically collected from each quarter into sterile plastic tubes (Falcon, BD Biosciences, Franklin Lakes, NJ) for both bacteriological and DCC analysis. Blood samples (10 mL) were collected by tail venipuncture into commercial EDTA-containing evacuated tubes (Vacutainer, BD Biosciences, San Jose, CA). Samples were refrigerated until arrival at laboratory facilities.

Trial 2. The calculated cutoff value was tested under field conditions in another herd located in Lombardy that was participating in a voluntary control program for contagious mastitis. The herd consisted of 180 lactating Holstein-Friesian dairy cows that were housed in freestalls and milked twice daily in a milking parlor. The herd had a history of high prevalence of *Staph. aureus* (approximately 50% prevalence at the beginning of the control program), and mammary infections caused by *Prothoteca zopfii* had recently been detected.

In total, 16 cows were randomly selected from the last milking group, which included animals previously diagnosed as infected by *Staph. aureus* or *P. zopfii* and other animals before culling. Of these, 9 cows were primiparous and 7 multiparous.

Quarter milk samples for bacteriological analysis were collected at 3 consecutive milkings. After cleaning and disinfection of the teat, the first 2 squirts of milk were discarded, and 10 mL of foremilk was aseptically collected in sterile plastic tubes (Bioster, Seriate, Italy). At the second milking, an additional 200 mL of quarter milk was sampled for DCC analysis. Samples were refrigerated until arrival at laboratory facilities.

SCC and Bacteriological Analysis

All samples were submitted to bacteriological analysis, which was performed as previously described (Oliver et al., 2004). Briefly, an aliquot of 10 µL of each sample was spread onto blood-agar plates (5% bovine blood, Oxoid, Basingstoke, UK) and plates were incubated at $37°C$. Plates were evaluated after 24 and 48 h, and colonies of growth were isolated. All colonies were identified by biochemical tests following Hogan et al. (1999). Somatic cells were counted on a Bentley Somacount 150 (Bentley Instruments, Chaska, MN).

DCC

Differential cell counts were performed on blood samples and on quarter milk samples by cytometry. Milk cells were isolated according to the protocol described by Koess and Hamann (2008), with modified centrifugation conditions. Briefly, 200 mL of milk was centrifuged for 30 min at 250 × *g* and 4°C. The cream layer and supernatant were discarded and the cell pellet was washed twice in 30 mL of PBS. Blood erythrocytes were lysed with Cell Lysis Solution (#A7933, Promega, Madison, WI) and leukocytes were collected by centrifugation at 500 × *g* for 10 min at room temperature.

Cell pellets were resuspended in 500 µL of RPMI 1640 with 10% fetal calf serum, and cells were counted in a haemocytometer; finally, the cell concentration was adjusted to 2×10^6 cells/mL. Aliquots of 100 µL of each sample were incubated with antibodies conjugated to fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE; Table 1) for 30 min at 4°C. Cells were then washed in PBS once and resuspended in PBS with 2% formalin. Fixed cells were kept at 4°C for 3 to 18 h and analyzed using a FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson, San Jose, CA). Eight thousand events were acquired per sample, and data were further analyzed using Cyflogic v. 1.2.1 free software (CyFlo Ltd., Turku, Finland).

Table 1. Antibodies used for cytometric analysis (all purchased from Ab Serotec, Oxford, UK)

CD molecule[1]	Antibody type	Specificity	Antibody clone
CD11b-FITC	Mouse IgG2b	Granulocytes	CC126
CD14-PE	Mouse IgG2a	Monocytes	TÜK4
CD21-PE	Mouse IgG1	B lymphocytes	CC21
CD5-FITC	Mouse IgG1	T lymphocytes	CC17

[1]FITC = fluorescein isothiocyanate; PE = R-phycoerythrin.

Percentages of PMNL, lymphocytes, and macrophages were calculated. In addition, to increase discrimination power of DCC, the ratios logarithmic PMNL:lymphocytes ratio (**log PMNL:Lym**) and logarithmic phagocyte:lymphocyte ratio (**log Phag:Lym**) were calculated as previously described (Pilla et al., 2012).

Statistical Analysis

Trial 1. Linear mixed models were applied on the data from the first trial to analyze the impact of fixed effects, random effects, and covariates on DCC in milk and blood. The statistical model was defined as follows:

$$y_{ijklm} = \mu + parity_i + DIM_j + quarter_k + cow_l + \alpha_1 day + DIM \times \alpha_1 day_{ijkl} + e_{ijklm}$$

where y_{ijklm} = DCC of cow *l*; μ = overall mean effect; *parity* = fixed effect of parity *i* (first parity or higher lactation no.); *DIM* = fixed effect for classes of days in milk *j* (early, mid, or late); *quarter* = fixed effect of udder quarter *k*; *cow* = random effect of cow *l*; *day* = consecutive no. of measurement within cow from d 1 to 5 (sampling number); α_1 = linear regression of sampling no. on DCC; *DIM* $\times \alpha_1 day_{ijkl}$ = interaction between DIM and sampling number, and e_{ijklm} = random residual effect belonging to observation y_{ijklm}.

For blood samples, the effect of udder quarter was excluded from the statistical model. Least square means for the covariate "day" stratified by DIM were generated by using the "at – statement" for sequenced data as implemented in the Proc Mixed of SAS (SAS Institute Inc., Cary, NC).

The cutoff value between healthy and diseased cows for log PMNL:Lym in milk was determined considering quarters samples with SCC $<10^5$ cells/mL and negative bacteriological results as healthy, and attributing them a score of 0. All other samples were considered diseased, and attributed a score of 1. The log PMNL:Lym values and attributed scores were then tested with receiver operating characteristic analysis using SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL), and a cutoff value was chosen to maximize sensitivity and specificity.

Trial 2. Quarter milk samples were classified as diseased or healthy as described in the previous section. Bacteriological analysis and SCC were considered the gold standard test. The cutoff was then applied to calculated log PMNL:Lym. All quarters with values below the cutoff were considered healthy, and those with values above the cutoff were considered diseased. A score of 0 was attributed to healthy samples and a score of 1 to the others. Sensitivity and specificity of the method were then calculated under field conditions.

Results

SCC and Bacteriological Analysis

Trial 1. Mean SCC of the 159 quarter milk samples considered was 77,770 cells/mL (SD 185,510). Thirty-four samples had SCC >100,000 cells/mL, with a maximum value of 1,512,000 cells/mL. Only 4 samples were bacteriologically positive: CNS were detected in 3 samples, and *Streptococcus* ssp. in 1 sample. Data from positive quarters were excluded from the statistical analysis but included for the cutoff determination.

Trial 2. Mean SCC of 192 milk samples considered was 543,230 cells/mL (SD 816,730). One hundred and two samples (53.1%) were bacteriologically positive: *Staph. aureus* was isolated from 71 samples, *Prototheca* ssp. was detected in 12 samples, CNS in 11 samples, *Enterococcus faecalis* in 7, and *Serratia* sp. in 1 sample. Twenty-seven quarters were considered healthy. Thirty-five quarters were considered diseased based on isolation of *Staph. aureus* or at least 10^3 cfu/mL of *Prototheca* in one or more samples, or on the detection of other pathogens in all 3 samples. Two quarters were considered diseased because mean SCC was >100,000 cells/mL.

Table 2. Mean values and standard deviations for individual cell populations as well as combinations of cell populations in milk.

Cell population or ratio[1]	Mean	Standard deviation
PMNL (%)	43.1	23.5
Lymphocytes (%)	30.1	19.4
Macrophages (%)	26.9	15.7
log PMNL:Lym	0.22	0.62
log Phag:Lym	0.48	0.53

[1]log PMNL:Lym = logarithmic PMNL:lymphocyte ratio; log Phag:Lym = logarithmic Phagocyte:lymphocyte ratio.

DCC

Trial 1. Overall (n = 155 samples), mean proportion of PMNL was 43.1% (SD 23.5%), that of lymphocytes was 30.1% (SD 19.4%), and that of macrophages was 26.9% (SD 15.7%). The ratios log PMNL:Lym and log Phag:Lym had mean values of 0.22 (SD 0.62) and 0.48 (SD 0.53), respectively (Table 2). Results of variance analysis of milk data are summarized in Table 3. Sampling day showed no significant effect on percentages of individual cell

populations (*P*-values from 0.68 to 0.99). Associating individual cell populations into ratios produced similar results, with *P*-values of 0.89 and 0.81 for log PMNL:Lym or log Phag:Lym, respectively. Even though some differences related to lactation stage were found for all variables, *P*-values ranged from 0.73 to 0.89. Finally, neither parity nor quarter position had a significant influence on percentages of cell populations or ratios. Variance analysis of blood data showed similar results (Table 4): sampling day, parity, and lactation stage had no significant effect on the percentages of individual cell populations or ratios.

Table 3. Probability values for testing significance of fixed effects of quarter position, parity, lactation stage, and the linear regression of sampling day on the percentage of individual cell populations and combinations of cell populations in milk[1]

Cell population or variable[2]	Quarter position	Parity	Lactation stage	Sampling day
PMNL	0.5349	0.8963	0.7550	0.7847
Lymphocytes	0.2268	0.2322	0.7863	0.9797
Macrophages	0.6276	0.2365	0.8891	0.6805
log PMNL:Lym	0.5645	0.5665	0.7319	0.8894
log Phag:Lym	0.6463	0.2980	0.7940	0.8127

[1]Analyzed factors were quarter position (front right, rear right, front left, and rear left), parity (1 or 2, and 3 or 4), lactation stage (early, mid, or late lactation), and sampling day (d 1 to 5).
[2]log PMNL:Lym = logarithmic PMNL:lymphocyte ratio; log Phag:Lym = logarithmic Phagocyte:lymphocyte ratio.

Table 4. Probability values for testing significance of fixed effects of parity, lactation stage, and the linear regression of sampling day on the percentage of individual cell populations as well as combinations of cell populations in blood[1]

Cell population or variable[2]	Parity	Lactation stage	Sampling day
PMNL	0.2232	0.8664	0.1931
Lymphocytes	0.1313	0.6460	0.1302
Macrophages	0.1088	0.0712	0.3317
log PMNL:Lym	0.1898	0.6354	0.0870
log Phag:Lym	0.1577	0.5415	0.0849

[1]Analyzed factors were parity (1 or 2, and 3 or 4), lactation stage (early, mid, or late lactation), and sampling day (d 1 to 5).
[2]log PMNL:Lym = logarithmic PMNL:Lymphocyte ratio; log Phag:Lym = logarithmic Phagocyte:Lymphocyte ratio.
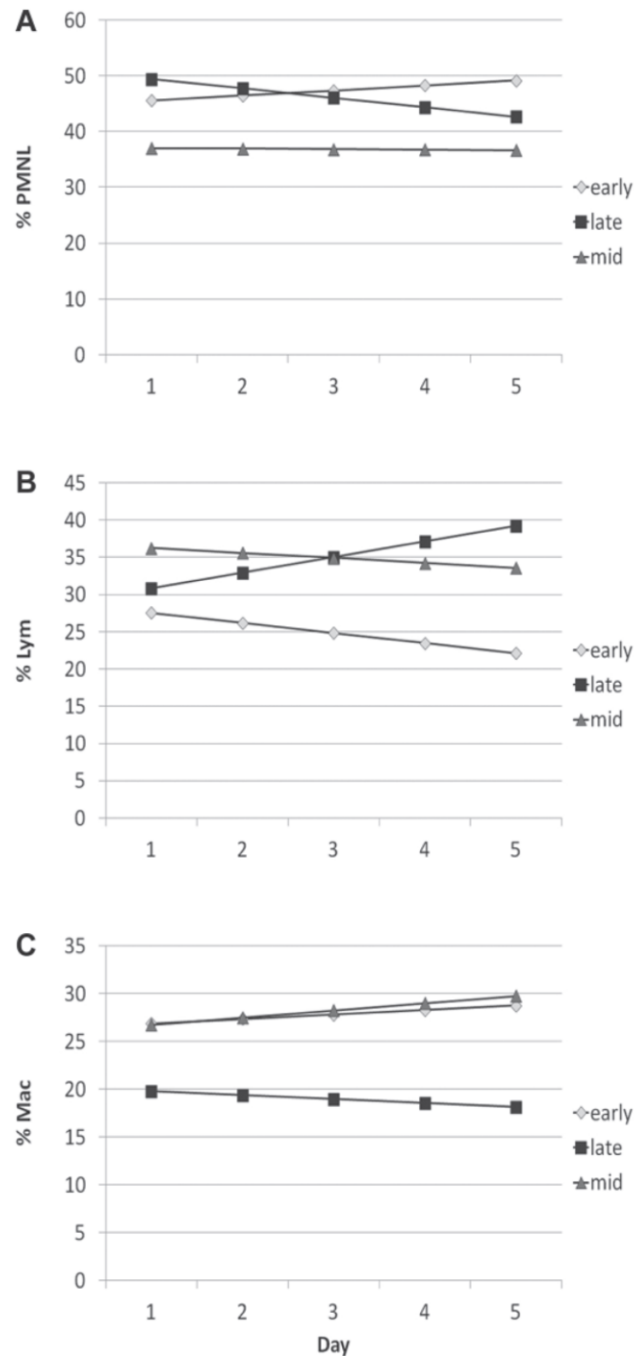
Figure 1. Least squares means of percentages (A) PMNL, (B) lymphocyte (Lym), and (C) macrophage (Mac) in milk, for each sampling day, separated by lactation stage (early, mid, or late).

Figures 1 and 2 show Least Square Mean values in milk for individual cell populations or ratios on each sampling day, corrected for quarter position and parity, and separated by lactation stage. Even though no significance could be attributed to lactation stage, a trend to increasing lymphocyte percentages and decreasing macrophage values was seen over the course of lactation (Figure 3). Analogously, older cows were more likely to have higher log PMNL:Lym compared with first- or second-parity cows, but the differences were

not significant (Figure 4). The results of blood analysis were similar to those of milk (Figure 5).
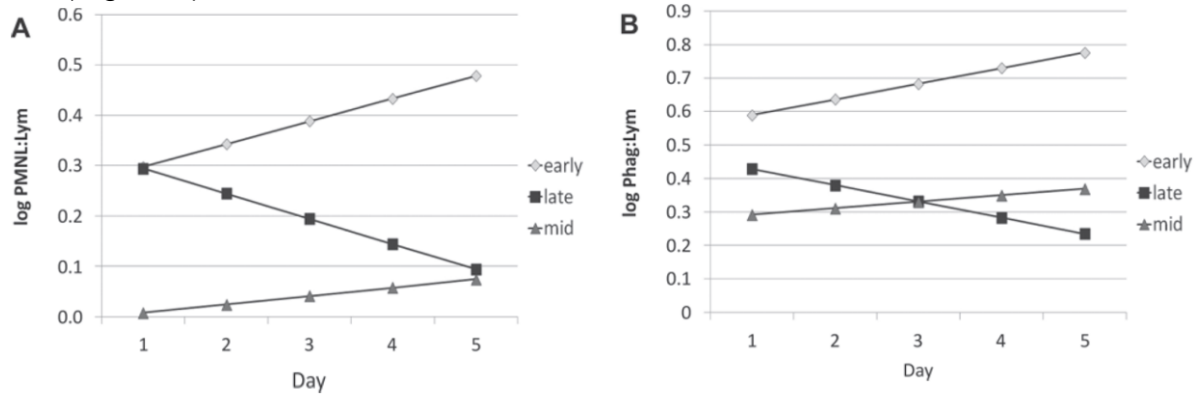

Figure 2. Least squares means of (A) logarithmic PMNL:lymphocyte (log PMNL:Lym) ratio and (B) logarithmic phagocyte:lymphocyte ratio (log Phag:Lym) in milk, for each sampling day, separated by lactation stage (early, mid, or late).
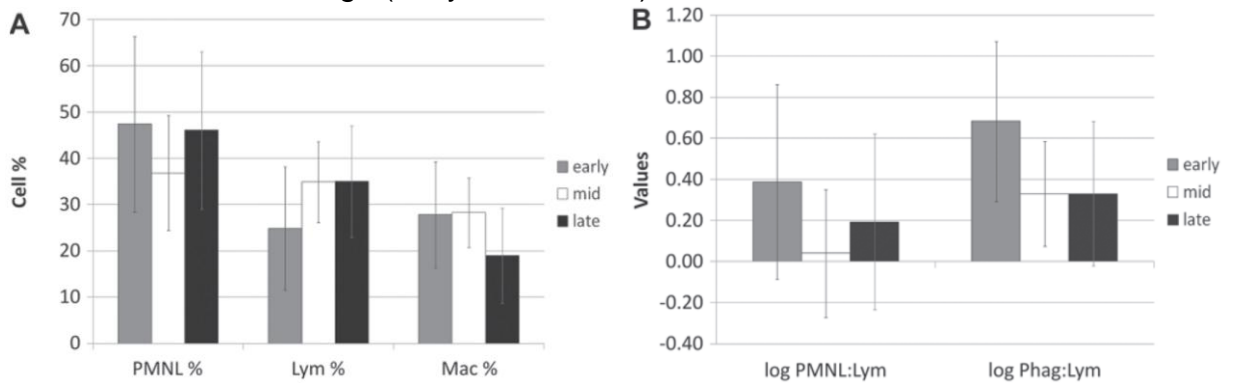

Figure 3. Least squares means and standard error of (A) PMNL, lymphocyte (Lym), and macrophage (Mac) percentages; and (B) logarithmic PMNL:lymphocyte (log PMNL:Lym) and logarithmic phagocyte: lymphocyte (log Phag:Lym) ratios (B) in milk, separated by lactation stage (early, mid, or late).
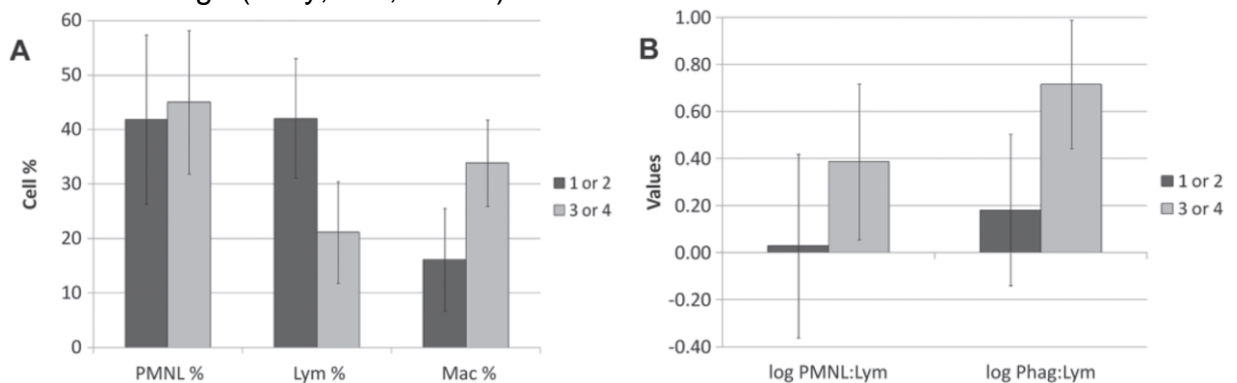

Figure 4. Least squares means and standard error of (A) PMNL, lymphocyte (Lym) and macrophage (Mac) percentages; and (B) logarithmic PMNL:lymphocyte (log PMNL:Lym) and logarithmic phagocyte: lymphocyte (log Phag:Lym) ratios in milk, separated by parity number. Dark grey bars represent animals that calved once or twice, light grey bars represent animals that calved 3 or 4 times.
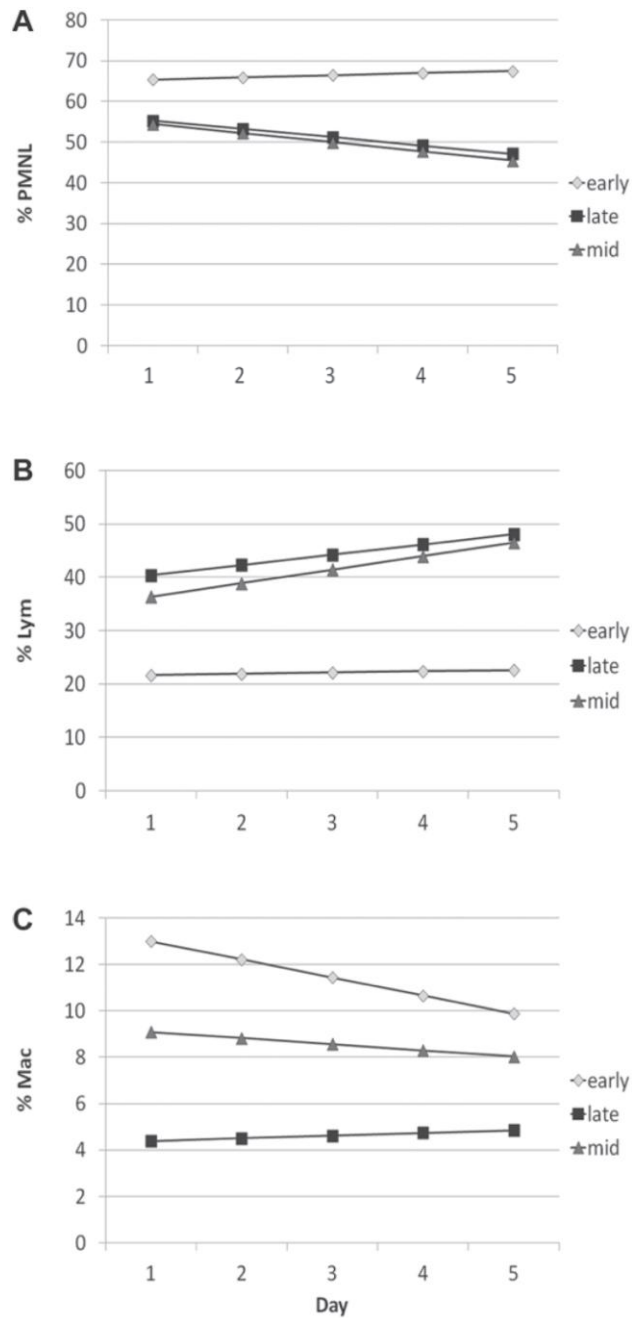
Figure 5. Least squares means of (A) PMNL, (B) lymphocyte (Lym), and (C) macrophage in blood for each sampling day, separated by lactation stage (early, mid, or late).

The area under the receiver operating characteristic curve used for the cutoff determination was 0.775. Choosing a cutoff value of 0.495 for log PMNL:Lym, sensitivity was 73.3% and specificity 73.6%.

Trial 2. When the cutoff value determined in trial 1 was applied to the data of the second trial, out of 64 quarters tested, 28 were correctly classified as positive, and 24 as negative, while two quarters were false positives, and

9 quarters were false negatives. Calculated sensitivity and specificity under field conditions were 75.7% and 92.3%, respectively.

Discussion

Differential cell count has been proposed as a valid tool for the identification of inflammatory processes in animals with low SCC (Rivas et al., 2001). Recent studies confirmed that DCC can be used to detect inflammatory processes in milk samples with extremely low SCC (Schwarz et al., 2011a, b; Pilla et al., 2012). Given the constant pressure in the lactating mammary gland and the dynamism of the immune system, information on the short-term repeatability of DCC is important to evaluate the applicability of the method as a tool in mastitis control programs.

The aims of the present study were to evaluate whether the results of DCC are consistent on subsequent days, using both individual cell population and the 2 ratios to increase the discrimination power of DCC as previously reported (Pilla et al., 2012). A further aim of the study was to establish a cutoff value for log PMNL:Lym, the ratio that best identified healthy and diseased quarters (Pilla et al., 2012), and to verify its applicability under field conditions.

Differential cell counts can be obtained by flow cytometer or light microscopy. Leitner et al. (2000b) reported a high correlation between the 2 methods for PMNL and lymphocytes and a lower correlation for macrophages and epithelial cells. While light microscopy is a cost-effective method, cytometry is more precise, allowing the evaluation of a higher number of cells per sample (Koess and Hamann, 2008). Therefore, cytometric analysis was chosen for DCC testing.

Animals considered in the first trial were selected from a herd free of contagious mastitis pathogens, with high health and hygiene standards. The choice of a commercial herd characterized by excellent management allowed us to reduce the influence of diseases or systemic pathologies unrelated to the mammary gland. To that end, blood samples were taken to verify that eventual fluctuations in milk data could be related to systemic conditions.

Fluctuations in SCC were observed in all but 3 quarters during the follow-up period, but no significant variation in DCC could be recorded. Even though

4 samples were bacteriologically positive, no bacteria could be detected in the following samples, thus infections were considered transient. Therefore, each positive milk samples was excluded from the analysis, but the animals were still considered healthy and were not excluded from the experiment. Even though no significant difference could be found among sampling days, variations in DCC could be seen in some quarters but were not correlated with SCC variation (data not shown).

Fluctuations of individual cell populations in milk were observed in different stages of lactation, but the differences were not significant, in agreement with Pilla et al. (2012) but in contrast to Dosogne et al. (2003), who reported higher lymphocytes and lower macrophages at the beginning of lactation.

These results suggest that DCC can be reliably applied in samples collected in different stages of lactation to evaluate the health status of the mammary gland, even though single variations observed in a few samples could indicate the possibility of misclassification.

The results obtained in the second trial using the cutoff value calculated in the first trial showed very high specificity and good sensitivity. Out of the 9 false-negative quarters, 2 were considered diseased based on SCC only, 4 were positive for *Staph. aureus*, 2 for *Prototheca* ssp., and 1 for CNS. If both SCC and DCC were considered, only 1 quarter would be missclassified, increasing sensitivity of the method to 97.3%, without any changes in specificity. That quarter had very low SCC (1,000 cells/mL in all samplings), and *Staph. aureus* was detected in low counts only in the first and second samplings (10^2 cfu/mL). Because the animal had 2 *Staph. aureus*-infected quarters shedding high numbers of bacteria, we speculated that bacteriological positivity of the other quarter could reflect a transient contamination of the teat canal that was adequately prevented from reaching the gland cistern by local defense mechanisms. Such teat canal contaminations have been previously reported and do not always correlate with intramammary infections (Zecconi et al., 1994).

Conclusions

Differential cell counting can identify inflammatory processes in quarters with low-SCC that are otherwise considered healthy. Information on the

consistency of this method is important to evaluate the applicability of DCC in mastitis control programs. In the present study, we considered healthy mammary quarters to establish test-retest reliability of a previously described combined variable used to identify healthy or diseased quarters and to establish a cutoff value to be used in the field. No influence of sampling day, parity, lactation stage, or quarter position could be found on either milk or blood DCC results; therefore, a cutoff value could be established to identify healthy or diseased quarters. Such value of 0.495, tested under field conditions, confirmed the previous results. Finally, data obtained in the field showed that combining SCC with the cutoff value of 0.495 for DCC, sensitivity and specificity increased to 97.3% and 92.3%, respectively. In conclusion, the use of both cytometric DCC and SCC could represent an excellent diagnostic method to identify inflammatory processes in the mammary gland while avoiding bacteriological analysis.

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7th Chapter

CD2/CD21 index: A new marker to evaluate udder health in dairy cows

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Abstract

Lymphocytes play a significant role in immunological processes of the bovine mammary gland and were found to be the dominant cell population in the milk of healthy udder quarters. The objective of this study was to investigate the quantitative relationship of $CD2^+$ T and $CD21^+$ B lymphocytes using flow cytometry. In a first study, quarter foremilk samples from apparently healthy udder quarters (somatic cell counts, SCC ≤100,000 cells/mL) were analyzed and compared with diseased quarters (SCC >100,000 cells/mL). Percentages of $CD2^+$ T cells were significantly higher in milk samples with SCC ≤100,000 cells/mL than in those with SCC >100,000 cells/mL, whereas the behavior of $CD21^+$ B cells was the contrary. As a result of this antidromic effect, a new variable, the CD2/CD21 index – representing the percentages of $CD2^+$ cells per $CD21^+$ cells – was defined. While diseased quarters generally revealed CD2/CD21 indices <10, values >10 were observed in apparently healthy quarters. Hence, a CD2/CD21 index of 10 may be suitable to aid differentiation between unsuspicious and suspicious or diseased udder quarters. To test whether CD2/CD21 indices <10 are primarily related to major pathogens, quarters with SCC ≤100,000 cells/mL, and >100,000 cells/mL with different kinds of the bacteriological status (culture-negative, minor or major pathogens), were selectively examined. Interestingly, CD2/CD21 indices <10 were found in quarters showing SCC ≤100,000 cells/mL and minor or major pathogens at the time of the current or previous bacteriological analysis. The results of our examinations indicated a clear connection between the CD2/CD21 index and the bacteriological status of the mammary gland. It offers a new possibility to distinguish unsuspicious from suspicious or diseased udder quarters.

Introduction

Mastitis is an inflammation of the mammary gland and a major cause of economic losses to the dairy industry in developed countries [1,2]. The most common cause of mastitis is infection with udder pathogenic microorganisms. To induce mastitis, the pathogens must enter the mammary gland by passing through the teat canal and overcoming the defense mechanisms of the udder [3]. For mastitis diagnosis, traditional and well-established tests including somatic cell counts (SCC) and microbial culture-based methods are standard [4]. According to current definitions of udder health in Germany, SCC ≤100,000 cells/mL in quarter foremilk samples are in the physiological range [5].

Lymphocytes were detected in high numbers in the mammary gland tissue of ruminants, in milk, and dry secretions [6–9]. The predominance of these cells in healthy mammary glands suggests that lymphocytes play a significant role in the maintaining the integrity of the mammary gland [10] and in host defense against infectious diseases of the mammary gland [11]. Data from literature indicates that mammary gland lymphocytes are capable of a broad range of effector functions including cytotoxic, suppressor, and antibacterial functions [11,12].

Studies involving monoclonal antibodies revealed that the majority of lymphocytes in mammary gland tissues and secretions were T lymphocytes, with the remaining population consisting of B lymphocytes and natural killer cells [7,13]. T lymphocytes, including α/β [14] and γ/δ cells [15–17], express CD2 molecules on their surface. α/β lymphocytes are made up of CD4$^+$ (T-helper) and CD8$^+$ (T-cytotoxic or T-suppressor) cells [18]. CD4$^+$ cells are activated in response to the recognition of antigen-MHC class II complexes on antigen-presenting cells, such as T lymphocytes and macrophages [19]. CD8$^+$ cells act by eliminating host cells expressing foreign antigens in association with MHC class I molecules, or they control the immune response by suppressing the activation of these cells during bacterial infection [19,20]. γ/δ lymphocytes are not as well characterized, but it has been suggested that they can be cytotoxic and may provide a unique line of defense against bacterial infections [19]. γ/δ lymphocytes migrate preferentially to epithelial surfaces and

do not circulate extensively [21]. At epithelial surfaces they destroy damaged epithelial cells [19]. B lymphocytes express the CD21 molecule and can serve as antigen-presenting cells, as well as secrete cytokines and differentiate into plasma cells that produce and secrete immunoglobulins [19,20,22,23]. Earlier studies [24,25] indicated a decrease in percentages of $CD2^+$ milk T lymphocytes one day after mammary glands of healthy dairy cows were experimentally challenged with *Staphylococcus* (*S.*) *aureus*.

In a recent study [26], lymphocytes were the only individual cell population showing statistically varying percentages between healthy and three kinds of diseased udder quarters; indicating that their percentages are susceptible to change. However, there is limited knowledge of the qualitative role of milk leukocytes in udders classified as healthy (SCC ≤100,000 cells/mL) [27]. The analysis of the relationship of immune cells (lymphocytes, macrophages, and granulocytes) in milk revealed inflammatory processes based on the predominance of granulocytes in apparently healthy mammary glands (SCC ≤100,000 cells/mL) [8,9]. Lymphocytes were discovered to be a dominant cell population in the milk of healthy udder quarters. The objective of this study was to analyze the quantitative relationship of $CD2^+$ T and $CD21^+$ B lymphocytes in quarter foremilk samples using flow cytometry to check early changes of the immunological status of the mammary gland. Therefore, udder quarters with SCC ≤100,000 cells/mL, as well as >100,000 cells/mL with different kinds of bacteriological status (culture-negative, minor or major pathogens) were selected.

Materials and Methods

Ethic statement

In Germany, it is not necessary to have specific ethical approval for collection of milk samples from dairy cows; all collection procedures were performed by veterinarians of Institute of Veterinary Medicine, Division of Microbiology and Animal Hygiene, Göttingen, Germany, or Regierungspräsidium Gießen, Milk Control, Wetzlar, Germany, following the German Veterinary Society standards [28] for aseptic collection of milk samples.

Farms and animals

Four German dairy farms (A, B, C, and D) were selected randomly. In the farms, 50 to 160 dairy cows were housed in pen barns and milked twice per day in milking parlors. Milking operations were similar in all farms. After forestripping into a foremilk cup, the milkers used damp cotton tissues for udder cleaning. Teats were dipped in iodine solution after milking. In all of the dairy farms, animals were fed with a TMR consisting of grass and maize silage, rape grist, and cereals. Water was available *ad libitum*. All farms were conventional milk producers and the average herd milk yields ranged from 8,000 to 10,000 kg/yr. The animals analyzed were kept under optimal conditions, according to national guidelines.

Study design

Two field studies were conducted. Initially, in study 1 the general udder health status of all lactating cows from farms A, B, and C was determined by analyzing the SCC and bacteriological status of quarter foremilk samples (sampling 1.1). Based on this data, 20 Holstein-Frisian cows in good condition were chosen ten days later for further detailed analysis of their health status by determining the SCC, bacteriological status, differential cell counts (DCC), and percentages of T and B lymphocytes (sampling 1.2). Since the aim of this study was to analyze apparently healthy mammary glands with low SCC values, 64 culture-negative quarters, as well as eight quarters containing minor pathogens with SCC ≤100,000 cells/mL, were selected. A control group of diseased quarters with SCC >100,000 cells/mL containing minor pathogens (n = 5) or major pathogens (n = 3) was also selected.

To confirm and refine the results of study 1, a second study was performed. For this reason, the udder health status of all lactating cows from dairy farm D was determined initially by analyzing the SCC and the bacteriological status of quarter foremilk samples (sampling 2.1). Based on this data, 16 Holstein-Frisian cows in good condition were chosen 18 days later to determine the SCC, bacteriological status, DCC, and percentages of T and B lymphocytes (sampling 2.2), as in study 1. One of the 16 cows selected was a three-quarter cow, meaning that in total 63 udder quarters were subject to analysis. Seventeen culture-negative quarters, 18 quarters containing minor

pathogens, and, in particular, six quarters with the detection of major pathogens were chosen in the SCC range ≤100,000 cells/mL. The selected control group (SCC >100,000 cells/mL) consisted of six culture-negative quarters, seven quarters containing minor pathogens, and nine quarters containing major pathogens. To evaluate the dynamics and repeatability of the results obtained from samplings 2.1 and 2.2, especially in quarters with SCC ≤100,000 cells/mL containing major pathogens, the SCC, bacteriological status, DCC, and percentages of T and B lymphocytes of all selected cows were determined again a further 13 days later (sampling 2.3). At sampling 2.3, samples of two cows could not be taken due to dry periods (n = 55 udder quarters).

In both studies, clinical mastitis symptoms such as flecks in the milk, swelling or redness of the udder quarters were only observed in quarters with SCC >100,000 cells/mL.

Milk sampling and processing

Quarter foremilk samples were obtained according to German Veterinary Society [28] standards. Before milking, teat ends were scrubbed with 70% ethanol and the first two squirts of milk were discarded. Aliquots of 110 mL of milk per udder quarter were collected aseptically in sterile 14-mL plastic tubes and two sterile 50-mL plastic tubes (Sarstedt AG & Co., Nümbrecht, Germany). Ten milliliters were determined for SCC and bacteriological examinations according to IDF [29] standards. Aliquots of 100 mL were necessary for the analysis of DCC and T and B lymphocytes. Cells were isolated from the milk using two centrifugation steps for 15 min at 200 × g and 4°C, respectively. Pellets were washed and resuspended in PBS to a final dilution of 1 × 10^6 cells/100 µL based on the predetermined SCC values for each milk sample.

Flow cytometry analysis

The determination of DCC was described in detail elsewhere [9]. Percentages of T and B lymphocytes were also established according to that method [9]. Briefly, 100 µL aliquots of the cell suspension were transferred into a 5-mL BD Falcon™ tube (BD, Heidelberg, Germany). 5 µL volumes of the unconjugated primary monoclonal antibodies, anti-CD2 (isotype: IgG2a) and anti-CD21 (isotype: IgM) (16-1E10 and BAQ15A, VMRD, Pullman, USA), both

diluted 1:10 in PBS, were added before the tubes were incubated on ice for 30 min. Cells were centrifuged (5 min at 250 × g and 4°C), resuspended in 3 mL of PBS, and allowed to stand for 5 min. After a second washing step, cells were resuspended in 50 µL of PBS and incubated with secondary monoclonal antibodies. Specifically, 2.5 µL of phycoerythrin-conjugated rat-anti-mouse IgM (553409, BD, Heidelberg, Germany) and 10 µL of a mix preincubated for 30 min were applied. This mix contained 5 µL of biotin-conjugated rat-anti-mouse IgG2a (553388, BD, Heidelberg, Germany) and 5 µL of PerCP-Cy™ 5.5 streptavidin (551419, BD, Heidelberg, Germany) – both diluted 1:10 in PBS. PerCP-Cy™ 5.5 streptavidin was used to visualize the biotin-labeled antibody. Following incubation for 30 min, cells were washed twice again and finally resuspended in 500 µL of PBS. Cells were kept on ice during all procedures, centrifuged at 4°C, and incubated in the dark.

Stained samples were analyzed using a FACSCalibur™ flow cytometer (BD, Heidelberg, Germany). CellQuest Pro software (BD, Heidelberg, Germany) was used for data collection and analysis. Five thousand events were evaluated from each sample [9]. Lymphocytes were identified by size and granularity characteristics in an analysis gate according to previous studies [9,30,31]. CD2+ T lymphocytes and CD21+ B lymphocytes were measured simultaneously as percentages of all lymphocytes. Gates enclosing the antibody-positive cells were placed outside the upper limit of background fluorescence. Cells without antibody labeling served as a negative control and were regarded to be a measure for background fluorescence. In addition, isotype control antibodies (rat-IgG1, κ isotype control, 553923 and rat-IgG2a, κ isotype control, 553930, BD, Heidelberg, Germany) were used to facilitate measurement of background staining.

Statistical analysis

Associations between lymphocytes, CD2+ T and CD21+ B lymphocytes, as well as between the CD2/CD21 index and SCC were analyzed by applying linear mixed models using the SAS program (version 9.1; SAS Institute Inc., Cary, NC) according to Schwarz et al. [8, 9]. The statistical model included the random effect of cow and the fixed effects of the farm, lactation number, position of the udder quarter, days in milk, as well as the four SCC groups

(group I: ≤6,250 cells/mL; group II: >6,250 to ≤25,000 cells/mL; group III: >25,000 to ≤100,000 cells/mL; group IV: >100,000 cells/mL) defined in a previous study [32]. In a further analysis, the bacteriological status was included and each of the four SCC groups were subdivided into culture-negative (-0), minor pathogens (-1), and major pathogens (-2) according to Reneau [33].

Results

Study 1: Somatic cell counts and bacteriological status of quarter foremilk samples

Based on the results of the initial determination of the cytobacteriological status (sampling 1.1) in three German dairy herds (A, B, and C), 80 quarter foremilk samples from 20 cows were taken for a thorough analysis of T and B lymphocytes at sampling 1.2. Variations of SCC and the bacteriological status between samplings 1.1 and 1.2 caused moderate deviations from initial selection criteria. In seven quarters, SCC increased from ≤100,000 cells/mL at sampling 1.1 to values >100,000 cells/mL at sampling 1.2. In total, the 80 quarters selected showed an SCC mean value of 85,780 cells/mL with an SD of 233,040 cells/mL at sampling 1.2 (Table 1).

In 11 of the 65 quarters with SCC ≤100,000 cells/mL, coagulase-negative staphylococci (CNS) were found, but no major pathogens. Thirteen of the 15 quarters with SCC >100,000 cells/mL were culture-positive, whereas two were culture-negative. While in five samples (SCC 104,000 to 624,000 cells/mL) CNS was detected, three samples (SCC 116,000 to 587,000 cells/mL) were tested positive for *Corynebacterium* spp. Major pathogens were found solely in five quarters. *S. aureus* was isolated in one of them with an SCC value of 454,000 cells/mL. In three quarters (SCC 181,000 to 1,394,000 cells/mL), *Streptococcus (Strep.) uberis* was detected. A double infection with *Strep. uberis* and *Strep. dysgalactiae* was diagnosed in an udder quarter with an SCC of 139,000 cells/mL.

Study 1: Lymphocytes and lymphocyte subpopulations of quarter foremilk samples

For a detailed analysis of T and B lymphocytes in the 80 quarter foremilk samples 5,000 events per sample were evaluated by flow cytometry at

sampling 1.2. The total number of lymphocytes was determined based on physical parameters. $CD2^+$ T and $CD21^+$ B cells were measured as percentages of all lymphocytes. Lymphocytes were detected in a wide range from 118 to 4,393 events (mean: 2,247 events; SD: 1,088 events). Proportions of $CD2^+$ T lymphocytes lay in a wide range between 4.26 and 92.75% (Table 1). The variation of $CD21^+$ B lymphocytes was narrower, ranging from 0.05 to 7.31% (Table 1).

Table 1. General overview of SCC, percentages of lymphocyte subpopulations and the CD2/CD21 index of the quarter foremilk samples analyzed using flow cytometry

| Item | SCC (× 1,000 cells/mL) | Lymphocyte subpopulations in % | | CD2/CD21 index |
		CD2	CD21	
Study 1 (n = 80, sampling 1.2)				
Mean	85.78	52.09	1.60	62.84
SD	233.04	22.31	1.39	63.50
Median	38	52.00	1.25	42.51
Minimum	2	4.26	0.05	0.74
Maximum	1,394	92.75	7.31	366.00
Study 2 (n = 63, sampling 2.2)				
Mean	589.21	62.47	15.17	15.95
SD	1,925.10	23.75	18.07	12.35
Median	105	74.57	7.97	8.28
Minimum	1	5.42	0.55	0.07
Maximum	10,927	95.78	44.54	48.79
Study 2 (n = 55, sampling 2.3)				
Mean	329.42	71.90	10.11	14.90
SD	759.61	17.63	9.47	15.17
Median	36	74.57	6.33	11.88
Minimum	3	17.70	1.08	1.36
Maximum	4,300	97.92	39.53	88.84

Due to the wide variations found within the total number of lymphocytes, as well as within the proportions of T lymphocytes, lymphocyte data was tested statistically for correlation with the SCC. Total numbers of lymphocytes were higher in milk with SCC ≤100,000 cells/mL than in milk with SCC >100,000 cells/mL (Figure 1a). The percentages of $CD2^+$ cells in milk samples with SCC ≤100,000 cells/mL were considerably higher than in samples with

SCC >100,000 cells/mL (Figure 1b). The profile of CD21$^+$ cells was the reverse: there were notably lower percentages in milk samples with SCC ≤100,000 cells/mL than in samples with SCC >100,000 cells/mL (Figure 1c).
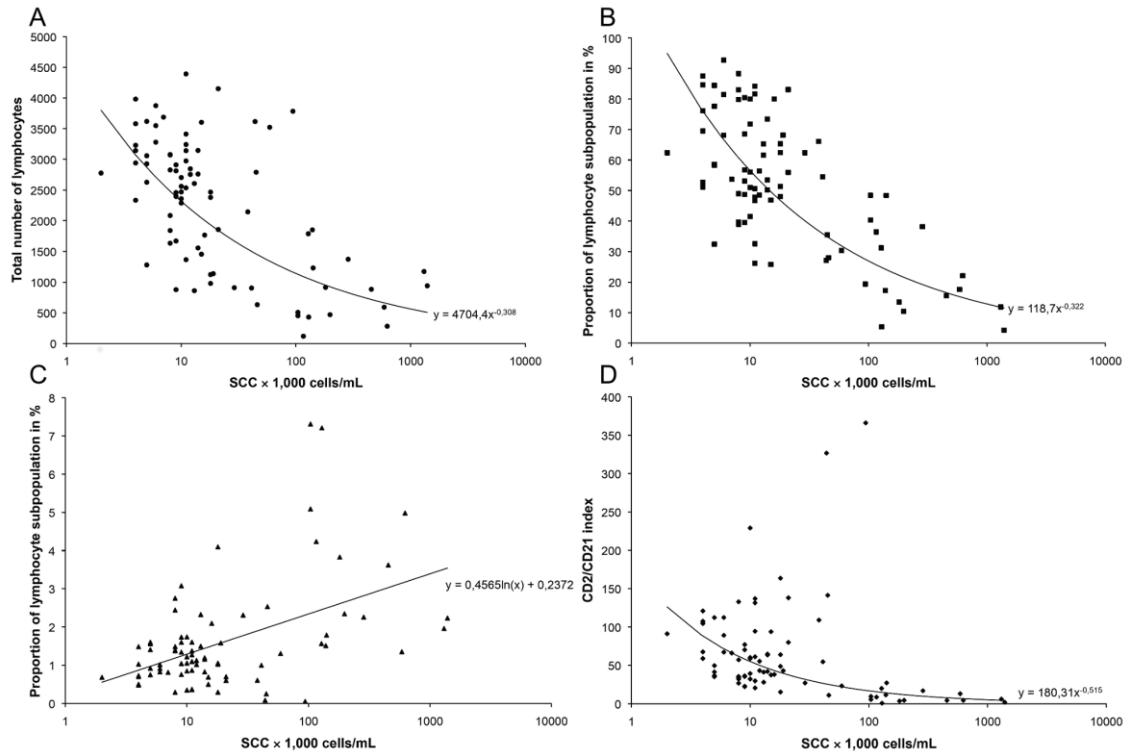

Figure 1. Lymphocyte subpopulations of 80 mammary glands (study 1) depending on SCC. The total number of cells/mL of milk (SCC) was determined for each sample. Cells were then isolated from the quarter foremilk samples and analyzed by flow cytometry. (a) Total number of lymphocytes (filled circle) pictured in combination with a calculated potential trendline; (b) Proportions of CD2$^+$ lymphocytes (filled square) pictured in combination with a calculated potential trendline; (c) Proportions of CD21$^+$ lymphocytes (filled triangle) pictured in combination with a calculated logarithmic trendline. (d) Values of the proportion of CD2$^+$ lymphocytes per CD21$^+$ lymphocytes (CD2/CD21 index) (filled rhomb) pictured in combination with a calculated potential trendline. Each symbol represents the result of one udder quarter analyzed, but overlapping is possible.

The statistical analysis indicated a significantly ($p < 0.001$) negative correlation between the total number of lymphocytes and SCC, as well as between the percentages of CD2$^+$ lymphocytes and SCC (Table 2). In contrast, the correlation between the percentages of CD21$^+$ cells and SCC was evidently ($p < 0.001$) positive. The statistical model considered fixed effects of the farm, lactation number and position of the udder quarter on lymphocytes and

lymphocyte subpopulations. The farm had a significant ($p < 0.05$) impact on the total number of lymphocytes, as well as on the percentages of CD2^{+} lymphocytes (Table 2). The total number of lymphocytes was further influenced significantly ($p < 0.05$) by the lactation number, but not by the days in milk or the position of the udder quarter. Percentages of CD2^{+} and CD21^{+} lymphocytes were affected by none of these parameters.

Table 2. Results of variance analysis[1] for the total number of lymphocytes, percentages of CD2^{+} and CD21^{+} lymphocytes, and CD2/CD21 index in the foremilk samples analyzed

| Item | Effect (p-value) | | | | |
	SCC	Quarter position	Lactation number	DIM	Farm
Study 1 (n = 80, sampling 1.2)					
Lymphocytes	0.0001	0.62	0.04	0.44	0.05
CD2^{+} lymphocytes	0.0001	0.62	0.70	0.79	0.0002
CD21^{+} lymphocytes	0.0001	0.82	0.10	0.36	0.22
CD2/CD21 index	0.0001	0.52	0.07	0.53	0.19
Study 2 (n = 63, sampling 2.2)					
Lymphocytes	0.0001	0.44	0.72	0.07	–
CD2^{+} lymphocytes	0.29	0.37	0.96	0.72	–
CD21^{+} lymphocytes	0.02	0.91	0.26	0.12	–
CD2/CD21 index	0.0005	0.56	0.37	0.22	–
Study 2 (n = 55, sampling 2.3)					
Lymphocytes	0.0001	0.69	0.59	0.56	–
CD2^{+} lymphocytes	0.30	0.22	0.97	0.50	–
CD21^{+} lymphocytes	0.0001	0.73	0.58	0.72	–
CD2/CD21 index	0.02	0.16	0.90	0.64	–

[1]Analyzed factors were SCC, quarter positions (front right, rear right, front left, and rear left), lactation number (1, 2, ≥ 3), days in milk (DIM) (61-99 days, 102-175 days, 246-360 days), and farm (A, B, and C).

As indicated by the calculated trendlines, percentages of CD2^{+} and CD21^{+} cells (Figures 1 b and c, respectively) emerged in contrary directions as SCC increased. Due to this antidromic trend of CD2^{+} and CD21^{+} cells, a new variable – the CD2/CD21 index – representing the ratio between the percentages of CD2^{+} cells per CD21^{+} cells, was defined. In the 80 quarter foremilk samples analyzed, this indicator showed values in a wide range from 0.74 to 366 (Table 1). The values of the CD2/CD21 index were considerably

higher in milk samples with SCC ≤100,000 cells/mL than in samples with SCC >100,000 cells/mL (Figure 1d). The statistical analysis indicated that the farm, position of the udder quarter, number of lactation, or days in milk did not notably influence the CD2/CD21 index (Table 2). However, the correlation between the CD2/CD21 index and SCC was significantly ($p < 0.001$) negative (Table 2), meaning that high SCC levels, which are generally considered to be associated with mastitis, were associated with low CD2/CD21 indices.

Study 1: Evaluation of the CD2/CD21 index depending on the udder health status

In a further statistical analysis, effects of both the SCC and bacteriological status on the CD2/CD21 index were investigated. In addition, differences of the CD2/CD21 index within the SCC range ≤100,000 cells/mL were analyzed. Based on the cytobacteriological status at sampling 1.2 the 80 udder quarters were classified into four SCC groups (group I: ≤6,250 cells/mL; group II: >6,250 to ≤25,000 cells/mL; group III: >25,000 to ≤100,000 cells/mL; group IV: >100,000 cells/mL), as defined in a previous study [31]. The bacteriological status was regarded by subdividing each SCC group into culture-negative (-0), minor pathogens (-1), or major pathogens (-2) (Figure 2). To consider the impact of variations in SCC and bacteriology between samplings 1.1 and 1.2 on the CD2/CD21 index, the cytobacteriological status at sampling 1.1 is also indicated in Figure 2.

At sampling 1.2, CD2/CD21 indices of quarters in groups I-0 (n = 11), II-0 (n = 33), and III-0 (n = 7) varied greatly between 11.06 and 366, without visible effects of the cytobacteriological status at sampling 1.1 (Figure 2). Samples from groups I-1 (n = 4), II-1 (n = 6), and III-1 (n = 1) showed CD2/CD21 indices ranging from 20.58 to 104.83; also without visible effects of the cytobacteriological status at sampling 1.1.

The CD2/CD21 indices revealed markedly lower values in groups IV-0 (n = 2), IV-1 (n = 8), and IV-2 (n = 5) than in the SCC range ≤100,000 cells/mL (Figure 2). While 11 of 15 quarters with SCC >100,000 cells/mL indicated values <10, values >10 were found in four quarters. One quarter of group IV-0, as well as one quarter of group IV-1 which were both culture-negative at sampling 1.1, showed CD2/CD21 indices of 27.09 and 19.93, respectively, at

sampling 1.2. One further quarter of group IV-1 that was positive for minor pathogens at sampling 1.1 indicated a CD2/CD21 index of 16.90. Major pathogens were detected at sampling 1.1 in the fourth quarter, showing a CD2/CD21 index of 13.00 (group IV-1). The CD2/CD21 index of samples in group IV-2 was generally <10.
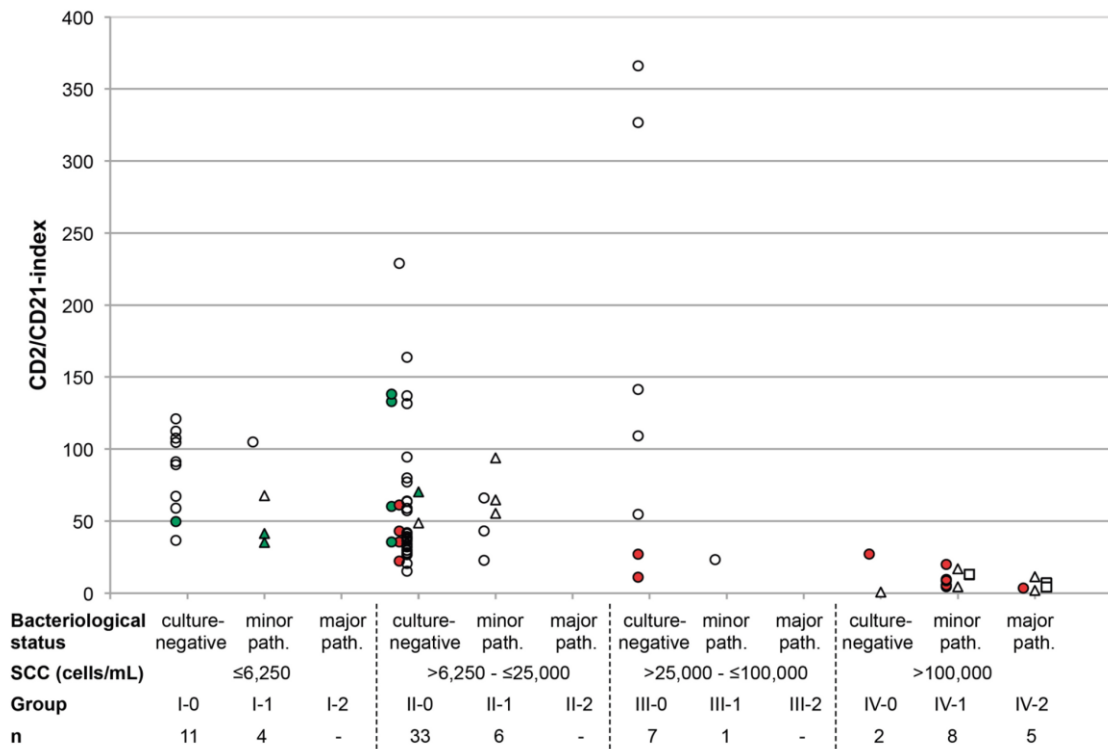

Figure 2. Associations between the CD2/CD21 index and the cyto-bacteriological status of the mammary glands in study 1 at sampling 1.2. Values of the CD2/CD21 index in four SCC groups (group I: ≤6,250 cells/mL; group II: >6,250 to ≤25,000 cells/mL; group III: >25,000 to ≤100,000 cells/mL; group IV: >100,000 cells/mL), with each group subdivided into culture-negative (-0), minor pathogens (-1), and major pathogens (-2). The 80 udder quarters analyzed were classified based on SCC values and the bacteriological status at sampling 1.2. To show the dynamics of the udder health status, the result of the previous bacteriological analysis (sampling 1.1) is indicated for each quarter the following by symbols: ○ = negative, △ = minor pathogens, □ = major pathogens. Moreover, the SCC variations are indicated by the color of the symbols: white = same SCC group at sampling 1.1 and 1.2, green = SCC was at least one SCC group higher at sampling 1.1, red = SCC was at least one SCC group lower at sampling 1.1. Each symbol represents the result of one udder quarter analyzed, but overlapping is possible. Path. = pathogens.

The statistical analysis indicated notably ($p < 0.05$) lower mean values of the CD2/CD21 index in groups I-0 (76.12) and II-0 (89.97) in comparison with group III-0 (146.26). Furthermore, the mean values in groups I-0, II-0, III-0, and I-1 (149.41) were considerably ($p < 0.05$) higher than in groups IV-0 (0.54), IV-1 (24.22), and IV-2 (8.88). However, the mean CD2/CD21 indices in groups II-1 (78.23) and III-1 (86.92) did not differ significantly from any of the other groups.

Study 2: Lymphocytes and lymphocyte subpopulations of quarter foremilk samples

The major finding of study 1 demonstrated that CD2/CD21 indices were generally >10 in quarters with SCC ≤100,000 cells/mL, independent of detection of minor pathogens, whereas almost all samples with SCC >100,000 cells/mL containing minor or major pathogens indicated values <10. Hence, to investigate whether a CD2/CD21 index of <10 is primarily related to major pathogens, a second study was conducted. Based on the results of the initial investigation of the udder health status in farm D (sampling 2.1), quarters with SCC ≤100,000 cells/mL and >100,000 cells/mL – either culture-negative or containing minor or major pathogens – were selected for a detailed analysis of T and B lymphocytes at sampling 2.2 using flow cytometry. In contrast to study 1, six quarters with SCC ≤100,000 cells/mL containing major pathogens were also selectively chosen.

At sampling 2.2, quarter foremilk samples were taken from 63 quarters of 16 cows housed in dairy farm D. In ten quarters, SCC increased from ≤100,000 cells/mL at sampling 2.1 to values >100,000 cells/mL at sampling 2.2. The SCC mean value of 589,210 cells/mL (Table 1), based on the samples at sampling 2.2, was clearly higher than in study 1 (sampling 1.2); especially due to the examination of five severely diseased quarters with an SCC between 1,054,000 and 10,927,000 cells/mL. As in study 1, lymphocytes were detected in a wide range from 99 to 4,556 events (mean value: 1,397; SD: 996). Sampling 2.2 confirmed the results of sampling 1.2 regarding the wide range of proportions of CD2$^+$ T lymphocytes between 5.42 and 95.78% (Table 1). However, at sampling 2.2, proportions of CD21$^+$ B lymphocytes varied between 0.55 and 44.54% (Table 1) and were clearly higher than those found at sampling 1.2. While the minimum value of the CD2/CD21 index of 0.07 at

sampling 2.2 was similar to the minimum value at sampling 1.2, the maximum value of 48.79 measured at sampling 2.2 was clearly lower (Table 1). Results of variance analysis of data from sampling 2.2 were similar to those observed at sampling 1.2, with the exception that lymphocytes were not significantly influenced by lactation number and percentages of $CD2^+$ cells did not correlate significantly with SCC (Table 2).

To evaluate the dynamics and repeatability of the results, 14 of the 16 animals could be sampled again (sampling 2.3) 13 days after sampling 2.2. Compared to sampling 2.2, the SCC mean value at sampling 2.3 dropped to 329,420 cells/mL (Table 1) because the SCC of the severely diseased quarters decreased to values between 848,000 and 4,300,000 cells/mL. Applying flow cytometry, lymphocytes were detected in a wide range from 119 to 4,151 events (mean value: 1,628, SD: 1,066), confirming the results of sampling 2.2. Proportions of both $CD2^+$ and $CD21^+$ cells at sampling 2.3 were similar to those observed at sampling 2.2 (Table 1). Compared to sampling 2.2, the CD2/CD21 index indicated slightly higher values ranging from 1.36 to 88.84 at sampling 2.3 (Table 1). Results of variance analysis of sampling 2.3 data were similar to those found at sampling 2.2 (Table 2).

Study 2: Evaluation of the CD2/CD21 index depending on the udder health status

As at sampling 1.2, data of sampling 2.2 revealed a significant correlation between the CD2/CD21 index and SCC. To test the effect of SCC and bacteriological status on the CD2/CD21 index, the same statistical analysis was performed as in study 1. For this analysis, the 63 udder quarters analyzed were grouped according to the cytobacteriological status at sampling 2.2 (Figure 3). Effects of variations in SCC and bacteriology between samplings 2.1 and 2.2 on the CD2/CD21 index were also considered. However, since SCC of the previous sampling showed no visible effects on the CD2/CD21 index in study 1, only the bacteriological status of sampling 2.1 is indicated in Figure 2.

At sampling 2.2, CD2/CD21 indices in quarters of groups I-0 (n = 7), II-0 (n = 7), and III-0 (n = 1) ranged between 5.64 and 27.96 (Figure 3). In groups I-1 (n = 10), II-1 (n = 3), and III-1 (n = 2) a wider range of values between 2.22 and 38.63 was observed. Interestingly, nine quarters of groups I-0, I-1, II-0, II-1,

III-1, and III-2 indicated CD2/CD21 indices of <10. In four of these quarters (groups I-0, I-1, II-0, and III-2), with CD2/CD21 indices ranging between 2.22 and 9.64, *S. aureus* was detected at sampling 2.1 (Figure 3). In one quarter of group II-0, with a CD2/CD21 index of 8.86 and two quarters of group II-1 with CD2/CD21 indices of 8.28 and 6.58, respectively, minor pathogens were detected at sampling 2.1. One further quarter of group II-0, with a CD2/CD21 index of 8.69, as well as one quarter of group III-1 with a CD2/CD21 index of 6.44, were culture-negative at sampling 2.1.
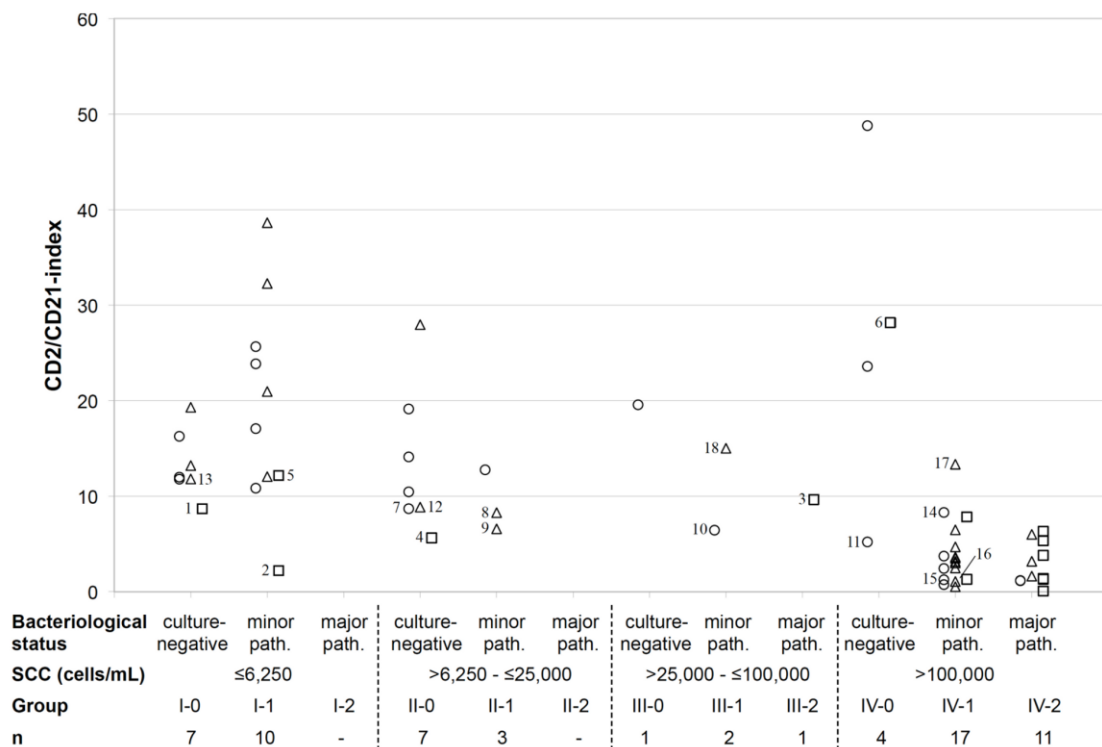

Figure 3. Associations between the CD2/CD21 index and the cyto-bacteriological status of the mammary glands in study 2 at sampling 2.2. Values of the CD2/CD21 index in four SCC groups (group I: ≤6,250 cells/mL; group II: >6,250 to ≤25,000 cells/mL; group III: >25,000 to ≤100,000 cells/mL; group IV: >100,000 cells/mL) with each group subdivided into culture-negative (-0), minor pathogens (-1), and major pathogens (-2). The 63 udder quarters were classified based on SCC values and the bacteriological status at sampling 2.2. For each quarter, the result of the previous bacteriological analysis (sampling 2.1) is indicated by the following symbols: O = negative, △ = minor pathogens, □ = major pathogens. Quarters no. 1-18 were marked for additional analysis of differential cell counts as their CD2/CD21 index differed from our working hypothesis (>10 in unsuspicious quarters and <10 in suspicious or diseased quarters) either at sampling 2.2 or 2.3. Each symbol represents the result of one udder quarter analyzed, but overlapping is possible. Path. = pathogens.

As in study 1 at sampling 1.2, 28 of 32 samples of groups IV-0 (n = 4), IV-1 (n = 17), and IV-2 (n = 11) clustered in a CD2/CD21 index range <10 (Figure 3). However, two quarters of group IV-0 indicated CD2/CD21 indices of 23.59 and 48.79 and they were culture-negative at sampling 2.1. In contrast, *S. aureus* was detected at sampling 2.1 in another quarter of group IV-0 with a CD2/CD21 index of 28.17. In the fourth quarter (group IV-1), with a CD2/CD21 index of 13.34, *Corynebacterium* spp. was already present at sampling 2.1. As at sampling 1.2, the CD2/CD21 index was generally <10 in all quarters of group IV-2 where major pathogens such as *S. aureus*, *Strep. uberis*, and *Strep. dysgalactiae* were isolated.

Statistical analysis revealed significant ($p < 0.05$) higher mean values of the CD2/CD21 index in groups I-0 (13.74), II-0 (16.30), III-0 (21.56), and I-1 (18.81) in comparison with group IV-1 (3.80). Furthermore, the mean values in groups II-0 and IV-0 (19.60) were notably ($p < 0.05$) higher than in group IV-2 (6.40). However, the mean CD2/CD21 indices in groups II-1 (12.50), III-1 (14.31), and III-2 (14.36) did not differ significantly from any of the other groups.

Data from sampling 2.2 indicated that CD2/CD21 indices in almost all quarters of groups IV-0, IV-1, and IV-2 were clearly lower than in quarters with SCC ≤100,000 cells/mL, as in study 1. Interestingly, in groups I-0, I-1, II-0, II-1, III-1 and III-2, values <10 of the CD2/CD21 index were also found at sampling 2.2. To investigate the further development of the CD2/CD21 index, sampling 2.3 was conducted 13 days later and the results were also tested in a statistical analysis (Figure 4).

At sampling 2.3, CD2/CD21 indices in groups I-0 (n = 8), II-0 (n = 7), and III-0 (n = 6) lay in a wider range between 1.46 and 57.00, than at sampling 2.2 (Figure 4). Samples in groups I-1 (n = 10), II-1 (n = 3), and III-1 (n = 2) showed CD2/CD21 indices between 1.44 and 37.65, as observed at sampling 2.2. Interestingly, as at sampling 2.2, six quarters in groups I-0, II-0, III-0, III-1, and III-2 revealed CD2/CD21 indices of <10. In three of these quarters (groups III-0, III-1, and III-2) with CD2/CD21 indices ranging between 1.44 and 6.43, *S. aureus* could be detected at sampling 2.1, whereas the quarter in group III-2 also indicated *S. aureus* at sampling 2.2. In the remaining three samples (groups I-0, II-0, and III-1), with CD2/CD21 indices ranging between 6.63 and

8.77, CNS were isolated at samplings 2.1 and 2.2 with the exception of the quarter in group I-0 that was culture-negative at sampling 2.2.

As observed at sampling 2.2, the CD2/CD21 index of 18 of 22 samples in groups IV-0 (n = 5), IV-1 (n = 11), and IV-2 (n = 6) clustered in the range <10 (Figure 4). One quarter in group IV-0, indicating a CD2/CD21 index of 88.84 at
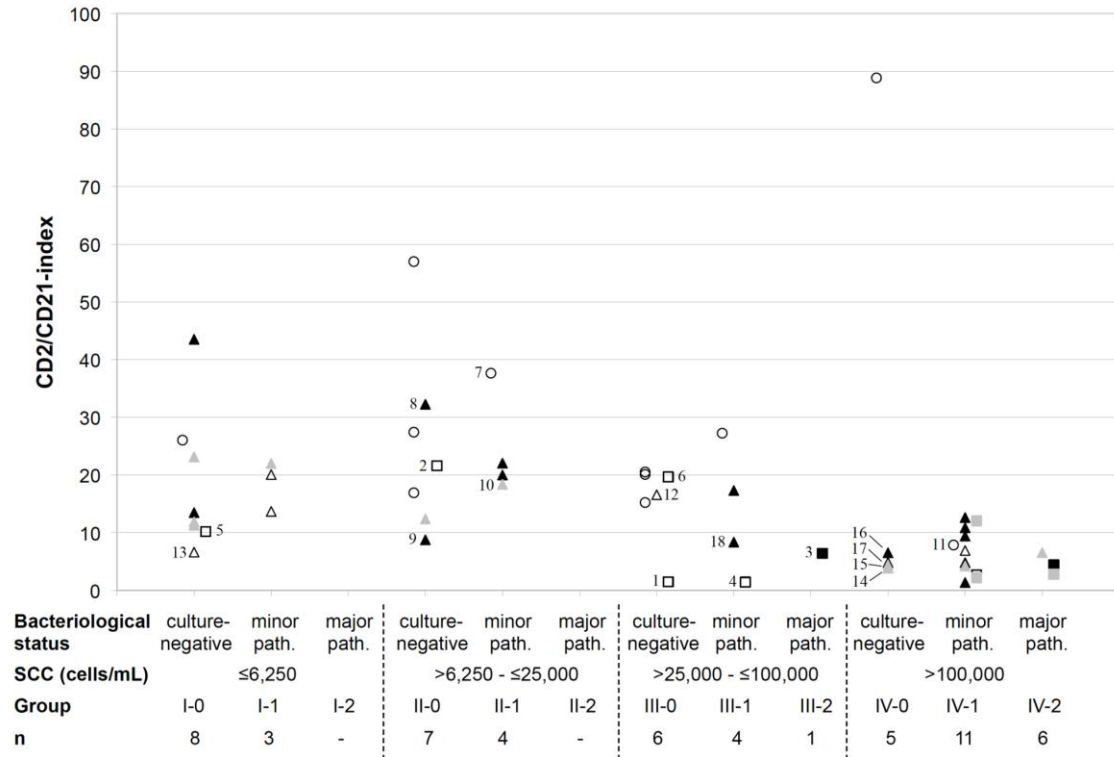

Figure 4. Associations between the CD2/CD21 index and the cyto-bacteriological status of the mammary glands in study 2 at sampling 2.3. Values of the CD2/CD21 index in four SCC groups (group I: ≤6,250 cells/mL; group II: >6,250 to ≤25,000 cells/mL; group III: >25,000 to ≤100,000 cells/mL; group IV: >100,000 cells/mL) with each group subdivided into culture-negative (-0), minor pathogens (-1), and major pathogens (-2). The 55 udder quarters were classified based on SCC values and the bacteriological status at sampling 2.3. For each quarter, the results of the two previous bacteriological analyses (samplings 2.1 and 2.2) is indicated by the following symbols: ○ = negative, △ = minor pathogens at sampling 2.1, □ = major pathogens at sampling 2.1, ▲ = minor pathogens at sampling 2.2, ■ = major pathogens at sampling 2.2, ▲ = minor pathogens at samplings 2.1 and 2.2, ■ = major pathogens at sampling 2.1 and 2.2. Quarters no. 1-18 were marked for additional analysis of differential cell counts as their CD2/CD21 index differed from our working hypothesis (>10 in unsuspicious quarters and <10 in suspicious or diseased quarters) either at sampling 2.2 or 2.3. Each symbol represents the result of one udder quarter analyzed, but overlapping is possible. Path. = pathogens.

sampling 2.3, was also culture-negative at both previous samplings (2.1 and 2.2). Three further quarters in group IV-1 revealed CD2/CD21 indices between 10.87 and 12.63. While in two of these quarters CNS were isolated previously at samplings 2.1 and 2.2, the remaining quarter was culture-positive for *Strep. uberis* at sampling 2.2. As at samplings 1.2 and 2.2, the CD2/CD21 index was <10 in all quarters of group IV-2 (n = 6) where the major pathogens *S. aureus*, *Strep. uberis*, and *Strep. dysgalactiae* were isolated.

Statistical analysis revealed a considerably ($p < 0.05$) higher mean value of the CD2/CD21 index in group II-0 (26.15) than in groups IV-1 (14.54) and IV-2 (6.58). However, the mean values in groups I-0 (19.35), III-0 (5.48), I-1 (19.45), II-1 (21.33), III-1 (9.61), III-2 (17.12), and IV-0 (21.31) did not differ significantly from any of the other groups.

Study 2: Associations between the CD2/CD21 index and differential cell counts

The data obtained so far suggests a connection between low CD2/CD21 indices and diseased udder quarters, as values <10 were measured in almost all quarters with a high SCC of >100,000 cells/mL (groups IV-0, IV-1, and IV-2) at samplings 1.2, 2.2 and 2.3. In contrast, apparently healthy udder quarters with SCC ≤100,000 cells/mL indicated values >10. However, at samplings 2.2 and 2.3, deviations from our working hypothesis were observed (Figures 3 and 4), and therefore the DCC of 18 quarters were further analyzed for a better interpretation of their CD2/CD21 indices.

In quarters no. 1-6, *S. aureus* was diagnosed at sampling 2.1 and SCC ranged from 5,000 to 34,000 cells/mL (Table 3). However, it was only in quarter no. 3 that *S. aureus* was detected at samplings 2.1, 2.2 and 2.3. With the exception of quarter no. 2 at sampling 2.3, CD2/CD21 indices were <10 in quarters no. 1-4 (Figure 5). The DCC analysis of quarters no. 1-4 indicated inflammatory reactions based on the predominance of granulocytes (≥48%) either at sampling 2.2 or 2.3 (Figure 5), which explains the low CD2/CD21 indices. However, in quarters no. 5 and 6, CD2/CD21 indices >10 were measured and inflammatory reactions were not detectable.
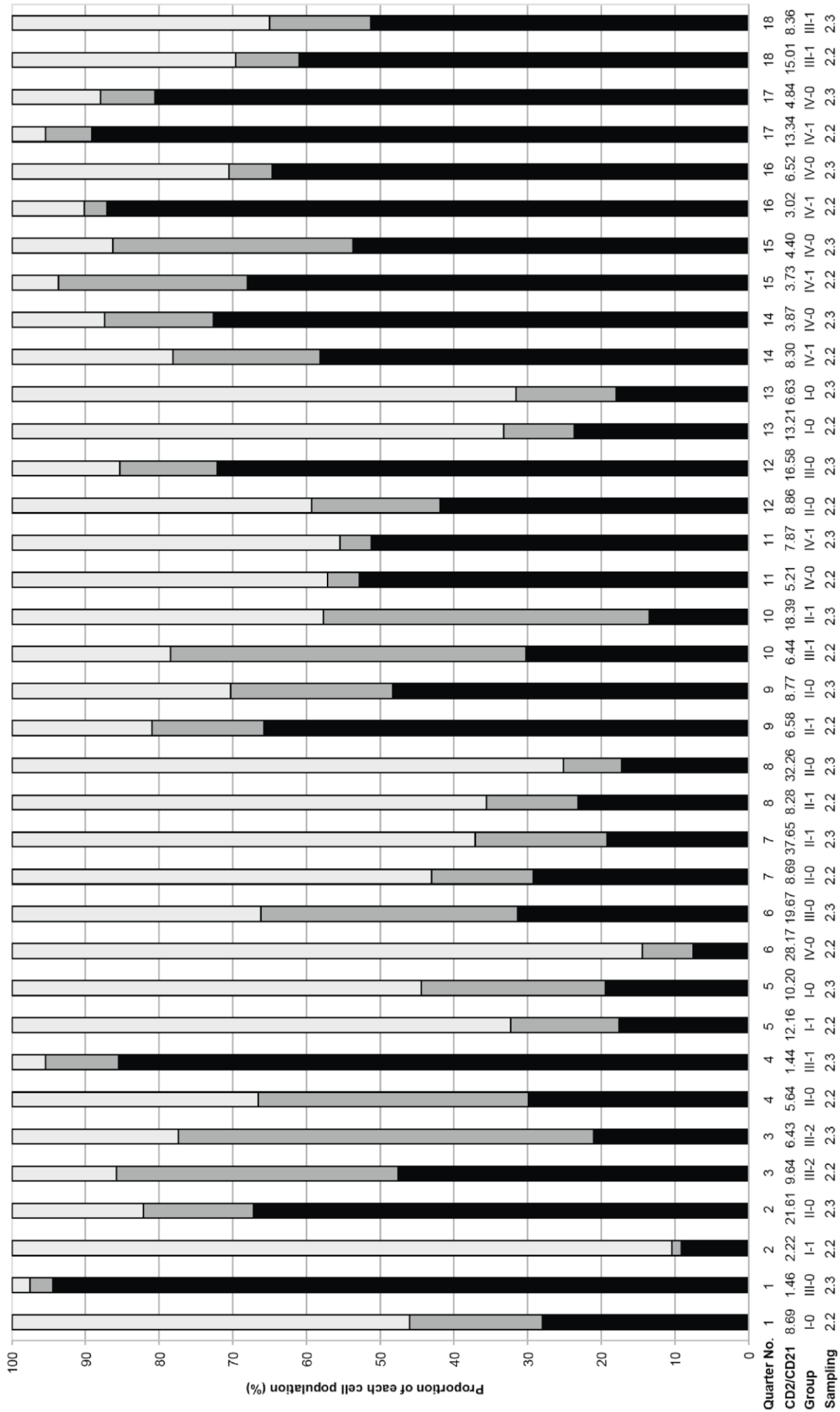
Figure 5. Associations between the CD2/CD21 index and differential cell counts of selected quarters numbered 1 to 18.
Differential cell count patterns (black bars = granulocytes, dark grey bars = macrophages, light grey bars = lymphocytes) and CD2/CD21 index at samplings 2.2 and 2.3 (study 2). Quarters no. 1-18 were selected as their CD2/CD21 index differed from our working hypothesis (>10 in unsuspicious quarters and <10 in suspicious or diseased quarters) either at sampling 2.2 or 2.3.

Table 3. Overview about SCC, bacteriological status, and CD2/CD21 index of 18 selected udder quarters from study 2 at samplings 2.1, 2.2, and 2.3. Quarters no. 1-18 were selected because their CD2/CD21 index differed from our working hypothesis (>10 in unsuspicious quarters and <10 in suspicious or diseased quarters) either at sampling 2.2 or 2.3.

No	Cow	Quarter position[2]	Sampling 2.1		Sampling 2.2			Sampling 2.3		
			SCC (× 1,000 cells/mL)	Bacteriological status[3]	SCC (× 1,000 cells/mL)	Bacteriological status[3]	CD2/CD21 index	SCC (× 1,000 cells/mL)	Bacteriological status[3]	CD2/CD21 index
1	401	RR	11	*S. aureus*	6	negative	8.69	62	negative	1.46
2	401	RL	11	*S. aureus*	3	CNS	2.22	12	negative	21.61
3	345	RR	23	*S. aureus*	53	*S. aureus*	9.64	36	*S. aureus*	6.43
4	345	FL	13	*S. aureus*	16	negative	5.64	34	*Coryneb.* spp.	1.44
5	390	RR	5	*S. aureus*	4	CNS	12.16	5	negative	10.20
6	445	RR	34	*S. aureus*	127	negative	28.17	68	negative	19.67
7	387	FL	25	negative	10	negative	8.69	7	*Coryneb.* spp.	37.65
8	387	RR	11	CNS	8	*Coryneb.* spp.	8.28	7	negative	32.26
9	345	RL	10	CNS	13	CNS	6.58	10	negative	8.77
10	345	FR	11	negative	42	CNS	6.44	19	CNS	18.39
11	278	FL	192	negative	1,596	negative	5.21	4,300	CNS	7.87
12	320	FR	2	CNS	9	negative	8.86	32	negative	16.58
13	223	RL	4	CNS	3	negative	13.21	4	negative	6.63
14	278	FR	185	negative	133	*Coryneb.* spp.	8.30	233	negative	3.87
15	278	RR	109	negative	138	CNS	3.73	155	negative	4.40
16	323	FL	118	*Coryneb.* spp.	588	*Coryneb.* spp.	3.02	705	negative	6.52
17	328	RL	127	*Coryneb.* spp.	144	*Coryneb.* spp.	13.34	132	negative	4.84
18	393	RR	57	CNS	68	CNS	15.01	45	CNS	8.36

[1]CD2/CD21 index not determined. [2]FR = front right; RR = rear right; FL = front left; RL = rear left. [2]*S. aureus* = *Staphylococcus aureus*; *Strep. uberis* = *Streptococcus uberis*; CNS = coagulase-negative staphylococci; *Coryneb.* spp. = *Corynebacterium* species; negative = no cultural growth of microorganisms.

In quarters no. 7-18, minor pathogens were isolated at least at one sampling and SCC ranged from 2,000 to 4,300,000 cells/mL (Table 3). While the CD2/CD21 indices were <10 in quarters no. 9, 11, 14, 15 and 16 at samplings 2.2 and 2.3, quarter no. 12 showed a CD2/CD21 index of 8.86 at sampling 2.2 and of 16.58 at sampling 2.3 (Table 3). Quarters no. 17 and 18 at sampling 2.2 revealed CD2/CD21 indices of 13.34 and 15.01, respectively, and values decreased to 4.84 and 8.36 at sampling 2.3. DCC analysis confirmed inflammatory reactions based on the predominance of granulocytes (≥43%) in quarters no. 9, 11, 12, 14, 15, 16, 17 and 18 at samplings 2.2 and 2.3 (Figure 5). However, quarters no. 7, 8, 10, and 13 did not indicate inflammatory reactions based on DCC analysis, although CD2/CD21 indices <10 were measured at least at one sampling (2.2 or 2.3, Figure 5).

Discussion

Lymphocytes play a significant role in immunological processes of the bovine mammary gland [10,11]. The induction and suppression of immune responses is regulated by lymphocytes [34]. Lymphocytes were found to be the predominant cell population in the milk of healthy udder quarters and consist predominantly of T cells, whereas B cells and NK cells are observed in low percentages [7,13]. Research on the interaction of lymphocyte subpopulations revealed that B cells require the help of T cells to produce specific antibodies [35]. However, the interaction of T and B lymphocytes in apparently healthy mammary glands with SCC ≤100,000 cells/mL remains poorly investigated, but mastitis pathogens [32] and inflammatory reactions based on DCC analysis [8,9,26] were already found in this SCC range. In this study, we analyzed the proportions of $CD2^+$ T and $CD21^+$ B lymphocytes purified from quarter foremilk samples to both improve our knowledge of quantitative relationships between these lymphocyte populations in clinically healthy and subclinically infected bovine mammary glands, and to observe early changes in the immunological status of the mammary glands.

The CD2/CD21 index, a new variable, was found empirically in study 1 due to the significantly varying percentages of $CD2^+$ T and $CD21^+$ B lymphocytes in the milk of apparently healthy quarters with SCC

≤100,000 cells/mL and diseased quarters (SCC >100,000 cells/mL). Moreover, recent studies [26,36,37] indicated that combinations of cell populations (e.g., ratios) are more suitable to differentiate between healthy and diseased udder quarters than percentages of individual cell populations alone. In our examinations, percentages of CD2^{+} lymphocytes were noticeably high in milk samples showing SCC ≤100,000 cells/mL and two culture-negative results. Information on the percentages of CD2^{+} lymphocytes in milk samples with SCC ≤100,000 cells/mL is not available in literature. Only a mean proportion of CD2^{+} lymphocytes of 88% for healthy udder quarters defined by SCC <450,000 cells/mL has been reported previously [38]. Further studies [15,18] have described the mean proportions of CD2^{+} cells to range from 83 to 85% in the milk of bacteriological negative udder quarters, but SCC values were not presented in this case. In contrast to the high percentages of CD2^{+} cells found in the milk of healthy quarters, the percentages of these lymphocytes were low in the milk of diseased quarters.

CD21^{+} lymphocytes were detected in low percentages in samples showing SCC ≤100,000 cells/mL and two culture-negative results. Information on the percentages of CD21^{+} cells in milk with SCC ≤100,000 cells/mL is not yet available in literature. In a field study [30], the mean proportions of CD21^{+} cells of 0.93% were found in the milk of 11 healthy cows with an SCC between 50,000 and 265,000 cells/mL. An experimental study [23] also revealed low mean proportions of CD21^{+} lymphocytes of 1% when analyzing milk of six cows with a mean SCC value of 53,000 cells/mL before inoculation with *Escherichia coli*. Contrary to the low proportions of B lymphocytes in the milk of healthy udder quarters, our data indicated an increase of up to 45% in diseased quarters, confirming results of previous studies [23,30]. This increase of B lymphocytes suggests that a humoral immune response developed [30].

The results of study 1 gave indications that a CD2/CD21 index of 10 may be suitable to differentiate between unsuspicious and suspicious or diseased udder quarters. Severely diseased quarters (group IV-2) generally revealed CD2/CD21 indices <10. In apparently healthy udder quarters (groups I-0, II-0, III-0, I-1, II-1, III-1), the CD2/CD21 index was generally >10. The results of study 1 tend to highlight a correlation between the CD2/CD21 index and the bacteriological status of the udder quarters, particularly when major pathogens

are detected. However, in order to investigate whether a CD2/CD21 index <10 is primarily related to major pathogens, a second study was conducted. Quarters with SCC ≤100,000 cells/mL and >100,000 cells/mL, with different kinds of bacteriological status (culture-negative, minor or major pathogens), were chosen. In this context, six quarters showing SCC <100,000 cells/mL and *S. aureus* were especially selected. The results of study 2 confirmed a CD2/CD21 index <10 in severely diseased quarters (group IV-2). However, we also observed CD2/CD21 indices <10 in nine and six quarters with SCC ≤100,000 cells/mL (groups I-0, I-1, II-0, II-1, III-1, and III-2) at samplings 2.2 and 2.3, respectively. The low CD2/CD21 indices in four of these quarters (no. 1-4) at sampling 2.2 and in three quarters (no. 1, 3, and 4) at sampling 2.3 may be attributed to the detection of *S. aureus* at sampling 2.1. The DCC data supported this hypothesis as these quarters indicated an inflammatory reaction based on the predominance of granulocytes either at sampling 2.2 or 2.3. However, the fifth quarter (no. 5) that was tested positive for *S. aureus* and showed SCC ≤100,000 cells/mL at sampling 2.1, revealed CD2/CD21 indices >10 at the following two samplings and did not show any inflammatory signs based on DCC analysis (granulocyte proportions 17-19%). Therefore, quarter no. 5 may be cured during our study. In four other quarters (no. 8-10, 12) at sampling 2.2 and in three quarters (no. 9, 13, 18) at sampling 2.3, the CD2/CD21 index <10 may be due to the detection of minor pathogens. If minor pathogens are detected, it is not clear whether the pathogens originate from teat canal colonization or whether they caused an intramammary infection [39,40]. In three of these quarters (no. 9, 12, 18) at samplings 2.2 and 2.3, inflammatory reactions could be detected based on DCC analysis, whereas the remaining three quarters (no. 8, 10, 13) did not show any signs of inflammation. Only one culture-negative quarter (no. 7) with SCC ≤100,000 cells/mL and without prior detection of udder pathogenic microorganisms, showed a CD2/CD21 index of 8.69 at sampling 2.2. While DCC data indicated no inflammation in this quarter (granulocyte proportions 19-29%), minor pathogens were detected and the CD2/CD21 index increased to 37.65 at sampling 2.3. This quarter was classified as false suspicious at sampling 2.2.

Interestingly, in both studies, CD2/CD21 indices >10 were found in quarters of groups IV-0 and IV-1, although values <10 would have been

expected. In studies 1 and 2, four quarters of group IV-0, that were culture-negative at two samplings indicated, CD2/CD21 indices >10 and seemed to be unsuspicious, which was supported by DCC analysis indicating no inflammatory reactions (granulocyte proportions: 10-15%). However, quarter no. 11, which showed CD2/CD21 indices of 5.21 and 7.87 at samplings 2.2 and 2.3, respectively, was an exception. In this quarter, culturing at sampling 2.2 could have been false negative because SCC values >1,000,000 cells/mL and inflammatory reactions (granulocyte proportions: 51-53%) were measured at samplings 2.2 and 2.3. Additionally, CNS were detected at sampling 2.3. One quarter of group IV-0 at sampling 1.2 and four quarters of group IV-0 at sampling 2.3 (quarters no. 14-17), in which minor pathogens were detected at least at one previous sampling, indicated CD2/CD21 indices <10. However, at sampling 2.3 culturing of samples from these quarters could have been false negative, as CD2/CD21 indices were <10 and DCC data revealed inflammatory reactions based on the predominance of granulocytes (54-81%). Interestingly, a further quarter (no. 6) of group IV-0 showing CD2/CD21 indices of 28.17 and 19.67 at sampling 2.2 and 2.3, respectively, cured during our study. In this quarter, bacteriological examinations were only positive at sampling 2.1 (*S. aureus*) and DCC data indicated no signs of inflammation (granulocyte proportions 7-31%).

Quarters in group IV-1 generally revealed CD2/CD21 indices <10. Indeed, in three of eight samples of group IV-1 at sampling 1.2, one of 17 samples of group IV-1 at sampling 2.2, and three of 11 samples of group IV-1 at sampling 2.3, CD2/CD21 indices >10 were found. It is not clear whether the minor pathogens detected in these quarters originated from teat canal colonization or whether they caused an intramammary infection [39,40]. The DCC data of four quarters supported an intramammary infection, since proportions of granulocytes were 77-89% (e.g., quarter no. 17). The remaining three quarters did not indicate any inflammatory reactions (granulocyte proportions 24-39%). However, the CD2/CD21 indices of these seven quarters ranged from 10.87 to 19.93 and should be seen as suspicious, at least in samples of group IV-1.

At sampling 2.1, *S. aureus* was found in six quarters with SCC ≤100,000 cells/mL. Triggered by either infection dynamics or the detection

method, only in one of these quarters, *S. aureus* could be confirmed at samplings 2.2 and 2.3. However, it is unlikely that results of culture were false positive at sampling 2.1, since *S. aureus* can occur at low SCC levels [32,41]. Therefore, it could be hypothesized that negative bacteriological results at samplings 2.2, and 2.3 depend on the intermittent shedding of *S. aureus* [42], presence of antimicrobials or other inhibitors in milk [43]. At the time of examination, pathogens could also be ingested by phagocytes or survive intracellularly in the host [44,45]. Shedding of amounts of pathogens, which are too low, or ceased growth may be further reasons for negative bacteriological results [42]. A recent study [46] described about 6-10% of quarters infected with *S. aureus* where the microorganism was not shed in the milk. In another survey [42], a percentage of false-negative results, obtained from milk bacteriological analysis in the case of quarters infected with *S. aureus*, of even 40% was determined. In any case, based on CD2/CD21 indices <10 at samplings 2.2 or 2.3 four of six quarters that indicated SCC ≤100,000 cells/mL and *S. aureus* at sampling 2.1 could be classified as suspicious. In total, suspicious CD2/CD21 indices <10 in 14 of 15 quarters with SCC ≤100,000 cells/mL were explainable by the detection of minor or major pathogens. Hence, it can be speculated that the CD2/CD21 index is connected with the current or former presence of pathogens in the mammary gland. Since we performed field studies and therefore do not know the stage of the infection in the quarters analyzed or the exact infection dynamics, specific infection studies with major pathogens (e.g., *S. aureus*) and minor pathogens (e.g., CNS) are necessary to obtain further understanding of the CD2/CD21 index.

Our statistical analysis indicated that the farms (study 1) had a significant impact on the percentages of CD2$^+$ lymphocytes. This effect may be due to a non-randomized selection of the cows within the farms and different numbers of cows selected per farm. While cows with healthy mammary glands were predominantly selected from farms A and C, samples from cows with diseased quarters were predominantly collected from farm B. In our examinations, the CD2/CD21 index was neither influenced statistically by the farm, quarter position, lactation number nor by days in milk.

The CD2/CD21 index provides a trend for the characterization of udder health. Our examinations showed that values of 10 may be suitable to aid

differentiation between unsuspicious and suspicious or diseased udder quarters. Compared to the well-established SCC indicator, the CD2/CD21 index confirmed our working hypothesis of values >10 in samples with SCC ≤100,000 cells/mL and values <10 in samples with SCC >100,000 cells/mL in 171 of 198 quarters analyzed. CD2/CD21 indices of 26 of the remaining 27 quarters were explainable by the detection of pathogens or DCC analysis. Our examinations led us to suspect a connection between the CD2/CD21 index and the current or former presence of mastitis pathogens. Further research in this field should concentrate on longitudinal examinations of $CD2^+$ and $CD21^+$ cells in the milk of mammary glands specifically infected with mastitis pathogens.

Acknowledgements

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8th Chapter

Feedback-based, system-level properties of vertebrate-microbial interactions

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Abstract

Background: Improved characterization of infectious disease dynamics is required. To that end, three-dimensional (3D) data analysis of feedback-like processes may be considered.

Methods: To detect infectious disease data patterns, a systems biology (SB) and evolutionary biology (EB) approach was evaluated, which utilizes leukocyte data structures designed to diminish data variability and enhance discrimination. Using data collected from one avian and two mammalian (human and bovine) species infected with viral, parasite, or bacterial agents (both sensitive and resistant to antimicrobials), four data structures were explored: (i) counts or percentages of a single leukocyte type, such as lymphocytes, neutrophils, or macrophages (the classic approach), and three levels of the SB/EB approach, which assessed (ii) 2D, (iii) 3D, and (iv) multi-dimensional (rotating 3D) host-microbial interactions.

Results: In all studies, no classic data structure discriminated disease-positive (D+, or observations in which a microbe was isolated) from disease-negative (D–, or microbial-negative) groups: D+ and D– data distributions overlapped. In contrast, multi-dimensional analysis of indicators designed to possess desirable features, such as a single line of observations, displayed a continuous, circular data structure, whose abrupt inflections facilitated partitioning into subsets statistically significantly different from one another. In all studies, the 3D, SB/EB approach distinguished three (steady, positive, and negative) feedback phases, in which D– data characterized the steady phase, and D+ data were found in the positive and negative phases. In humans, spatial patterns revealed false-negative observations and three malaria-positive data classes. In both humans and bovines, methicillin-resistant *Staphylococcus aureus* (MRSA) infections were discriminated from non-MRSA infections.

Conclusions: More information can be extracted, from the same data, provided that data are structured, their 3D relationships are considered, and well-conserved from recently developed host-microbial interactions. Applications include early diagnosis, error detection, and modeling.

Introduction

The rate of undetected infections remains markedly elevated and may be increasing [1-3].

Pathogens that develop resistance to antimicrobials pose new challenges, such as methicillin- or multidrug-resistant *Staphyloccocus aureus* (MRSA) infections, which, in the USA, cause more deaths than tuberculosis, AIDS, and viral hepatitis combined [4]. Macro-parasite-mediated diseases are also associated with high levels of drug resistance [5]. To enhance the detection of infectious disease-related data patterns, new approaches are required.

To that end, systems biology (SB) and evolutionary biology (EB) may be considered. To diminish data variability, EB focuses on biological features well conserved in evolution [6-12]. However, in infectious diseases, EB has not yet provided usable methods [6]. Unlike reductionistic approaches, which only consider a few or static variables, SB focuses on systems and their dynamics – a feature that may extract more information from the same data [13-18].

However, before SB/EB concepts are explored within the context of infectious diseases, we need to remind ourselves that we live in a three-dimensional (3D) environment [19]. And yet, the data we are exposed to are mainly 'flat', such as anything reported on a page or screen. Such formats are bi-dimensional: they lack the third dimension (depth). Bi-dimensional(2D) are poor (if not also, biased) descriptions of three- (four- and/or multi-) dimensional data structures. Only 3D plots (volumes) can express all the combinations (points, lines, or surfaces) biological data can generate [20]. Furthermore, rotating 3D plots could inform whether perspective (the angle under which the data are assessed) influences pattern detection [21].

In spite of such possibilities, 3D data analysis seems to be under-utilized in the area of infectious diseases. In October of 2012, a search conducted in the Web of Science[©] yielded >18,000 hits when 'three-dimensional' and 'data analysis' were queried, but less than 100 hits were retrieved when 'infection' was added.

While feedback is a function of interest in both SB and EB and it has been known for at least half a century in medicine and two millennia in physics

[22-25], feedback has only marginally been explored in infections. In October of 2012, more than 200,000 bibliographic hits could be retrieved under 'feedback' and ~1700 hits were yielded when 'feedback' and 'definition' were searched for, but less than 50 hits were found when 'infection' was added. Even though the precursor of feedback ('homeostasis') was first proposed in 1932 [26], and, in 1956, the phrase 'negative feedback' was first published in biology [27], only after the concept was introduced in engineering, feedback was fully adopted in biology. After the emergence of system dynamics, non-linear approaches have been applied to study feedback phases [28].

In its simplest version, *feedback* can be defined as the ability of a system to adjust its output in response to monitoring itself [29]. An expanded definition, which defines as dynamic any situation in which some quantity increases or decreases over time [30, 31], regards feedback as a process that involves an interaction between two or more elements (e.g., a microbe and a host) which is designated *positive* when the activation or accumulation of one component leads to the activation or accumulation of the other component, and *negative* when the activation or accumulation of one component leads to the deactivation or depletion of the other component [29]. *Positive* feedback occurs when a signal induces more of itself, or of another molecule that amplifies the initial signal, and this serves to stabilize, amplify or prolong signaling. *Negative* feedback occurs when a signal induces its own inhibition [29].

Feedback exhibits loops or closed chains in which change in one component is fed back to its origin [31, 32]. Other feedback structures are: (i) nodes, (ii) cyclic data patterns, (iii) directionality, and (iv) connectivity [24, 31, 33]. 'Nodes' refer to data groups where processes begin and end, and/or where data inflections may occur. Thus, feedback is a deterministic process, characterized by abrupt transitions from low to high (or high to low) activity [24, 31]. When high-level structures are assembled, feedback also reveals emergent properties [7-9].

Feedback emergent properties (the result of combinatorial theory and organizational complexity) can be explained with a mundane example that involves language. When we consider any list of letters, no meaning is obtained. However, when a few letters are combined, words emerge – and, with them, meaning emerges. When we combine words, sentences emerge, which

elicit more information. Information does not depend on any one letter: it depends on combinations of letters (words). While low-level data (letters) lack information, information is created (and increases) when higher levels (words, sentences, paragraphs, and so forth) are used. Typically, rich (interpretable and usable) information emerges from the highest of such levels.

Similarly, the ability of a biological system to perform many functions with a few resources depends on its combinatorial potential, which is expressed as multiple structural levels [34]. Therefore, to design a method that discriminates infectious disease-related data patterns, at least three aspects or features should be considered: 1) multi-dimensionality, 2) combinatorial theory, and 3) various levels or scales.

However, 'level' is an elusive concept. On the one hand, it may be synonymous with 'organizational complexity', which may be a dimensionless concept. On the other hand, 'level' may be measurable and synonymous with 'scale', as in the continuum that includes molecular, cellular, multi-cellular, organ, individual, population (group of individuals), species, groups of species (e.g., vertebrates), and ecological scales. Because both connotations may apply, new methods should adopt indicators inherently combinable, which are applicable across biological scales and can assess relationships, such as those created by multi-cellularity [35,36].

Such relationships, to be detected, require 'functional data integrity.' By that we refer to the fact that the anti-microbial immune system is indivisible and, consequently, no leukocyte type ever works alone. 'Functional data integrity' alludes to the ability of measuring interactions (multi-factor relationships), not just one element [14]. Unlike 'elementary variables', 'structured indicators' can estimate functions, e.g., early anti-microbial responses.

The difference between 'elementary variables' and 'structured indicators' has been described before. While an 'indicator' possesses *links* – which establish a *temporal connectivity* and, therefore, reveal *directionality* and *causality* –, a simple variable, such as the percentage of neutrophils, lacks such information [31]. Hence, 'functional data integrity' summarizes all previous concepts with an observable set of properties: (i) it is the opposite of 'fragmentation' – it includes data from all cells of the immune system, i.e., it possesses 'integrity', (ii) it is inherently combinatorial, that is, it may generate a

large number of 'words', and probably 'sentences', even though its primary components (cell types) are as few as or fewer than the letters of any language; and (iii) such combinations may ultimately gauge critical biological functions, such as feedback functions – which may emerge from interactions that involve several biological scales and, to be optimally detected, should be measured in 3D space. That translates as measuring not the percentage of a single cell type but, for instance, the ratio between lymphocytes and macrophages – a multi-cellular interaction essential in antigen recognition [37].

To measure interactions, compositional data may be considered. Compositional data can provide relative information (information on one factor in relation to another). Such information is based on the use of ratios [38, 39]. Leukocyte data are compositional: their relationships can be expressed as relative ratios [40]. Compositional data possess scale invariance: information (interpretable and usable) data patterns can be expressed, regardless of the (molecular/ cellular/ multi-cellular/ organ/ population/ species/ ecological) scale of the data [41-43].

To complete the list of desirable criteria an informative method should possess, data variability ('noise') should be reduced and pattern recognition should be enhanced. Noise is reducec, if not eliminated, when a single linge of observations is generated. Data patterns, if present, are likely to be detected when a single line of data points are observed.

Informative patterns, such as data inflections, as well as a single line of data points, can be generated when these conditions are met: (i) functional data integrity is applied (data from all cell types are considered), (ii) a 2D plot is created in which, on one axis, the percentage of one cell type is expressed, and a ratio is recorded on the second axis, and (iii) the denominator of such ratio is the same percentage expressed on the first axis. We call such indicators 'anchors', e.g., the 2D set that includes the lymphocyte (L) % (axis 1) and the phagocyte (macrophage [M] and neutrophil [N])/L ratio (axis 2). Because, in this structure, all data points are 'anchored' along a single line, noise is substantially reduced. Because, to build 'anchors', only two axes are required, a third axis remains available, in a 3D plot, to assess any additional variable.

Discrimination is also improved when bio-numerical properties are considered in the design of the indicators, as when two ratios are plotted

together, and the numerator of one ratio is the denominator of the other ratio (e.g., the neutrophil per lymphocyte ratio [N/L ratio] vs. the mononuclear cell [MC, or L and M]/N [MC/N ratio]). In such a structure, when one ratio increases, the other ratio decreases. This structure acts as an 'amplifier': even when changes are quantitatively small, distinct (usually orthogonal) patterns can be revealed.

When 'amplifiers' are used and biological knowledge is included in the design, temporal changes can be assessed. That can be achieved when one ratio estimates early host-microbial responses and the other ratio expresses late responses. For example, a 2D plot that includes N/L and MC/N ratios expresses early responses when the N/L ratio is high (e.g., much greater than 1), or late responses when the MC/N ratio is >1 [44-46]. Such structure can distinguish the temporal sequence of biological responses regardless of chronological scales (minutes/hours/days) and is robust to the absence (or presence) of slow (or fast) immune responders [47].

While the cyclic nature of feedback features is useful to describe dynamics [48-50], to detect infectious disease dynamics, logical aspects should also be addressed. Fallacies may occur at the earliest stage of an investigation, when a hypothesis is postulated. For instance, when the hypothesis assumes that only two alternatives are possible (e.g., one disease-positive [D+] and one disease-negative [D–] data class [51]), but three or more alternatives exist, errors will follow.

Hence, using assumption-free, structured indicators (designed to possess functional data integrity and reduce noise), the multi-dimensional patterns of host-microbial interactions were explored. Two questions were asked: 1) can SB/EB indicators reveal feedback phases?, and 2) can such indicators be used to enhance the detection of infectious disease-related data patterns?

Materials and Methods

Materials

Leukocyte data and microbial test results were collected in: 1) bacterial infections induced by methicillin- or multidrug-resistant *Staphylococcus aureus*

(MRSA) and non-MRSA bacterial infections of bovines and humans, 2) parasite (*Plasmodium falciparum*) infections that affected humans; and 3) viral (West Nile virus) infections experimentally induced in chickens. Six evaluations – three longitudinal and three cross-sectional studies – were conducted.

Method

Leukocyte data (heterophils, granulocytes, or neutrophils [N]; macrophages or monocytes [M]; and lymphocytes [L]) were structured as described earlier. Leukocyte and microbial procedures are described in Text S1 of Supporting Information, which includes a glossary [52-64]. Briefly, tables and generic, and goal-related analyses were created or processed as follows:

A. Data organization and table building.
 i. A table was created in which columns included primary variables (L%, N%, M%, their counts, as well as microbial test results).
 ii. Additional columns included secondary variables, e.g., the percentages of (a) phagocytes (P, or N+M), (b) mononuclear cells (MC, or L+M), and (c) the remaining alternative, here named 'small leukocyte %' (SL, or L+N).
 iii. Later, tertiary variables were added to new columns, which denoted interactions, such as the N/L, M/L, M/N, P/L, MC/N, and SL/M ratios; e.g, the N/L ratio was calculated by dividing the N% over the L%. Hence, 12 leukocyte-related variables were created out of the 3 original percentages (through combinations, the number of variables was expanded four times). However, more combinations were created when the analysis was conducted.

B. Generic analysis.
 i. When the goal was to produce a single line of data points, 'anchors' were selected.
 ii. When enhanced discrimination was pursued, 'amplifiers' were chosen; for instance, if a 2D plots was used, the N/L ratio was plotted on one axis and the M/N ratio on the other.
 iii. When both effects were pursued, a 3D plot was utilized and one variable performed two roles, e.g., the set that includes the N%, the MC/N and N/L

ratios is both an 'anchor' (MC/N vs. N%) and an 'amplifier' (the N/L vs. the MC/N).

iv. Because a ratio has two expressions (such as the L/M and the M/L ratio), both versions of each ratio were analyzed (a strategy that doubled the number of possible analyses).

C. Applications or goal-depending analysis.

i. To enhance discrimination, 3D plots were rotated until a data inflection was displayed and one corner of the plot displayed the zero value of all the three axes, as shown in Figure 1.

ii. To determine directionality, temporal data were assessed.

iii. To detect emergent properties, microbial data were considered.

iv. To determine the role of perspective, 3D plots were rotated.

v. To explore robustness, different species/pathogens were explored under the same angle.

vi. To detect different subsets of the same data class, the 3D plot was rotated until the highest values of two indicators were observed on opposite corners (as shown in Figure 1). In addition, the size of symbols representing non-relevant features was decreased, so only the features of interest were emphasized, e.g., if the goal was to detect false negatives, D+ symbols were reduced; if the goal was to detect ≥2 D+ stages, D− symbols were reduced.

Results

<u>Feedback-related patterns</u>

The use of SB/EB indicators in an experimental study of virally-infected chickens revealed 10 properties or features: 1) functional data integrity, 2) a single line of data points, 3) data inflections, 4) a circular data structure, 5) directionality of the temporal responses, 6) patterns that suggested three feedback phases, 7) two distinct D+ subsets, 8) overshooting (a D+ subset with higher MC/N values than D− observations), 9) information of prognostic value, and 10) low data variability (Figure 1 A).

Functional data integrity was achieved because each observation expressed values contributed by all cell types. Each data point estimated three interactions: 1) the relationship between neutrophils and lymphocytes, 2) that between mononuclear cells and neutrophils, and 3) the overall or 'high-level' interaction, generated by the two interactions mentioned above.

The observed *single line of observations* revealed *circularity*, which was characterized by three major *data inflections*: the first inflection was observed within one day post-inoculation (1 dpi) with West Nile virus (WNV, green arrow, Figure 1 A); around 5 day post-inoculation (5 dpi), a second data inflection was observed (red arrow, Figure 1 A); which was followed, almost immediately, by the last inflection (blue arrow, Figure 1 A). Because temporal observations displayed *directionality*, three data ranges were distinguished in Figure 1 A: 1) that of the *steady feedback* phase (0 dpi or D– data [green symbols, of which 80% were within the range indicated by the green box]); 2) away from the steady state phase (between 1 and 5 dpi), in which D+ data predominated (*positive feedback* phase [red symbols]); and 3) the *negative feedback* phase (after 5 dpi), in which, over time, D+ data (blue symbols) approached the data range of the steady phase. The end of the feedback function was signaled when the latest (14-dpi) observations reached values similar to those they started with (sky blue symbols).

Hence, *two D+ subsets* (early D+ and late D+ observations) were distinguished. The late D+ subset was characterized by high MC/N values – observed around 5 dpi –, which displayed *overshooting*, that is, greater MC/N values than those of D– data points. Because the latest (14 dpi) D+ data points did not differ from D– values, it was concluded that high MC/N, D+ individuals had a favorable *prognosis*: such pattern indicated the beginning of the return to the steady status. Because most early and late observations were located on opposite sides of the plot analyzed, both *low variability* and *enhanced discrimination* were documented (Figure 1 A). To facilitate visualization, each feedback phases is emphasized in Figures 1 B-D.
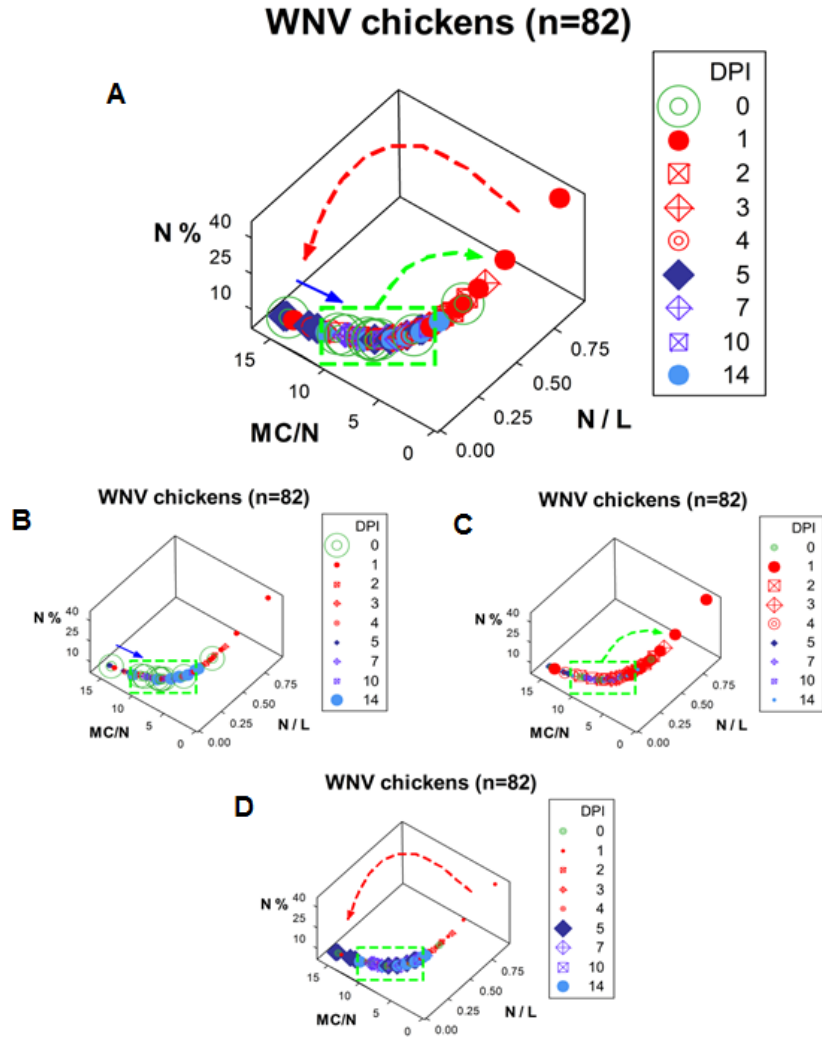
Fig. 1. Feedback patterns of avian longitudinal-experimental immune responses against West Nile Virus. **A**: Leukocyte and microbial test results of 10 chickens (shown to be West Nile virus [WNV] negative at day 0) were inoculated with WNV and followed over two weeks (total: 82 longitudinal observations). The 3D relationship that included the heterophil (N) %, the ratio of N per lymphocyte (N/L), and the mononuclear cell/N (macrophage plus lymphocyte/N or MC/N) ratio showed three major data inflections: 1) a double 90-degree inflection was observed between pre-inoculation (0 dpi) and one day post-inoculation (1 dpi) data points (green arrow), indicating that the N/L ratio increased within a 24-hour period, and that high N/L observations were D+ (red symbols); 2) at, approximately, 5 dpi, a second data inflection was observed (red arrow), which was associated with high MC/N and low N/L values; and 3) soon after 5 dpi, the third data inflection took place, indicating the beginning of the return to the steady phase (blue arrow). The third phase was characterized by the gradual decrease of MC/N values (deep blue symbols). The last phase ended when 14-dpi observations (sky blue symbols) displayed leukocyte values similar to those of 0 dpi (D−) data (green symbols, of which 80% were within the data range indicated by the green box).

Together, a quasi-circular, closed, temporal progression was detected, in which three feedback phases were differentiated: 1) the steady phase (green symbols), 2) the positive phase (red symbols), and 3) the negative phase (blue symbols). Because observations that differed less than 24 hours (0- vs. 1-dpi data) were clearly separated, these patterns could detect early inflammatory responses, even in the absence of microbial data. These patterns distinguished two D+ classes (red and blue symbols). Because D+ observations that revealed 'overshooting' (higher MC/N values than those of D– data) later approached the D– stage, D+ individuals showing high MC/N values may have a favorable prognosis. **B-D**: To facilitate visual detection of patterns specific of each feedback phase, the same data displayed in A are shown emphasizing: only the feedback steady phase (**B**), only the early (positive feedback) phase (**C**), and only the late (negative feedback) phase (**D**). Utilizing a different quantitative method, these data have been partially reported elsewhere [52].

Reproducibility of feedback-like patterns

The reproducibility of feedback patterns was investigated across species and diseases (Figures 2 A-H). Longitudinal bovine leukocyte profiles, assessed together with bacteriological test results (Figures 2 A, B), showed patterns similar to those observed in birds, such as a single line of data points, circularity and directionality (arrows, Figure 2 A). While the, predominantly, cross-sectional nature of the human data prevented the full determination of temporal features (Figures 2 C, D), a subset of 5 D+ children, who were tested twice, also revealed, partially, the directionality shown by birds and cows: a group of high MC/N, D+ children, when tested two weeks later, was D– (blue arrow, Figure 2 C). The fact that 5 children (D+ at their first test) were D– two weeks later, suggested, again, that leukocyte-microbial profiles (high MC/N, D+ data) can have prognostic applications. Even though the spontaneous nature of bovine MRSA infections could not show a D– profile (no 'day 0' data were available, Figures E, F), the bovine MRSA data revealed the same (early vs. late) temporal patterns observed in other studies (arrows, Figures 2 E). Although longitudinal data were not available in study in which humans were infected with bacteria, a distinct pattern was observed: MRSA observations did not express overshooting (no MRSA infection displayed high MC/N values), while non-MRSA data did (Figure 2 G). False negative results were suggested by human and bovine data: some high N/L values were associated with microbial-negative results (black boxes, Figures 2 C-F). The false negative hypothesis was

confirmed in humans: 8 microbial-test negative, high N/L children were febrile (8 black circular symbols, one within a black box, Figures 2 C, D, H).
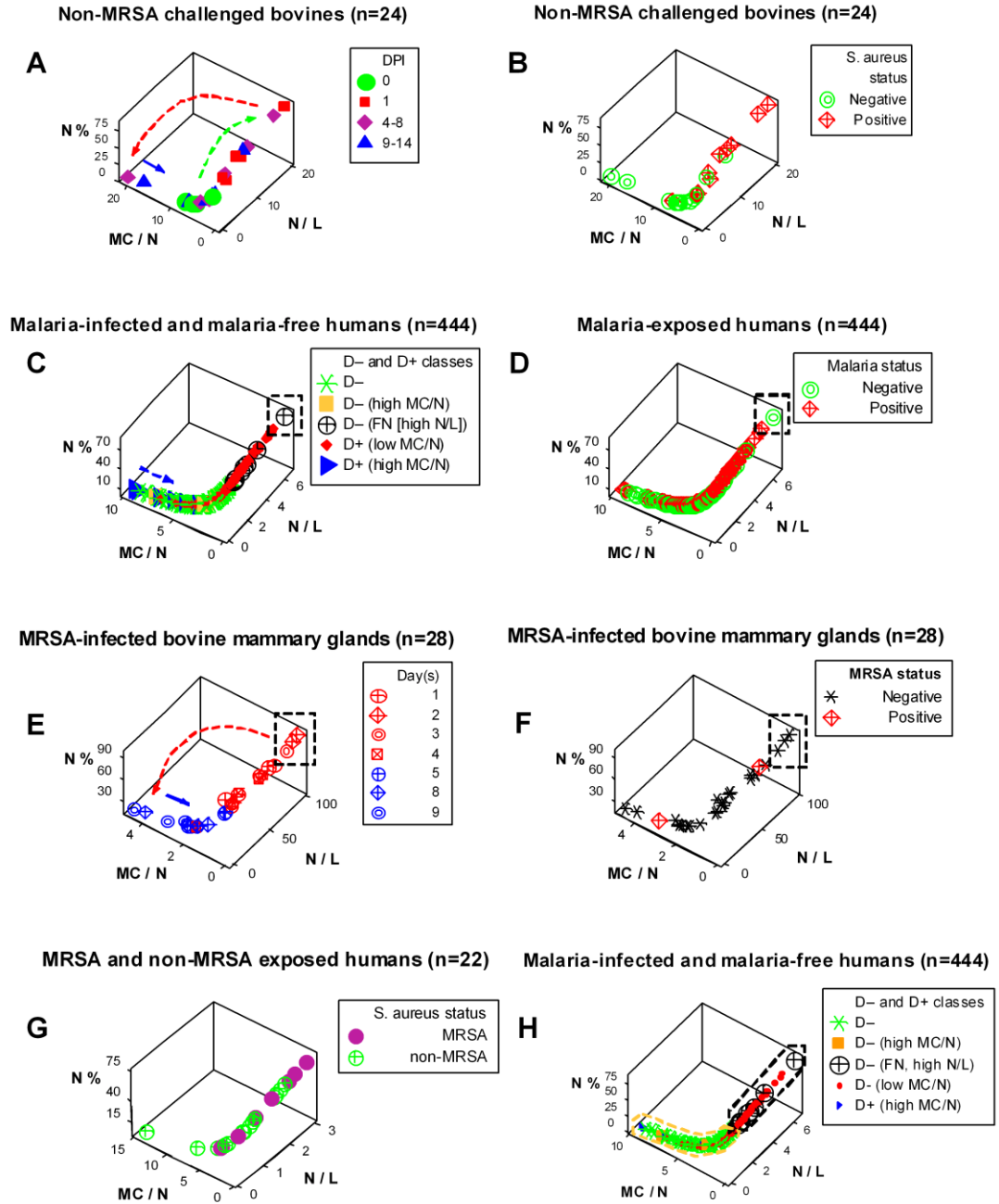

Fig. 2. Reproducibility of feedback patterns across species and pathogen types. Bovines and humans exposed to either bacteria (both sensitive and resistant to anti-microbials) or parasites showed patterns similar to those displayed by birds (**A-F**). **A, B**: Leukocyte profiles and microbial test results of 6 dairy cows inoculated at day 0 with non-methicillin resistant (non-MRSA) *Staphylococcus aureus*, followed over two weeks, are reported (total: 24 longitudinal observations, data previously reported, using a different analytical method [44]). **C, D**: Leukocyte

profiles of 439 humans non-infected or infected by malaria, tested once, of whom five displayed high MC/N values and malaria-positive test results, and were tested twice (two weeks apart), becoming malaria-negative in the later test (total: 444 observations, data previously reported, using a different analytical method [61]). **E, F:** Longitudinal profiles of bovine mammary gland leukocytes collected from a cow spontaneously infected with methicillin-resistant *S. aureus* (MRSA, total: 28 longitudinal observations or 7 daily tests per mammary gland, a study previously reported, in which a different analytical method, a different technology, and different samples were utilized [54]). **G:** Cross-sectional leukocyte profiles of humans infected by either MRSA (n=7) or non-MRSA (n=15) bacterial isolates (data not previously reported). DPI: day(s) post-inoculation with non-MRSA. DAYS: consecutive days since MRSA was isolated (day 1= day of first isolation). Left columns show temporal data, in longitudinal studies (**A, E**); or disease-positive (D+) and disease-negative (D–) malaria-related data subsets (**C**). Right columns display microbial test results (**B, D, F, G**). Microbial-negative results that revealed high N/L values were suspected to be false negative (boxes, **C-F**). In the malaria study, 8 false negatives were detected (8 black circular symbols, of which one is shown within a box, **C**), which were associated with fever. Arrows indicate the directionality of temporal responses (**A, C, E**). **H**: To facilitate visual detection of patterns, the same data displayed in plot C are shown again, with emphasis on D– data (the symbols of D+ data are reduced in size). A data infection is observed, which distinguishes two D– subsets. The high N/L subset (black polygon), as indicated in the main text, was suspected (and later confirmed) to include false negatives.

From no discrimination to discrimination of host-microbial interactions

Discrimination of health status was lost when individuals, not populations, were analyzed (Figures 3 A-J). Some birds were fast responders – they showed patterns typical of late D+ responses as early as one day after challenge (boxes, Figures 3 A, G), while one bird did not display high MC/N values at any time (oval, Figure 3 C). Such differences in responsiveness were observed even though the birds included in this study were randomly selected.

Discrimination was also lost when 'functional data integrity' was not considered (when each leukocyte type was assessed alone). When only the percentage of neutrophils (lymphocytes, or macrophages) was assessed, bovine MRSA and non-MRSA data overlapped (Figure 4 A).
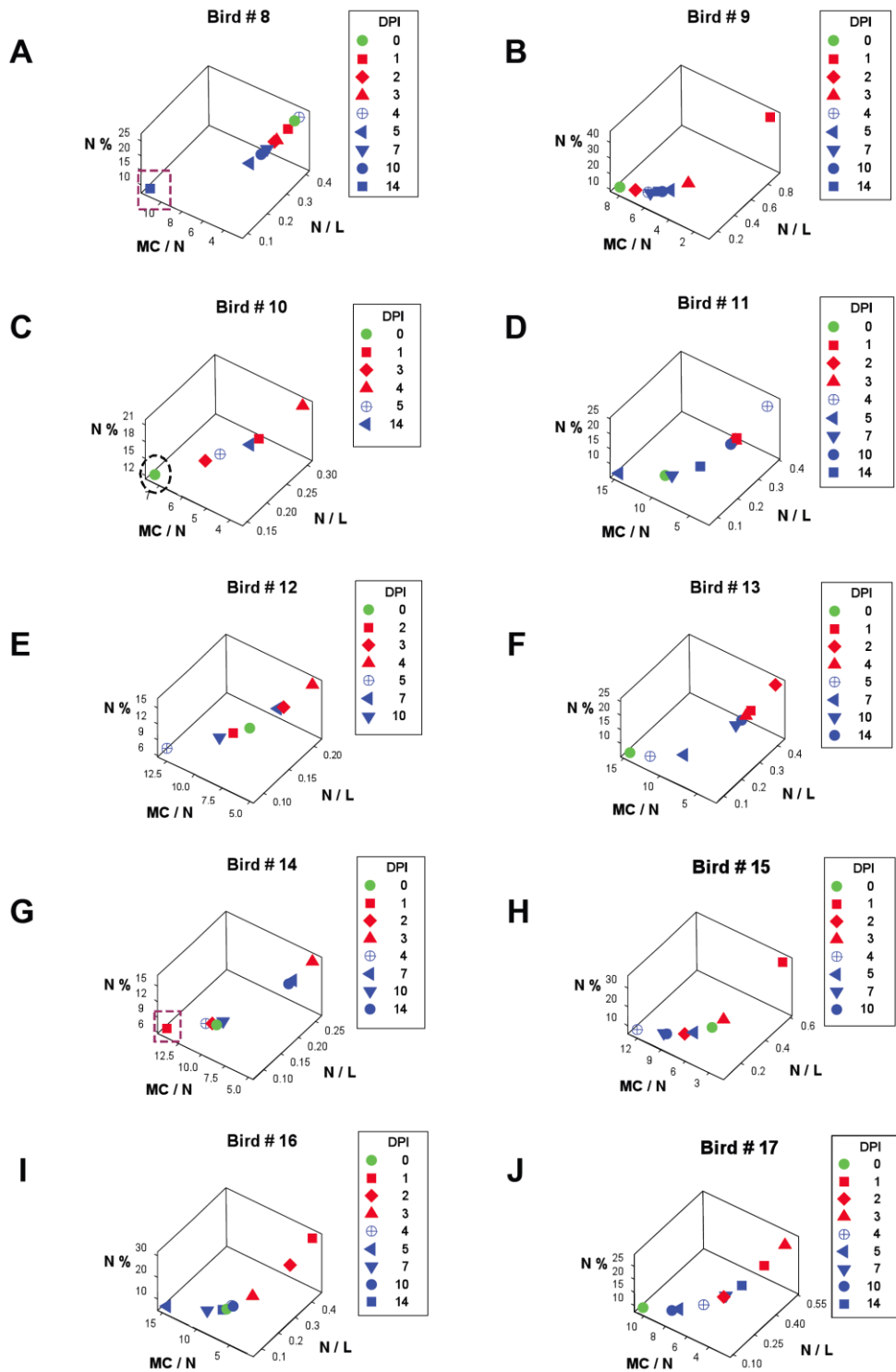
Fig. 3. **Responsiveness of individuals – avian examples.** The same avian data previously analyzed at the population scale (Figures 1 A-D) were assessed at the individual scale (**A-J**). Even though chickens were selected through randomization, high variability was found. For instance, two birds (#8 and 14) were fast responders: as early as 1 day-post inoculation (dpi) with West Nile Virus, they showed leukocyte profiles typical of the late or negative feedback phase (square boxes, **A, G**), In contrast, at least one bird did not display overshooting (no D+ observation of that bird #10 displayed MC/N values greater than the D– [0-dpi] data point, oval, **C**).

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Table 1. Comparisons between MRSA and non-MRSA profiles, and among MRSA subsets

Data classes or subsets	Variables	*P* value (Mann-Whitney test)
Non-MRSA (all observations) vs. MRSA	N / L	<0.01
Non-MRSA (all observations) vs. MRSA	MC / N	<0.01
Non-MRSA post-challenge vs. MRSA	N / L	<0.01
Non-MRSA post-challenge vs. MRSA	P / L	<0.01
MRSA class A vs. MRSA class B	SL / M	<0.01
MRSA class A vs. MRSA class B	M / N	<0.01
MRSA class A vs. MRSA class B	P / L	<0.01
MRSA class A vs. MRSA class C	N / L	<0.01
MRSA class A vs. MRSA class C	P / L	<0.01
MRSA class B vs. MRSA class C	N / L	<0.01
MRSA class B vs. MRSA class C	MC / N	<0.01

Table 2. Cross-sectional bovine non-MRSA infections

Population	Prevalence (%)		Examples of bacterial species isolated	
	Major pathogens	Minor pathogens	Major pathogens	Minor pathogens
CS I (n=120)	27.5 %	13.3 %	- *Staphylococcus aureus*	- *Staphylococcus chromogenes*
CS II (n=500)[1]	9.8 %	5.2 %	- *Escherichia coli*	
CS III (n=429)[2]	9.9 %	5.3 %	- *Streptococcus dysgalactiae*	- *Staphylococcus hyicus*
CS IV (n=80)[3]	2.5 %	2.5 %	- *Streptococcus uberis*	- *Corynebacterium ssp.*
CS V (n=80)[4]	6.5 %	23.8 %	- *Klebsiella pneumoniae*	
CS VI (n=188)[5]	13.3 %	11.2 %		

[1]Raw data partially or totally reported elsewhere [55].
[2]Raw data partially or totally reported elsewhere [56].
[3]Raw data partially or totally reported elsewhere [57].
[4]Raw data partially or totally reported elsewhere [58].
[5]Raw data partially or totally reported elsewhere [59].

In contrast, SB/EB indicators distinguished non-MRSA from MRSA data: while non-MRSA data displayed 'left overshooting' – higher MC/N values than than those of MRSA data points –, all four mammary glands of the MRSA cow showed 'right overshooting' (higher N/L values than those of non-MRSA infections, Figure 4 B). The MRSA profile was detected even when compared against a large, cross-sectional, non-MRSA dataset (Figure 4 C, Table 1; see Table 2 for further details on the non-MRSA, cross-sectional data). Even though no MRSA was isolated in three bovine mammary glands, all four mammary glands of the MRSA cow showed similar leukocyte profiles, which revealed
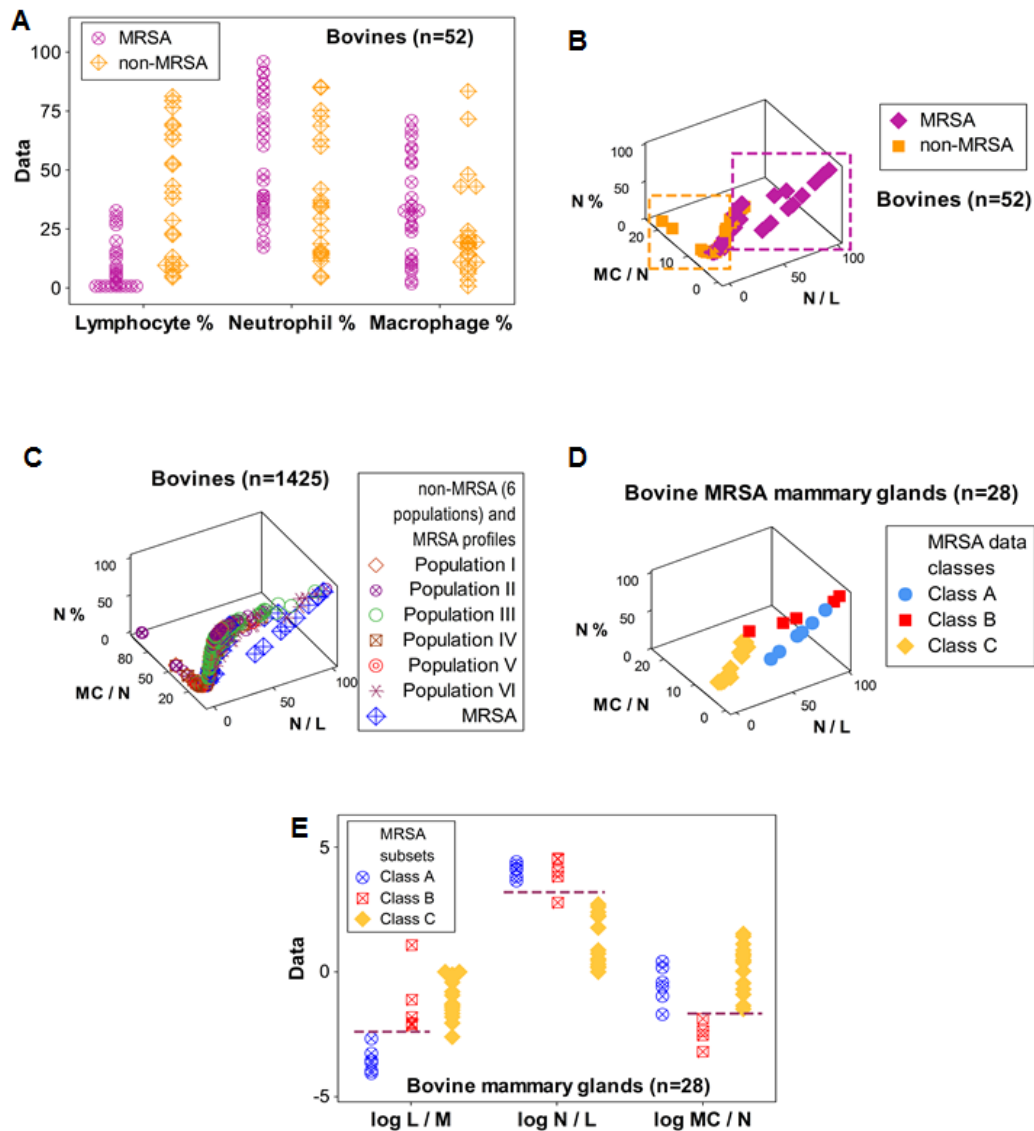
Fig. 4. Discrimination between bovine MRSA and non-MRSA patterns. While no leukocyte percentage discriminated between methicillin- or multidrug-resistant *S. aureus*–infected (MRSA) and non-MRSA bovines (**A**), a three-dimensional (3D) plot that utilized SB/EB indicators distinguished MRSA from non-MRSA patterns, e.g., MRSA observations displayed higher N/L values than non-MRSA data points, while higher MC/N values were revealed by non-MRSA observations (**B**). The MRSA profile was differentiated even when compared against a large, cross-sectional bovine dataset (**C**). Regardless of microbial test results, 3 MRSA data classes were detected (**D**). When, based on 3D patterns, the MRSA data were partitioned, each MRSA data class (A, B, C) was distinguished by one or more indicators, and non-overlapping distributions were observed, which differed from one another at statistically significant levels (*P*<0.01, Mann-Whitney test, Table 1, **E**). Horizontal lines indicate full discrimination (non-overlapping data distributions) between two or more MRSA classes (**E**). Although utilizing a different quantitative method, bovine cross-sectional data of populations II-VI (**C**) have been partially or totally reported before [55-59].

3 data classes (A, B, and C). Figure 4 D shows that the distributions of classes A-C did not overlap. All three MRSA data subsets were statistically distinguished by, at least, one indicator ($P<0.01$, Mann-Whitney test, Table 1; and Figure 4 E).

To determine whether perspective influences pattern detection, both human and bovine *S. aureus*-positive data were analyzed under different angles. The set that included leukocyte counts (human blood white cells or WBC, and bovine somatic cells or SCC [milk cells mainly composed of leukocytes]), the percentage of mononuclear cells (MC %) and the N/L ratio revealed a subset of data points that was only or mainly composed by MRSA observations (red polygons or circles, Figures 5 A-F). This subset did not overlap with the remaining (MRSA and non-MRSA) data points. When the data were analyzed under two different angles, between three and five data points were found within the human MRSA-only cluster (Figures 5 E, F). Hence, perspective may indeed alter the number of observations detected with a particular feature.

Because the human and bovine MRSA-only clusters revealed similar values (Figures 5 C-F) and the bovine cluster included the earliest observations (days 1-4, Figures 5 C, D), the MRSA-only cluster was suspected to express early infections. The early (MRSA-only) cluster differed statistically from the remaining data points ($P<0.01$, Mann-Whitney test, Figure 5 G).

Other indicators (that possessed functional data integrity but did not meet 'anchoring' criteria) confirmed patterns shown by the indicators described above. For instance, in the malaria study, the indicator set that measured the M/N (not the MC/N) ratio identified the same 8 data points regarded to be false negatives (arrows, Figure 6 A; data also shown in Figure 2 H).

When a different 'anchor' (composed of the SL/M ratio and the M%) was used to analyze the malaria data, two D+ subsets were distinguished (Figure 6 B). Because the D+ subset with the highest M% and lowest SL/M values indicated a recovery profile, children in that subset were examined 14 days later. At that time point, all previously D+ children were D– and showed a distinct, non-overlapping leukocyte profile (Figure 6 C). The changing pattern observed over two weeks, which supported a favorable prognosis, displayed a 3D data inflection (Figure 6 D).
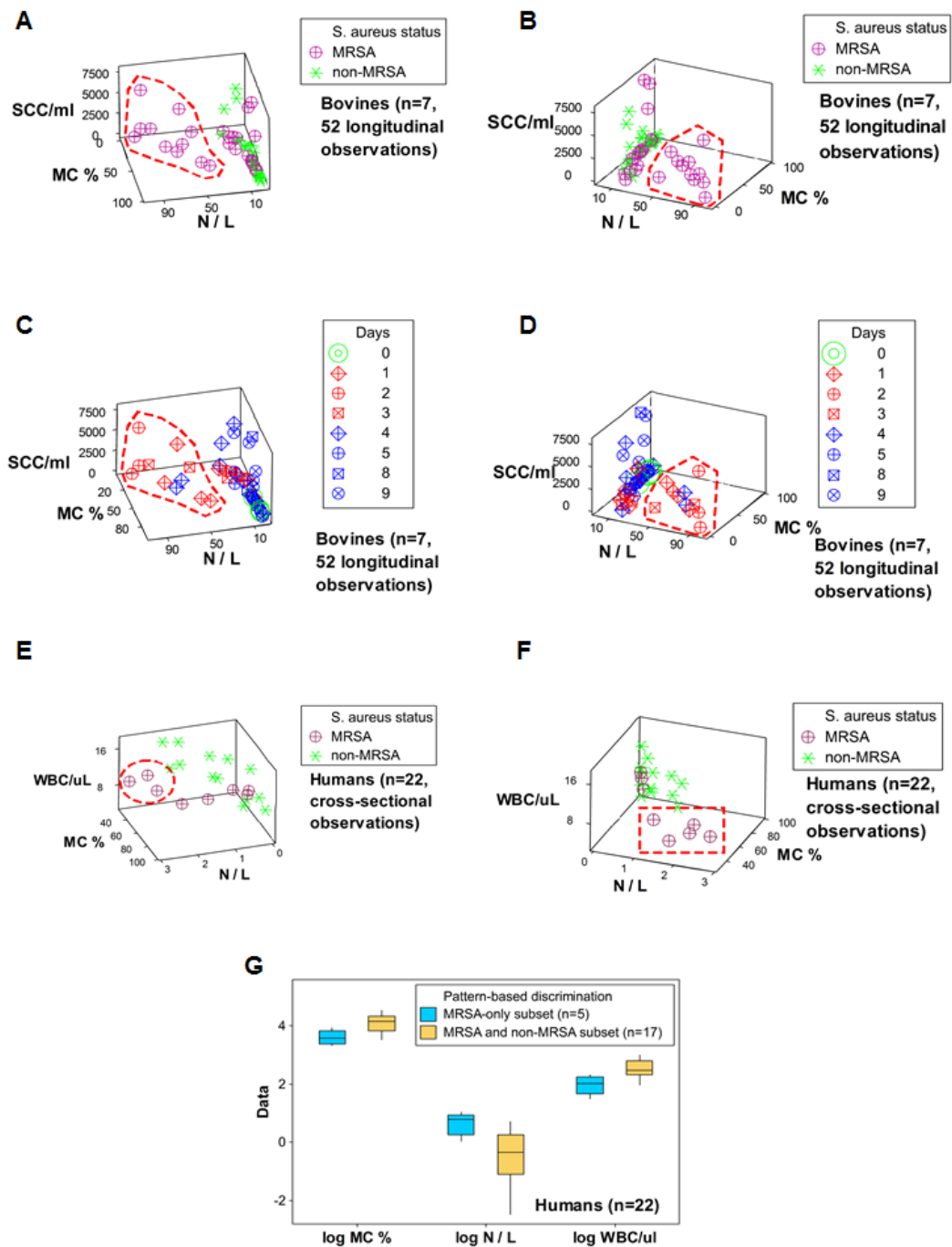
Fig. 5. The role of perspective: discrimination between early MRSA and other (MRSA and non-MRSA) patterns, in bovines and humans. MRSA and non-MRSA induced leukocyte profiles were investigated in bovines and humans. In both species, non-MRSA individuals were infected by methicillin-susceptible *S aureus*. Two 3D perspectives of the same data were analyzed in MRSA and non-MRSA bovine infections (**A, B**). When the total leukocyte count (thousands of milk cells or 'somatic cell counts' [SCC]) was assessed together with the mononuclear cell (MC) percent and the N/L ratio, two data subsets were differentiated: one was characterized by MRSA-only observations, while the other data subset included both MRSA and non-MRSA observations

(**A, B**). The MRSA-only subset was predominantly composed of early observations (days 1-4, red polygon, **C, D**). When human blood leukocyte counts (hundreds of white blood cell [WBC] counts), collected from MRSA and non-MRSA infected humans were investigated, a MRSA-only subset was observed, which was defined by the same parameters utilized with the bovine data: low MC% and high N/L values (**E, F**). Because the number of observations found within the subset that only included MRSA observations ranged between three (**E**) and five data points (**F**), it was demonstrated that the angle under which the data are analyzed is relevant: if perspective is considered, greater discrimination may be achieved. Three leukocyte indicators distinguished the two human (MRSA-only vs. MRSA and non-MRSA) subsets ($P<0.01$, Mann-Whitney test, **G**).

A third D+ subset was found in the malaria data with a 'hybrid' set that included both 'anchor' (the P/L ratio vs. the L%) and 'amplifier' features (the P/L and L/M ratios, Figure 6 E). Such structure facilitated data partitioning into subsets. Statistically significant differences were found: 1) between FN and all D– observations, 2) between every D+ subset and every D– subset, and 3) among the 3 D+ subsets ($P≤0.03$, Mann-Whitney test, Table 3).

Because statistical significance may be found even in the absence of discrimination (D+ and D– data overlapping may occur, even when median D+ and D– values differ statistically), the SB/EB approach was also assessed spatially. No data overlapping was found among: 1) the three D+ subsets (Figure 6 F); and 2) all three D– and two D+ (and FN) stages (Figure 6 G). The overlapping rate (percentage of observations assigned to one disease stage which showed values typical of another disease class) ranged between 0 (Figure 6 G) and 0.0002 (1/336, one medium L/M D+ data point, arrow, Figure 6 F). While spatial patterns did not distinguish some D+ (low or medium L/M) data points from D– data, such classes were differentiated on the basis of parasite test results (Figures 6 F, G).

Assessment of percentages, ratios, counts, and hypothesis-related assumptions

When SB/EB concepts were not applied, neither the L%, the N%, nor the M%, alone, discriminated, in any study conducted, D+ from D– data (Figures 7 A-D). When SB/EB concepts were not applied, neither log-transformations nor ratios distinguished data classes (Figures 7 E-H). In two species, cell counts did

not distinguish MRSA from non-MRSA subjects (Figures 7 I, J). Hence, without the SB/EB approach, no primary variable, per se, could discriminate.
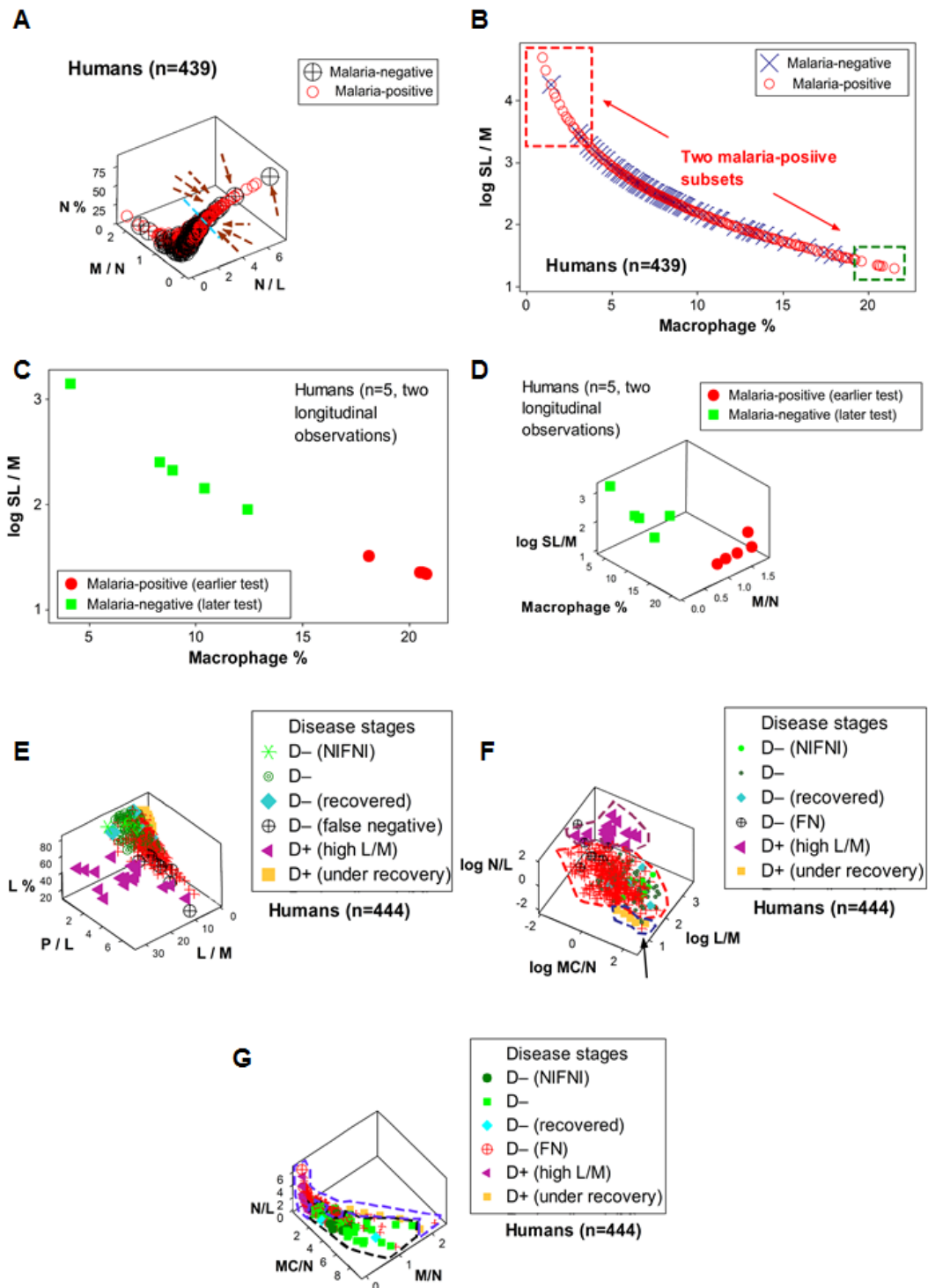

Fig. 6. Detection of false negatives, prognosis, and three D+ data subsets in human malaria. The process by which false negative results were assessed is illustrated with data collected from humans infected or not infected by malaria. Arrows indicate 8 parasite-negative results, which

were associated with high N/L values (**A,** also shown in Figures 2 C, D, H). Clinical data corresponding to the 8 children revealed that all of them were febrile. Spatial data patterns facilitated the detection of the 8 false negative (FN) results: an orthogonal data inflection (sky blue line) separated the data range in which the 8 FN points were found from the area in which D+ data predominated (**A**). Other spatial data patterns identified a subset associated with a favorable prognosis: two D+ subsets (arrows, **B**) were separated from one another by a segment in which both D+ and D− data points were observed, suggesting that the two D+ subsets observed at both ends of the plot could differ functionally (boxes, **B**). The subset with the higher M% was suspected to be under recovery. When the 5 individuals within the high M% subset were tested again, two weeks later, all of them were D− (**C**). The change in health status, which took place within two weeks, revealed an orthogonal 3D data inflection (**D**). An additional set of indicators (the L%, P/L and L/M ratios) detected a third D+ subset, which showed high L/M values and differed from all other subsets (purple triangles vs. other symbols, **E**). Based on spatial patterns (shown in Figures 1-3 and here), the data were partitioned into subsets, which differed from one another at statistical significant levels (all comparisons with *P*<0.03, Mann-Whitney test). The degree of non-overlapping data distributions between two or more subsets (discrimination) was 1/336 (arrow indicates the overlapping point, **F**) when the 3 D+ stages (characterized by high MC/N or under recovery [n=5], medium L/M [n=314], or high L/M [n=17]) were assessed. Total discrimination (no overlapping or 0/130) was seen when 3 D+ data classes (under recovery [n=5], high L/M [n=17], and FN [n=8]) were assessed vs. the 3 D− classes [n=100]) and the set that included the N/L, MC/N, and M/N ratios was utilized (**G**).

Table 3. Differentiation of malaria classes. The statistical results of human data reported in Figures 6 E-G (n=444) are shown, where pairs of data classes are compared. The *P* values of analysis of medians (Mann-Whitney test) were determined by the MC/N ratio, the SL/M ratio (*), or the P/L ratio (#). NS: not significant at *P*=0.05. The D− NIFNI group (neither infected, febrile, nor inflamed) is not a separate class, it is a reference for the overall D− class. See legend of Figures 6 E-G for further details.

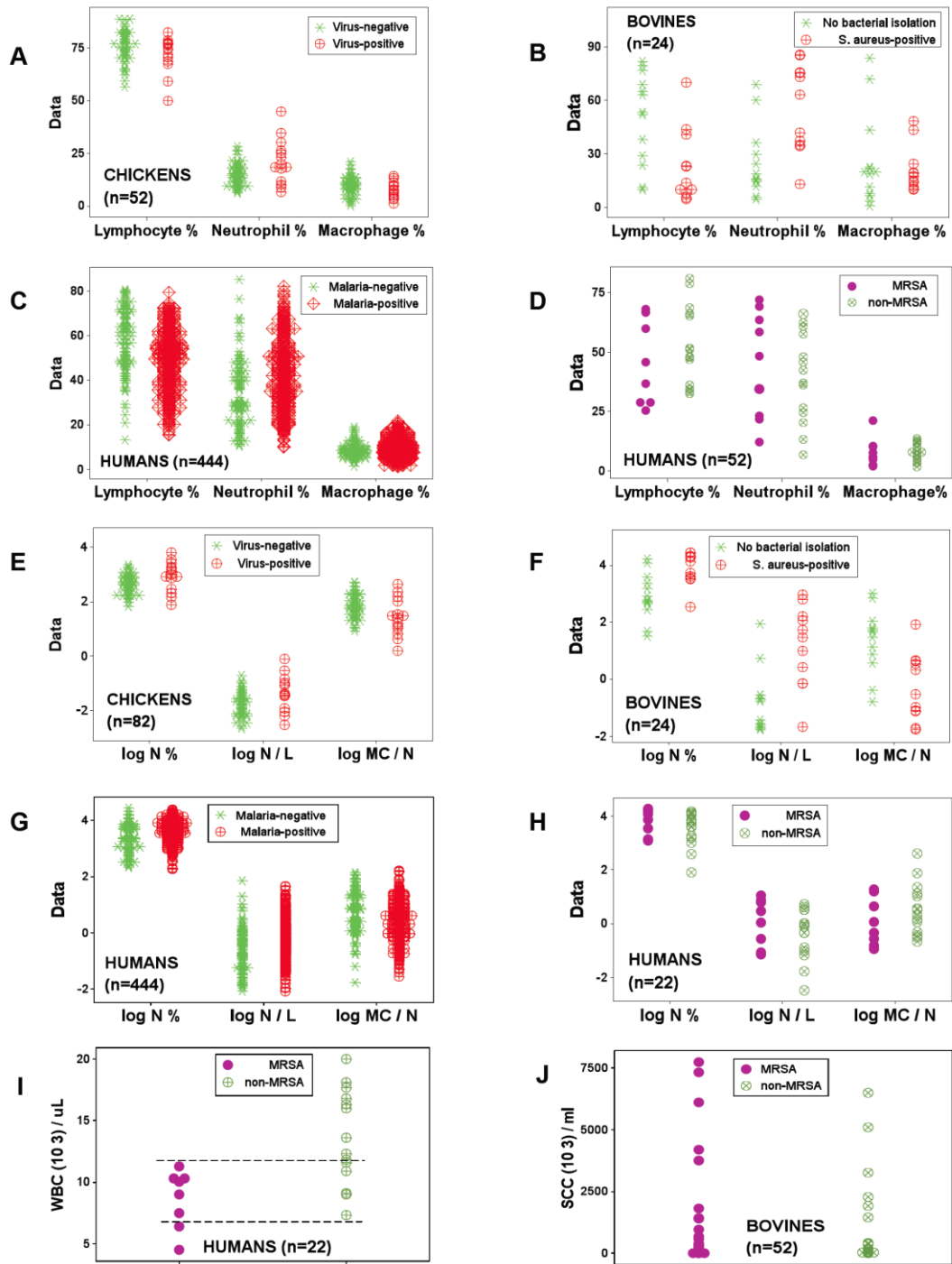| Data classes	D− (n=83)	D− NIFNI (n=12)	D− recovered (n=5)	False D− (febrile, n=8)	D+ high MCN/N (n=5)	D+ low SL/M (n=314)
D− NIFNI (n=12)	NS					
D− recovered (n=5)	NS	NS				
False D− (febrile, n=8)	<0.01	<0.01	<0.01			
D+ high MC/N (n=5)	<0.01	<0.01	<0.01*	<0.01		
D+ medium L/M (n=314)	<0.01	<0.01	<0.03#	<0.01	<0.01	
D+ high L/M) (n=17)	<0.01	<0.01	<0.01	<0.01*	<0.01	<0.01

Fig. 7. Assessment of ratios, counts, percentages, and hypothesis-related assumptions. When the SB/EB approach was not applied the percentage of lymphocytes, neutrophils, or macrophages did not distinguish, in any study, D– from D+ data (**A-D**). Indicators that, together, detected patterns (the N%, the N/L and MC/N ratios), did not discriminate D– from D+ data when assessed individually (**E-H**). Total leukocyte counts also failed to distinguish health status: neither the human white blood cell count (WBC) nor the bovine milk total cell count ('somatic cell count' or SCC) differentiated D– from D+ data (**I, J**). Hence, findings supported several Systems Biology principles: 1) data

integrity is necessary (because the immune system is indivisible, discrimination is lost when any leukocyte type is measured alone, **A-C**), 2) the format utilized is relevant: to detect 'high-level' interactions (those involving at least two interactions), 2D or 3D plots are required (as Figures 1-6 show); and 3) emergence was demonstrated: while, individually, no indicator distinguished D– from D+ data (**A-H**), when 3D structures were assembled, D– and D+ data were distinguished (as shown, for instance, in Figure 2 H). Findings also demonstrated that statistical significance is not synonymous with discrimination: the median WBC count of human MRSA infections differed from the median WBC count of non-MRSA individuals (*P*<0.03, Mann-Whitney test), even though D– and D+ data overlapping was observed (**I**). However, when the data were structured as SB/EB indicators, both statistical significance and discrimination were achieved (as shown, for instance, in Figures 4, 5, 6). SCC: somatic cell counts (thousands)/ml. WBC: white blood cells (hundreds)/µl.

The validity of the 'gold standard' (the assumption that there is an ideal microbial test) was not supported in the human study on malaria: 8 children regarded as D– by microbial tests were febrile (false negatives or FN, Figure 2 C). In the bovine MRSA study, only two out of 7 tests (performed with milk collected from the same mammary gland) yielded MRSA, that is, the 'gold standard' hypothesis failed 5 out og 7 times (a 71.3% false negative rate, see Figures 2 E, F). In contrast, in humans, SB/EB spatial patterns identified data points suspected to be FN: they were spatially distant from D– observations (Figures 2 C, H; and 6 E)

Discussion

Major findings

The SB/EB approach revealed similarities across vertebrate species (e.g., data circularity, Figures 1 and 2). Such approach also demonstrated differences within the same species and disease. For instance, high L/M values distinguished one malaria-positive subset [65] from other D+ subsets (Figure 6 E). Findings rejected: 1) the 'gold standard' hypothesis; 2) the binary hypothesis (only two, one D+ and one D–, data classes); and 3) the hypothesis that postulates randomization reduces variability. To interpret the findings, biological, statistical and methodological aspects are considered and their influence on theory is outlined.

Biological and statistical considerations

In agreement with the theory that postulates the immune system is indivisible [66], no cell type, alone, discriminated D+ from D– subsets (Figure 7). It was also confirmed that dichotomizing approaches (which attempt to convert data inherently continuous into discontinuous data classes) are associated with D+ and D– data overlapping [67].

In contrast, discrimination was enhanced when interactions among all leukocytes were explored in 3D space. Such approach measured or revealed *hierarchy*, *feedback, and emergence* [66]. '*Hierarchy*' was assessed by focusing on the trans-vertebrate species set that also included several pathogen types. Such system revealed *feedback* loops. '*Emergence*' was not revealed by any one primary component. Emergent properties, such as false negative patterns, were only detected when several levels of the biological system were assembled.

While SB/EB properties have been regarded to reveal low variability [12], avian data seemed to contradict such expectation. In spite of randomization [68], high data variability was shown by the fact that both low and fast responders were observed (Figure 3). High variability co-existed with low variability, as Figure 1 reveals.

To explain such an apparent contradiction, we could pose the following question: 'how old are you: 44 million years old, or four years old?' The answer is not 'neither' but, probably, 'both.' All vertebrates are '44 million years old' because many of their critical structures are that old, if not older, such as mitochondria and the complement system [69-71]. Yet, a particular species (and an individual of a particular species) is much 'younger', e.g., the first chickens (*Gallus domesticus*) and hominids emerged in the last 3.6 million years [72, 73]. That means that individuals express biological functions that precede their own species and their own birth.

On the other hand, because the responsiveness of an individual can be shaped by unique experiences and pathogens can undergo mutations (such as MRSA), 'new' situations may arise. Because methicillin was introduced in 1960 [4], MRSA infections are recent evolutionary phenomena. Because vertebrates have not yet had enough time to adapt to MRSA, it is not surprising that the immune response against MRSA differs from that against well-

conserved (non-MRSA) pathogens (as observed, here, in two species). Because vertebrates participate in both 'old' and 'new' interactions, there is no contradiction between the variability shown by individual birds and the similarity displayed by well-conserved functions, such as feedback.

Because MRSA pathogens, in addition to be resistant to anti-microbials, also induce immune failure, such pathogens may result in abnormally high – although ineffective– N/L ratios [74-79], as found in bovines and humans. The highest values of such dysfunctional relationships were observed at the earliest observations (Figures 5 A-F). Because a immune response can only be sustained for a limited time, such pattern could be used to distinguish early MRSA from late (MRSA and non-MRSA) responses.

Methodological considerations

Methodological issues were also evaluated [80]. Because false negatives were documented, the hypothesis that there is an ideal test ('gold standard') was rejected [81, 82]. Because two or more D+ stages were distinguished, both the binary hypothesis ('only two data classes') and the assumption that all D+ data points have similar meaning were negated (Figures 1 and 6 B-E). One possible reason why those hypotheses were not empirically supported is that they do not account for dynamics and/or data circularity [83, 84].

Findings also addressed a circular problem, described as follows: in order to identify an infecting microbe, a specific test is needed; however, in order to choose such test, before, the identity of the pathogen should be known in advance. While the 'gold standard' could not solve this conundrum, the SB/EB approach provided an alternative for its solution [85].

Consequences on theory

Findings may be used to rectify a concept previously espoused. Feedback loops do not differ in directionality, as suggested before [24]. The apparent change in directionality is an artifact due to earlier analyses, which did not consider 3D space. In 3D space, feedback loops reveal a single (circular) directionality.

Because feedback loops expressed temporal changes, causality was supported [24]. Thus, the 3D feedback-oriented analysis provided both descriptive and explanatory information.

Unlike approaches that dichotomize continuous data and generate D+ and D− data overlapping [62], the 3D analysis of feedback loops displayed data inflections, which resulted in minimal D+ and D− data overlapping. Such feature could be used to facilitate data partitioning.

Data structured to express feedback dynamics overcame the limitations of static approaches, as when Principal Component Analysis is used to assess compositional data [86]. Unlike prevalence – a static index [87] –, the proportion of subjects within early vs. late responses (informative on dynamics) could distinguish populations with similar prevalence levels. Findings also showed that SB models can be applied across scales [88].

Replications and applications

Across species, the SB/EB approach helped to recognize infectious disease data patterns. Because this study did not focus on the pathogenesis of any disease, the reproducibility of the findings should be investigated in future studies. Potential applications include: 1) early diagnosis, 2) error detection, 3) differentiation of D+ classes, 4) prognosis, 5) evaluation of interventions, and 6) modeling.

For instance, two or more D+ classes may be distinguished [89, 90]. High MC/N values ('left overshooting') could be used to predict recovery. When 'right overshooting' is observed (high N/L or P/L values) but no microbe is isolated, an infection cannot be ruled out (a false negative result may be suspected). To prevent delayed detection of MRSA cases [91], the SB/EB approach, which seemed to reveal early MRSA data patterns, could be considered.

The SB/EB approach may also be used to evaluate interventions and support modeling. For instance, the evaluation of interventions may distinguish the influence of feedback from the responsiveness of individuals: when an intervention seems to be a 'success', it could be asked whether such outcome is due to fast responders (a 'false positive' result), or, when a 'failure' appears to occur, whether it was due to slow responders (a 'false negative' result). In

mathematical modeling, analyses that focus on MRSA-like infections could be optimized if the cyclic nature and directionality of feedback processes were addressed [92-94].

Conclusions

More information related to infectious diseases can be extracted, using the same data, when some conditions are met. Findings document the influence of data structure on the amount and explanatory content of infectious disease-related information. Feedback-related patterns of 3D leukocyte structures may have broad applications, including earlier diagnosis and lower rates of false results.

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Supporting Materials

Institutional approvals

Prior to data collection, all studies, whether published or not, were approved by the appropriate ethics committees of the institutions here identified.

Avian studies

Specific pathogen free (SPF) white leghorn chicken embryos were obtained from Charles River Laboratories (Chicago, IL), hatched and raised until 6-weeks old at the University of Wisconsin-Madison, then transferred to BSL-3 facilities at the U.S. Geological Survey National Wildlife Health Center and acclimated for three weeks prior to infection experiments. Ten nine-week old chickens were randomized for subcutaneous injection with 100-µl bovine

albumin-1 (BA-1) containing 10^5 plaque-forming units of American crow isolate 16399-3 WNV and subsequently bled on days 1-5, 7, 10, and 14 DPI [52]. While not analyzed with the quantitative method here evaluated, the raw data here reported have been previously published [52]. The chickens utilized for this study were treated humanely with due consideration to the alleviation of their distress and discomfort, and according to University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) protocol #A01059 and US Geological Survey National Wildlife Health Center IACUC protocol #EP040811.

Laboratory techniques. Vero cells were used to detect the presence of virus in serum by plaque formation according to standard procedures [53]. Viremia (plaque forming units (PFU)/ml serum) was calculated from the serum dilution that produced between 5 and 30 plaques per well. Blood smears were created on the blood sampling days indicated above, treated with Wright-Giemsa stain, and cell ratios were calculated once 100 WBCs were counted per slide via light microscopy at 1000X. West Nile virus-infected chickens produced WNV- specific IgG and. especially IgM titers, were positively correlated with viremia. Uninfected chickens remained antibody and virus-negative (i.e., D–) throughout the duration of the study [52].

Bovine studies

Three studies were conducted with cows, from which microbial tests were conducted with milk samples: one longitudinal and experimental study (LE), one longitudinal study of an animal spontaneously infected with methicillin-resistant *S. aureus* (MRSA), and a cross-sectional study (CS) that included 6 populations located in 4 countries (CS I to VI, Table 2). In the LE study (conducted in the US), 6 lactating cows were inoculated intra-mammarily with *S. aureus*, and milk samples were investigated before and up to 14 days after challenge with *S. aureus* [44]. While not analyzed with the quantitative method here evaluated, the raw data here reported have been previously published [44].

In the MRSA study, conducted in Italy, all mammary glands of one infected cow were investigated at days 1-5, 8, and 9 (7 tests), where day 1 was

the first day MRSA was isolated in milk cultures. While neither analyzed with the quantitative method here evaluated nor based on the samples here measured, the variables here assessed have been previously published [54].

All CS studies except CS II were conducted with non-periparturient cows. Table 3 describes the size, examples of bacterial species isolated, and the bacterial prevalence found in each CS study. CS I data are original. CS II-VI data, while not analyzed with the quantitative method here evaluated, have been partially or totally reported before [55-59], as described in the legend of Figure 4.

All bovines were treated according to protocols approved by the US Institutional and Animal Care and Use Committee or similar German, Israeli, and Italian agencies.

Laboratory techniques. The total milk leukocyte count/ml was quantified with a Fossomatic 5000 (Hillerød, Denmark), DeLaval DCC (Tumba, Sweden), or a Bentley Somacount 150 (Bentley Instruments, Chaska, USA). Microbial cultures were performed in compliance with guidelines described elsewhere [60]. Ten µL(CS III and CS VI), 50 µL (MRSA, CS IV, and V), or 100 µL (CS I and II studies) of milk were cultured. Bovine leukocytes were identified and counted by cytology (all studies except CS III), flow cytometry (CS III), or both methods (LE study), as described elsewhere [44, 55-59]. Susceptibility patterns of bacterial isolates were determined using the disk diffusion method indicated below (see human studies).

Human studies

A – Malaria

Participants. Children aged 3-36 months (n=439) were recruited at Siaya District Hospital, Kenya, a holoendemic *P. falciparum* transmission area where residents may receive up to 300 infective mosquito bites per annum [61]. After the parent or guardian of the child provided written informed consent to participate in the study, a questionnaire was conducted to collect demographic and clinical information, including the signs and symptoms of the present illness. In order to minimize the effect of previous malarial infections and/or recent anti-malarial use, no child with either prior hospitalizations (for any

reason) or treatment for malaria within the previous two weeks was investigated. None of the recruited children had cerebral malaria, non-falciparum malarial infections, bacteremia, HIV-1, or hookworm infections [62].

After enrollment, children were divided into three (two malaria-negative [M–] and one malaria-positive [M+]) groups. Both M– groups were defined by absence of *P. falciparum* parasitemia. One M– subset was defined as neither infected, febrile, nor inflammed (NIFNI). The NIFNI subset acted as an internal control for the overall M– class. The M+ group was defined by presence of *P. falciparum* parasitemia (any density) on thin and thick peripheral blood smears. Children were then re-examined two weeks later for the presence of malaria parasitemia and hemoglobin concentrations. After the data were collected and based on data patterns, both the M– and the M+ data were divided into additional subsets, e.g., M– results not suspected or suspected to be false negative, and M+ results suspected or not suspected to be under recovery. Five children, suspected to be under recovery, were tested twice over two weeks, and their data included in some analyses, so the total number of observations, in such cases, was 444. Children were treated according to guidelines of the Ministry of Health, Kenya. The study was approved by the University of New Mexico and the Kenya Medical Research Institute.

B – Bacterial infections

Participants. Because bacteremia is highly prevalent among Kenyan children with malaria, 22 bacterial infections were evaluated in malaria-positive children. Based on the holoendemicity of malaria in this region, children with bacterial infections in the absence of malaria were not obtained. To control for the confounding effects of malaria, children with bacterial infections were matched according to age, gender, parasitemia, and hemoglobin concentrations. The subset of children co-infected with bacteremia and malaria included 7 multi-drug resistant *S. aureus* (MRSA) and 15 non-MRSA isolates sensitive to, at least, oxacillin.

Laboratory techniques. No child received any intervention before samples were collected. Asexual malaria trophozoites were determined as described before [62]. Thick and thin peripheral blood smears were prepared

from venous blood samples and stained with Giemsa reagent for malaria parasite identification, and quantified by microscopy. Asexual malaria trophozoites were counted against 300 leukocytes based on absolute counts of white blood cells (WBC)/mL in whole blood. Parasite density was estimated as follows: parasites/mL = WBC count/mL x trophozoites/300. Complete blood counts were performed with a Beckman Coulter[©] Ac-T diff2™ (Beckman Coulter, Inc.).

Bacterial cultures were performed as previously described [63]. Briefly, blood cultures were performed for all children upon enrollment into the study, and in suspected cases of bacteremia at acute febrile visits. Approximately, 1.0 mL of venipuncture blood was collected aseptically into sterile pediatric Isolator[®] microbial tubes (Wampole Laboratories, Princeton, USA) or directly inoculated into the pediatric blood culture bottle (Pediplus, Becton-Dickinson, Franklin Lakes, USA). Blood samples in the pediatric Isolator[®] microbial tubes were inoculated directly onto chocolate agar plates, while pediatric blood culture bottles were incubated in an automated BACTEC 9050 system (Becton-Dickinson) for 4 days. Positive cultures were examined by Gram stain and sub-cultured on blood agar, chocolate agar or MacConkey agar plates based on the Gram stain results.

Susceptibility patterns of the bacterial isolates were determined using the disk diffusion method according to the Clinical Laboratory Standards Institute guidelines [64]. Bacterial isolates were tested against disks of erythromycin (15 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), nalidixic acid (30 µg), tetracycline (30 µg), ampicillin-salbactum (10/10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), oxacillin (1 µg), amoxicillin-clavulanic acid (20/10 µg), doxycycline (30 µg), cefotaxime-clavulanic acid (30/10 µg) and gentamicin (10 µg). Methicillin-resistant *S. aureus* (MRSA) was detected using oxacillin disk followed by vancomycin testing. Control *S. aureus* (ATCC 25923 and 29213) strains were run concomitantly with the test organisms. For all of the microorganisms tested, resistance was defined according to the Clinical Laboratory Standards Institute guidelines [64]. The study was approved by the Kenya Medical Research Institute.

Glossary

Three major constructs were assessed as defined:

i. <u>Functional data integrity</u>: a 2D/3D data structure that included at least two variables which, together, included data from all cell types and described at least one biological function. This means that, collectively, the set (i) included data from L, M, and N, (ii) the data were structured in a way such that biological functions generated by multi-cellular interactions could be assessed (e.g., the L/M ratio), and (iii) higher-level biological functions (those that may result from interactions among interactions, such as the interplay between early and late responses could be measured (e.g., the simultaneous assessment of the N/L and M/N ratios).

ii. <u>Amplification</u>: a data structure with numerical properties that enhancepattern detection by virtue of measuring two ratios at the same time, in which the same variable is assessed twice (the numerator of one ratio is the denominator of the other ratio). For instance, the simultaneous assessment of the N/L and M/N ratios includes the same variable twice (the N%, in this example). Notice that 'amplifier' indicators do not necessarily possess 'functional data integrity' (in this example, there is no L data).

iii. <u>Anchoring</u>: a subset of functional data integrity in which two variables suffice to produce a single line of observations, when one leukocyte ratio and one leukocyte percentage are measured, in which the denominator of the ratio is the percentage being measured in another axis (e.g., the P/L vs. L %). Notice that 'amplifier' indicators, in 2D and, depending on the perspective considered, also in 3D plots, generate a single line of observations but do not necessarily produce amplification.

Data analysis

To generate a double-blind assessment, no researcher participated in all studies, and no researcher involved in data collection participated in data analysis. Plots and descriptive statistical tests were produced with *Minitab 15*, Minitab Inc., State College, PA, USA.

9th Chapter

General discussion

Mastitis is one of the most prevalent infections in the dairy cattle industry, worldwide. Besides causing substantial economic losses, mastitis adversely affects dairy cow welfare (Menzies et al., 1995). Since approximately 70 to 80% of financial losses of this disease are caused by subclinical mastitis, this type of mastitis has a particularly high significance. The annual loss due to subclinical mastitis aggregated across all U.S. dairy cows was calculated to be approximately one billion dollar (Ott, 1999). Assuming that continuously increasing milk yields lead to more susceptible cows, the actual annual loss is probably even higher. Currently, the evaluation of udder health is based on somatic cell count (SCC) and bacteriological examination (Viguier et al., 2009). In this regard, a SCC threshold of 100,000 cells/ml in quarter foremilk samples is used to differentiate between healthy and diseased mammary glands (Harmon, 1994; DVG, 2002). However, it is well-known that SCC vary with status of lactation, age, stress of the animals, time and frequency of milking, season, and, mainly, udder infection status (Dohoo and Meek, 1982; Harmon, 1994). In addition, bacteriological culture of foremilk samples can be false negative although the quarters are infected. Reasons could be, for example, intermittent pathogen shedding, presence of antimicrobials or other inhibitors in milk or ceased growth of the pathogens (Newbould and Neave, 1965; Hill et al., 1978; Reiter, 1978; Sears et al., 1990). Recently, polymerase chain reaction has been proposed as an alternative to culture (Koskinen et al., 2009). However, even though polymerase chain reaction is a rapid test, this method is expensive and less sensitivite than bacterial culture (Paradis et al., 2012). The major problem of subclinical mastitis is that neither symptoms of the involved quarters (e.g., swelling or redness, flecks in milk) nor routine diagnostic methods (e.g., SCC, bacteriological examination) indicate the disease.

The primary objective of this thesis was to analyze the immunological status of clinically healthy and subclinically infected bovine mammary glands by cell differentiation methods. This thesis should contribute to a more detailed understanding of subclinical mastitis by providing data regarding the relationship of immune cells in milk of affected quarters.

The implementation of classical mastitis prevention programs (Neave et al., 1969) in combination with the introduction of penalty limits for bulk milk SCC

have led to substantial progress in controlling subclinical mastitis worldwide. For instance, the geometric bulk milk SCC in Hesse, Germany, decreased from 255,000 to 200,000 cells/mL between 1993 and 2008 (HVL, 1994, 2009). The occurence of mastitis pathogens also changed. In Hesse, the prevalence of *Staphylococcus aureus* was reduced from 13.0% in 1995 to 2.9% in 2008 (LHL, 2009). In contrast, coagulase-negative staphylococci are currently the most isolated pathogens from milk samples in many countries (Pitkälä et al., 2004; Piepers et al., 2007; Sampimon et al., 2009). Hence, it is worth knowing the current udder health situation in the dairy cow population.

This thesis (**2nd chapter**) gave information about the udder health situation in a representative part of the dairy cow population in Hesse, Germany. The distribution of SCC, prevalence of mastitis pathogens, and the prevalence of mastitis pathogens in dependence of SCC were analyzed on the basis of quarter foremilk samples taken from 2000 to 2008. The results of this study demonstrated a proper level of udder health. Two-thirds of all quarter foremilk samples indicated SCC ≤100,000 cells/mL. About 52% of the samples analyzed were culture-positive. Prevalences of the mastitis pathogens detected in the Hessian survey were comparable to those obtained from Belgium (Piepers et al., 2007), Finland (Pitkälä et al., 2004), and the Netherlands (Sampimon et al., 2009). For the first time, detailed results about prevalences of mastitis pathogens in dependence of SCC were presented in the literature. Prevalences of mastitis pathogens were clearly lower in samples with SCC ≤100,000 cells/mL than in samples with >100,000 cells/mL. Interestingly, major pathogens (*Staphylococcus aureus*, *Streptococcus uberis*) as well as minor pathogens (coagulase-negative staphylococci, *Corynebacterium* species) were detectable in milk samples with SCC starting from a level of 1,000 cells/mL. In a Dutch study, similar data were found but neither described nor discussed specifically (ten Napel et al., 2009). However, it is not clear whether the pathogens isolated from quarter foremilk samples with SCC values from 1,000 to 100,000 cells/mL originated from contamination or whether they caused an intramammary infection. In any case, no signs of an intrammamary inflammation were visible based on SCC. Hence, differential cell counts (DCC) might be a better indicator than SCC for a profound evaluation of inflammation, especially at SCC levels ≤100,000 cells/mL, because the analysis of the

relationship of different cell types in milk (lymphocytes, macrophages, and polymorphonuclear neutrophilic neutrophils (PMNL)) is suitable for a detailed evaluation of the udder health status (Pillai et al., 2001; Rivas et al., 2001).

The literature shows that milk DCC differ clearly between healthy and diseased quarters. In normal milk without any symptoms of mastitis lymphocyte proportions between 14 and 80%, macrophage proportions between 12 and 46%, and those of PMNL between 6 and 50% were described (Rivas et al., 2001; Merle et al., 2007; Koess and Hamann, 2008). In mastitis milk, PMNL proportions of up to 95% have been reported (Paape et al., 1979; Kehrli and Shuster, 1994). During various phases of inflammation SCC differs in total numbers, whereas DCC varies in composition of the cell populations involved (Nickerson, 1989). Therefore, in addition to SCC, determination of different types of immune cells present in milk is beneficial for describing the udder health status (Pillai et al., 2001; Rivas et al., 2001). However, till now little is known about DCC and the qualitative role of milk leukocytes in healthy mammary glands. One reason is probably that DCC in low-SCC milk is difficult to perform (Dosogne et al., 2003).

Since inflammatory processes were suspected even within the SCC range of udder quarters classified as healthy according to current definitions (2nd chapter), a further objective of this thesis (**3rd chapter**) was the detailed evaluation of such quarters by microscopic DCC analysis. Reviewing the literature, this was the first study investigating cell populations in low-SCC milk in detail. Lymphocytes were found as the predominant cell population in milk of healthy mammary glands with proportions of up to 92%. In contrast, a previous study reported macrophages to be the predominant cell population in milk of healthy mammary glands (Lee et al., 1980). In our examinations, macrophages were the second dominant cell population in almost all samples tested in relationship to lymphocytes and PMNL. However, the differences to the results of Lee et al. (1980) might be explainable by different definitions of healthy mammary glands. While in our study milk with very low SCC values was analyzed, Lee et al. (1980) defined udder quarters as healthy based on negative bacteriological examinations and did not present any SCC values. Our data revealed PMNL as the dominant cell population in milk of diseased

quarters, as published previously (Leitner et al., 2000a; Merle et al., 2005; Koess and Hamann, 2008). The predominance of PMNL is an important indicator of inflammatory reactions (Pillai et al., 2001; Paape et al., 2002). Interestingly, in our examinations PMNL dominated already at SCC ≥43,000 cells/mL suggesting that inflammatory processes appear already in the SCC range of healthy quarters. Factors that might have triggered the elevated PMNL proportions might be manifold. The mammary gland of a dairy cow is under constant pressure from udder pathogenic microorganisms in the environment. The elevated PMNL proportion could be evidence for the initial phase of an inflammation. In this regard it is also possible that PMNL are able to defend pathogens successfully and prevent mastitis. Although pathogens could not be isolated in such quarters, they might be infected nonetheless. Reasons for false negative bacteriological examinations could be, for example, intermittent pathogen shedding, presence of antimicrobials or other inhibitors in milk or ceased growth of the pathogens (Newbould and Neave, 1965; Hill et al., 1978; Reiter, 1978; Sears et al., 1990). The interdependence of the individual udder quarters might also have triggered the elevated proportions of PMNL. Some authors suggested that individual udder quarters within a cow can be influenced by infections of neighboring quarters (Merle et al., 2007), whereas others did not find any evidence for an interdependence of udder quarters (Wever and Emanuelson, 1989) because they did not find DCC to be affected by the bacteriological status of adjacent quarters. Our data indicated no interactions between the individual udder quarters. According to Davis et al. (2008) stress could also be a factor triggering elevated PMNL percentages. Although we did not measure parameters related to stress, the influence in our study might be minimal because the animals analyzed were kept under optimal conditions and according to national guidelines. In addition, no obvious symptoms of stress (e.g., kicking during pre-milking preparation) were observed. Hence, this was the first study analyzing DCC of milk samples taken from mammary glands classified as healthy by SCC ≤100,000 cells/mL that indicated inflammatory reactions in those quarters.

SCC is an undisputed and well-established criterion for the evaluation of udder health and milk quality. In addition to SCC, DCC can be used for a more

detailed analysis of udder health status, as shown in chapter 3. However, the literature shows variations in the distribution of leukocytes in milk of non-infected mammary glands. These variations were probably dependent on differences in methods, sampling, investigators (Schröder and Hamann, 2005), breeds (Leitner et al., 2003), stages of lactation (Vangroenweghe et al., 2001; Dosogne et al., 2003), and variable SCC. To reduce the influences of method and investigator, flow cytometry should be applied for cell differentiation. Due to the rapid characterization of a large number of cells as well as the definite identification of individual cell populations using specific antibodies, flow cytometric analysis gives more accurate results compared with microscopic analysis (Loken and Stall, 1982; Rivas et al., 2001; Dosogne et al., 2003; Koess and Hamann, 2008). Hence, another aim of this thesis (**4**[th] **chapter**) was to differentiate immune cells in milk of udder quarters classified as healthy based on SCC values of ≤100,000 cells/mL using flow cytometry. Reviewing the literature, this was the first study measuring simultaneously percentages of lymphocytes, macrophages, and PMNL by a flow cytometric method. Results of the microscopic cell differentiation study (chapter 3) were confirmed applying the advandced analysis technique. Lymphocytes also dominated with proportions of up to 88% in milk of healthy mammary glands. Again, macrophages were found as second predominant cell population in almost all samples tested in relation to lymphocytes and PMNL. Interestingly, in this study, inflammatory reactions could already be detected starting at an SCC level of 9,000 cells/mL based on predominant percentages of PMNL. As discussed in chapter 3, the factors that might have triggered the elevated PMNL percentages might be manifold. At the time of examination pathogens could not be isolated from the involved quarters. As demonstrated above, bacteriological analysis could be false negative for different reasons. The data of the flow cytometric study indicated immunological interdependence as well as independence between the four udder quarters at low and high SCC levels. The factor stress that also might have triggered the elevated proportions of PMNL, might be minimal in this study for the same reasons as discussed above. Further analysis of the data revealed significant differences of cellular components in milk between culture-positive and culture-negative udder quarters. DCC results of quarters with the detection of major pathogens indicated an intramammary

infection and were in agreement with previous observations (Piccinini et al., 1999). A new finding of our study was that even in case of the detection of minor pathogens, the percentages of lymphocytes and PMNL differed significantly from those in culture-negative quarters. Hence, DCC could be a helpful tool for interpretation whether the bacteria detected originated from contaminations of the skin, teat canal, or environment or whether they cause an intramammary infection. This is particularly relevant in quarters that are not noticeable by elevated SCC (>100,000 cells/mL). As suspected in the Hessian survey (chapter 2), the microscopic (chapter 3) as well as the flow cytometric analyses (chapter 4) could detect inflammatory reactions in udder quarters with SCC that were clearly below the current threshold of 100,000 cells/mL.

At present, SCC and bacteriological analysis are the standard techniques for the evaluation of udder health (Viguier et al., 2009). However, SCC is low in the initial stage of inflammatory reaction, until the invading pathogen is recognized by immune and epithelial cells that release chemoattractants, thus stimulating migration of PMNL (Paape et al., 2002; Oviedo-Boyso et al., 2007; Koess and Hamann, 2008). This thesis confirmed that DCC are beneficial for describing the udder health status, even in low SCC milk (chapters 3 and 4), as suggested by previous studies (Pillai et al., 2001; Rivas et al., 2001). It is known that the course of mastitis has an influence on DCC. In the presence of acute mastitis (e.g., caused by *Staphylococcus aureus* or *Escherichia coli*) PMNL were found as the predominant cell type (Leitner et al., 2000a; Merle et al., 2007; Koess and Hamann, 2008). In contrast, in chronic mastitis caused by *Staphylococcus aureus* or coagulase-negative staphylococci percentages of PMNL can be as low as that in uninfected quarters, whereas macrophage percentages are higher (Leitner et al., 2000a). Also, the analysis of DCC patterns in the presence of various pathogens revealed differences (Leitner et al., 2000a). While percentages of PMNL were high (73%) in quarters chronically infected with *Streptococcus uberis*, quarters with a chronic *Staphylococcus aureus* infection showed clearly lower PMNL percentages (42%). Therefore, another aim of this thesis (**5th chapter**) was to identify cytological parameters that could be used easily in the field for classifying udder quarters as healthy or diseased by comparion of cytobacteriological results with DCC.

Although flow cytometric analysis gives more accurate results compared with microscopic analysis (Loken and Stall, 1982; Rivas et al., 2001; Dosogne et al., 2003; Koess and Hamann, 2008), in this study DCC were determined by the cost-effective and routinely used light microscopic method. Animals were selected from three dairy herds with different prevalences of intramammary infection, which were caused by different etiological agents. While environmental pathogens were the causative agents of mastitis in herd A, contagious bacteria dominated in herds B and C with low and high diffusiveness, respectively. DCC patterns were significantly different among the three herds, as expected and in accordance with previous reports (chapter 4). Relative higher values of PMNL followed by lymphocytes and macrophages were found in herds A and B, suggesting that most infections were acute. In contrast, macrophages were the main cell population in herd C indicating chronic infections, as shown in a previous study (Sladek and Rysanek, 2009). To enable comparisons between cytobacteriological and DCC results, the current recommendations of DVG (2002) were used. Consequently, all samples analyzed were clustered into four health groups (N = normal secretion, SCC ≤100,000 cells/mL, bacteriological negative; LM = latent mastitis, SCC ≤100,000 cells/mL, bacteriological positive; UM = unspecific mastitis, SCC >100,000 cells/mL, bacteriological negative; M = mastitis, SCC >100,000 cells/mL, bacteriological positive). Of the three cell populations, PMNL are known to strongly increase during the course of infection. Accordingly, PMNL were statistically lower in group N than in groups UM and M, but no difference was demonstrated between groups N and LM. The macrophage percentage was very similar in the four groups, because macrophages are associated with the late phase of infection (Leitner et al., 2000a; Sladek and Rysanek, 2009) and are expected to increase in chronic infections, which were presumably at low levels in the cows of this study. Lymphocytes were the only individual cell population showing statistically different percentages between the healthy group (N) and all diseased groups (LM, UM, M). Especially, combinations of cell populations were evaluated to increase the discrimination power of DCC. Indeed, combining populations increased the F-values, indicating that a larger percentage of quarters would be correctly classified when that parameter was considered. Combining PMNL and

macrophages into phagocytes (Phag variable) increased F-values from 1.65 and 15.54, respectively, to 32.64. Evaluating the logarithmic (log) Phag:Lym ratio, which includes all three cell populations, an F-value of 45.90 was found. All combinations of individual cell populations showed statistically significant differences between groups N and D. However, the best F-value of 48.23 was related to log PMNL:Lym ratio. One explanation would be that only the log PMNL:Lym ratio includes both cell populations which are statistically influenced by health groups but excludes macrophages. Chapter 5 clearly showed that combinations of cell populations are more suitable for differentiation between healthy and diseased udder quarters than single cell populations. Nevertheless, further studies for determination and validation of cutoff values of the individual cell populations as well as combined variables differentiating between healthy and diseased quarters should be conducted.

As shown above, the percentage of each cell type can be widely variable in milk samples of healthy udder quarters. It is known that DCC patterns are mainly influenced by the course of mastitis and the causative agent (Leitner et al., 2000a). In addition, the effect of lactation stage and parity number should be taken into account (Vangroenweghe et al., 2001; Dosogne et al., 2003). Leitner et al. (2000b) evidenced a high repeatability for samples taken from the same cow in different stages of lactation and suggested that the leukocyte pattern in uninfected mammary glands is genetically controlled. However, so far, no information on short-term repeatability of DCC is available. Since the immune system is dynamic and the mammary gland is subjected to persistent stress during lactation, a basic knowledge of the cellular profile in healthy glands is fundamental to evaluate the applicability of DCC as tool in mastitis control programs. Hence, another aim of this thesis (**6th chapter**) was to investigate DCC in milk from healthy mammary quarters and to test whether the results are consistent on subsequent days. In addition, a cutoff value for log PMNL:Lym, which was the best ratio for differentiation between healthy and diseased quarters (chapter 5), should be established and verified under field conditions.

In the first trial, quarter milk and blood samples were taken from eight healthy cows for five consecutive days. Milk samples were tested by SCC and bacteriological analysis, whereas DCC was performed on all blood and milk

samples by the more precise flow cytometric method. To reduce the influence of diseases or systemic pathologies unrelated to the mammary gland, in the first trial only animals from a herd with high health and hygiene standards considered to be free of contagious mastitis pathogens were selected. Additionally, blood samples were taken to check if eventual fluctuations in milk data could be related to systemic conditions. No influence of sampling day, parity, stage of lactation or quarter position could be found on either milk or blood DCC patterns. These findings suggest that DCC can be reliably applied in samples collected at different points in time in lactation to evaluate the health status of the mammary gland, even though single variations observed in a few samples may indicate misclassification. Hence, a cutoff value of 0.495 for log PMNL:Lym could be established to identify healthy or diseased quarters. For verification of this cutoff value, in a second trial 16 animals were randomly selected from a different herd and quarter milk samples were taken on three consecutive days. When the cutoff value was applied to the data along with SCC, high specificity and good sensitivity of 97.3% and 92.3%, respectively, were calculated. Out of the nine false-negative quarters, two had been considered as diseased based only on SCC, four were positive for *Staphylococcus aureus*, two for *Prototheca* species, and one for coagulase-negative staphylococci. On the other hand, considering both SCC and DCC, only one quarter would be misclassified, increasing sensitivity of the method to 97.3%, without any changes in specificity. Such quarter had very low SCC (1,000 cells/mL in all samplings), and *Staphylococcus aureus* was detected in low counts only at the first and second sampling (10^2 UFC/mL). Since the animal presented with two different *Staphylococcus aureus*-infected quarters shedding high numbers of bacteria, we speculated that bacteriological positivity of the other quarter could reflect a transient contamination of the teat canal that was adequately prevented from reaching the gland cistern by local defence mechanisms. Such teat canal contaminations have been previously reported and did not always correlate with intramammary infections (Zecconi et al., 1994). In chapter 6 short-term repeatability of DCC patterns was shown and a cutoff value for log PMNL:Lym working under field conditions was found to differentiate between healthy and diseased udder quarters.

Lymphocytes were found as predominant cell population in milk of healthy udder quarters (Chapters 3, 4, 5, and 6). The predominance of these cells in healthy mammary glands suggests that lymphocytes play a significant role in the maintenance of the integrity of the mammary gland (Shafer-Weaver et al., 1996) and in host defense against infectious diseases of the mammary gland (Sordillo et al., 1991). There are two distinct subsets of lymphocytes that differ in function: T and B lymphocytes. While T lymphocytes regulate induction and suppression of immune responses (Nickerson, 1989), the main task of B cells is the production of antibodies against invading pathogens (Oviedo-Boyso et al., 2007). In one presented study (chapter 5) lymphocytes were the only individual cell population showing statistically different percentages between healthy quarters and all three kinds of diseased quarters; indicating that their percentages are susceptible to change. However, little is known about the immunological status of apparently healthy udder quarters classified by SCC ≤100,000 cells/mL. Due to the predominance of lymphocytes in milk of healthy udder quarters and the clearly different functions of T and B lymphocytes, a further aim of this thesis (**7th chapter**) was the detailed analysis of the relationship of CD2^{+} T and CD21^{+} B lymphocytes in foremilk samples of clinically healthy and subclinically infected udder quarters using flow cytometry to check early changes of the immunological status of the mammary gland.

Our examinations revealed noticeable high percentages of CD2^{+} T lymphocytes in milk of quarters showing SCC ≤100,000 cells/mL and two culture-negative results. In contrast, percentages in milk of diseased udder quarters were low. Percentages of CD21^{+} B lymphocytes were low in milk of culture-negative quarters with SCC ≤100,000 cells/mL and increased in milk of diseased quarters. The antidromic trend of T and B lymphocyte percentages in milk of healthy and diseased udder quarters led us to define a completely new variable – the CD2/CD21 index. Due to the specific functions of T and B lymphocytes, the percentage of CD2^{+} cells per CD21^{+} cells represents the interaction between cellular and humoral immune responses. While in view of lymphocytes cellular immune mechanisms dominate in milk of apparently healthy udder quarters, the role of the humoral immune response became intensified in diseased quarters. Furthermore, chapter 5 indicated that combinations of single cell populations increase the power to discriminate

between healthy and diseased udder quarters. Based on the results of our examinations a CD2/CD21 index of 10 was detected to be suitable for differentiation between unsuspicious and suspicious or diseased udder quarters. Severely diseased quarters with SCC >100,000 cells/mL containing major pathogens generally revealed CD2/CD21 indices <10. In contrast, CD2/CD21 indices >10 were generally found in quarters showing SCC ≤100,000 cells/mL and two culture-negative results. In udder quarters containing minor pathogens (SCC ≤100,000 cells/mL or >100,000 cells/mL) CD2/CD21 indices <10 (n = 29) as well as >10 (n = 7) were found. However, it is not clear whether the minor pathogens detected in these quarters originated from teat canal colonization or whether they caused an intramammary infection (Devriese and De Keyser, 1980; Linde et al., 1980). The DCC data of four of the seven quarters with CD2/CD21 indices >10 supported an intramammary infection because proportions of granulocytes were 77-89%. The remaining three quarters indicated no inflammatory reactions (granulocyte proportions 24-39%). However, the CD2/CD21 index of these seven quarters ranged between 10.87 and 19.93 and should be seen as suspicious, at least in samples showing SCC >100,000 cells/mL and minor pathogens. The analysis of selectively chosen quarters that had SCC ≤100,000 cells/mL and major pathogens (*Staphylococcus aureus*) also showed CD2/CD21 indices <10. DCC patterns of these quarters revealed inflammatory reactions based on the predominance of PMNL. Hence, it can be speculated that the CD2/CD21 index is connected with the current or previous presence of pathogens in the mammary gland. However, we performed field studies and do not know the exact time of infection. Even though, in chapter 7 a potential new indicator for differentiation of unsuspicious and suspicious or diseased udder quarters was found.

The literature shows that the rate of undetected infections remains markedly elevated and may be increasing (McBryde et al., 2009; Rerknimitr et al., 2010; Tàrnok et al., 2010). Pathogens that develop resistance to antimicrobials pose new challenges, such as methicillin- or multidrug-resistant *Staphylococcus aureus* (MRSA) infections, which, in the United States, cause more deaths than tuberculosis, AIDS, and viral hepatitis combined (Boucher and Corey, 2008). Therefore, improved characterization of infectious disease-

related data patterns is required. Here, system biology and evolutionary biology may be considered. System biology may extract more information from the same data focusing on systems and their dynamics (Kitano, 2002a, b; Cedersund and Roll, 2009; Villoslada et al., 2009; Drack and Wolkenhauer, 2011). To diminish data variability, evolutionary biology focuses on biological features well conserved in evolution (Macklem, 2008; Nesse and Stearns, 2008; Johnson, 2010; Luni et al., 2010). In this context, the investigation of *feedback* defined as process that involves an interaction between two or more elements (e.g., a microbe and a host) is crucial. Feedback can be designated *positive* when the activation or accumulation of one component leads to the activation or accumulation of another component, and *negative* when the activation or accumulation of the earliest component leads to the deactivation or depletion of the later component (Freeman, 2000).

A further aim of this thesis (**8th chapter**) was to explore DCC data patterns of host-microbial interactions for improvement of disease diagnosis. Data collected in six bovine studies (chapters 3, 4, and 5; Leitner et al., 2000a; Anderson et al., 2010; Pilla et al., 2012), two human studies (not published yet), and one avian study (Jankowski, 2010) with viral, parasite, or bacterial agents were analyzed. In all studies the classic approach (e.g., percentages of an individual cell population) did not differentiate disease-positive from disease-negative groups without overlapping. In contrast, the 3D, system biology:evolutionary biology-approach distinguished three (steady, positive, and negative) feedback phases, in which disease-negative data characterized the steady phase, and disease-positive data were found in the positive as well as in the negative phase. Furthermore, based on clear inflammatory DCC profiles the advanced data analysis identified cases of false negatives. This finding rejects the 'gold standard' hypothesis that there is an ideal microbial test, in accordance with previous studies (Feinstein, 1990; Grimes and Schulz, 2002). Also, 3D analyses distinguished two or more disease-positive stages negating both the binary hypothesis ('only two data classes') and the assumption that all disease-positive data points have similar meaning. One possible reason why those hypotheses were not empirically supported is that they do not account for temporal dynamics and/or data circularity (Milton et al., 1989; Hu et al., 2011). For prevention of false negative results, a specific test is needed to identify the

infecting microbe. However, while the 'gold standard' cannot solve this conundrum, the system biology:evolutionary biology-approach provides an alternative for its solution. In chapter 8 DCC patterns were structured and host-microbial interactions could be assessed.

In conclusion, this thesis contributed to a more detailed understanding of subclinical mastitis by providing new data regarding the relationship of milk immune cells in clinically healthy and subclinically infected bovine mammary glands. In addition, new concepts for data analysis and potential new tools for diagnosis of subclinical mastitis were described in this thesis.

Conclusions

This thesis indicated a high standard of udder health in the Hessian dairy cow population analyzed because in 62% of all udder quarters SCC ≤100,000 cells/mL were detected. In view of mastitis pathogens, prevalences were clearly lower in samples with SCC ≤100,000 cells/mL than in samples with >100,000 cells/mL confirming the 100,000 cells/mL threshold for differentiation between healthy and diseased mammary glands. Minor and major pathogens were detected even at a minimum of 1,000 cells/mL suggesting that inflammatory reactions appear already in the SCC range ≤100,000 cells/mL. Although SCC is an undisputed and well-established criterion for the evaluation of udder health, in addition to SCC, DCC can be used for a more detailed analysis of the udder health status. Analyzing DCC of udder quarters classified as healthy by SCC ≤100,000 cells/mL, inflammatory reactions were detectable at an SCC level of ≥43,000 cells/mL or ≥9,000 cells/mL due to predominating PMNL proportions in foremilk samples of the corresponding quarters applying microscopic or flow cytometric methods, respectively. These were the first examinations indicating inflammatory reactions in udder quarters with SCC that were clearly below the current threshold of 100,000 cells/mL. Further analyses revealed that DCC patterns differed significantly between herds depending on the causative mastitis pathogen as well as the diffusiveness of the bacteria. It was also shown that combinations of the individual cell populations improved the power of DCC to differentiate between healthy and diseased udder quarters. In this context, the best results being achieved using log PMNL:Lym ratio as variable. A further study revealed a cutoff value of 0.495 for log PMNL:Lym differentiating between healthy and diseased mammary glands with a high specificity and good sensitivity of 97.3% and 92.3%, respectively. The use of both cytometric DCC, in particular the log PMNL:Lym ratio, and SCC could represent an excellent diagnostic method to identify inflammatory processes in the mammary gland, avoiding bacteriological analysis. However, all DCC examinations discovered lymphocytes to be the predominant cell population in milk of healthy udder quarters. The detailed analysis of the lymphocyte subpopulations $CD2^+$ T and $CD21^+$ B lymphocytes revealed

significant different percentages of these cell populations in milk of healthy and diseased udder quarters. Hence, a new variable – the CD2/CD21 index – provides a trend for the characterization of udder health. Our examinations showed that a CD2/CD21 index of 10 may aid differentiation between unsuspicious and suspicious or diseased udder quarters. Detailed analyses indicated a connection between the CD2/CD21 index and the current or former presence of mastitis pathogens. Finally, applying advanced data analysis methods host-microbial interactions could be assessed. 3D data analysis distinguished three feedback phases: steady, positive, and negative. Furthermore, false-negative results of bacteriological analysis were detected based on spatial data patterns. The findings of this study may have broad applications including earlier diagnosis, differentiation of disease-positive classes, and lower rates of false-negative results.

Future prospects

Further research in this field should monitor the development of udder health to detect changes of SCC levels and the spectrum of pathogens causing mastitis. In view of DCC analysis further studies should focus on longitudinal examinations of immune cells in milk of udder quarters with SCC ≤100,000 cells/mL to investigate physiological variations of the DCC patterns. In this context a detailed analysis of quarters with SCC ≤100,000 cells/mL that reveal high percentages of PMNL is also crucial for a detailed characterization of factors triggering the elevated percentages of inflammatory cells. To confirm and refine the results gained in the studies on combinations of cell populations and cutoff values for an improved differentiation of healthy and diseased mammary glands, further studies with higher numbers of samples should be performed. Regarding the CD2/CD21 index longitudinal examinations with udder quarters specifically infected with major (e.g., *Staphylococcus aureus*) and minor pathogens (e.g., coagulase-negative staphylococci), respectively, are necessary for a more detailed characterization of the CD2/CD21 index. Concerning the advanced data analysis methods the data illustration may be improved by applying rotating 3D figures. Moreover, this study did not focus on the pathogenesis of any disease and therefore reproducibility of the findings should be explored in further studies.

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10th Chapter

Appendix

List of Publications

This thesis is based on the publications marked bold:

I   **D. Schwarz, U. S. Diesterbeck, K. Failing, S. König, K. Brügemann, M. Zschöck, W. Wolter, and C.-P. Czerny. 2010. Somatic cell counts and bacteriological status in quarter foremilk samples of cows in Hesse, Germany – A longitudinal study. Journal of Dairy Science 93:5716–5728. DOI: 10.3168/jds.2010-3223.**

II   **D. Schwarz, U. S. Diesterbeck, S. König, K. Brügemann, K. Schlez, M. Zschöck, W. Wolter, and C.-P. Czerny. 2011. Microscopic differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. Journal of Dairy Research 78:448–455. DOI: 10. 1017/S0022029911000574.**

III   **D. Schwarz, U. S. Diesterbeck, S. König, K. Brügemann, K. Schlez, M. Zschöck, W. Wolter, and C.-P. Czerny. 2011. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. Journal of Dairy Science 94:5033–5044. DOI: 10. 3168/jds.2011-4348.**

IV   P. Münster, I. Völkel, W. Wemheuer, D. Schwarz, S. Döring, and C.-P. Czerny. 2012. A longitudinal study to characterize the distribution patterns of *Mycobacterium avium* ssp. *paratuberculosis* in semen, blood and faeces of a naturally infected bull by IS900 semi-nesterd and quantitative real-time PCR. Transboundary and Emerging Diseases DOI: 10.1111/j.1865-1682.2012.01336.x.

V   **R. Pilla, D. Schwarz, S. König, and R. Piccinini. 2012. Microscopic differential cell counting to identify inflammatory reactions in dairy**

cow quarter milk samples. **Journal of Dairy Science 95:4410–4420. DOI: 10.3168/jds.2012-5331.**

VI **R. Pilla, M. Malvisi, G. G. M. Snel, D. Schwarz, C.-P. Czerny, S. König, and R. Piccinini. 2012. Differential cell count as a new method to diagnose dairy cow mastitis. Journal of Dairy Science DOI: 10.3168/jds.2012-6298.**

VII **D. Schwarz, A. L. Rivas, U. S. Diesterbeck, S. König, K. Schlez, M. Zschöck, W. Wolter, and C.-P. Czerny. 2012. CD2/CD21 index: A new marker to evaluate udder health in dairy cows. (submitted to PLOS ONE).**

VIII **A. L. Rivas, M. Jankowski, R. Piccinini, G. Leitner, D. Schwarz, K. L. Anderson, J. M. Fair, A. L. Hoogesteijn, W. Wolter, M. Chaffer, S. Blum, T. Were, S. N. Konah, P. Kempaiah, J. M. Ong'echa, U. S. Diesterbeck, R. Pilla, C.-P. Czerny, J. B. Hittner, J. M. Hyman, and D. J. Perkins. 2012. Feedback-based, system-level properties of vertebrate-microbial interactions. PLOS ONE DOI: 10.1371/ journal.pone.0053984.**

Non-peer reviewed publications:

I D. Schwarz and C.-P. Czerny. 2012. Vorsicht: Mastitis auch bei geringem Zellgehalt. Elite – Magazin für Milcherzeuger 1/2012.

II D. Schwarz and C.-P. Czerny. 2012. Mastitserreger auch bei niedrigen Zellgehalten. top agrar 2/2012.

List of Presentations

I D. Schwarz, K. Failing, W. Wolter, and C.-P. Czerny. Cell differentiation in milk of cows with healthy udders. XXV. World Buiatrics Congress. Budapest, Hungary. July 7, 2008.

II A. L. Rivas, K. L. Anderson, G. Leitner, D. Schwarz, R. Lyman, R. R. Rodriguez, M. Chaffer, A. Zecconi, O. Krifucks, C.-P. Czerny, and W. Wolter. Multi-dimensional diagnosis of intramammary infections: a 3-herd evaluation. International Conference on Mastitis Control. The Hague, The Netherlands. October 2, 2008.

III D. Schwarz, U. S. Diesterbeck, A. L. Rivas, K. Schlez, M. Zschöck, W. Wolter, and C.-P. Czerny. Looking for counts or looking for proportions? Looking for counts and proportions of leukocyte-microbial interactions. Workshop on quantitative models to asses infectious bacterial disease stages. Los Alamos, NM, USA. December 9, 2009.

IV U. S. Diesterbeck, A. L. Rivas, D. Schwarz, and C.-P. Czerny. Are all infections identical? Leukocyte-microbial disease staging. Workshop on Workshop on quantitative models to asses infectious bacterial disease stages. Los Alamos, NM, USA. December 9, 2009.

V R. Piccinini, R. Pilla, and D. Schwarz. The "first line of defense" does…not exist (there are many). Workshop on quantitative models to asses infectious bacterial disease stages. Los Alamos, NM, USA. December 10, 2009.

VI D. Schwarz, U. S. Diesterbeck, A.L. Rivas, K. Schlez, M. Zschöck, W. Wolter, and C.-P. Czerny. Molecular basis of disease staging: the role of CD2 and CD21. Workshop on quantitative models to asses infectious bacterial disease stages. Los Alamos, NM, USA. December 10, 2009.

VII   A. L. Rivas, R. Piccinini, R. Pilla, and D. Schwarz. Quantitative indicators of infections affecting vertebrates. Second workshop on quantitative models to asses infectious bacterial disease stages. Milan, Italy. October 8, 2010.

VIII   R. Piccinini, R. Pilla, and D. Schwarz. How differential cell counting can help sustainable herd management. IDF World Dairy Summit. Parma, Italy. October 17, 2011.

IX   P. Münster, S. Urstadt, I. Völkel, D. Schwarz, A. T. Kuhlen, S. Döring, and C.-P. Czerny. A longitudinal study to characterize the shedding patterns of *Mycobacterium avium* ssp. *paratuberculosis* in a naturally infected breeding bull by polymerase chain reaction assays. 11th International Colloquium on Paratuberculosis. Sidney, Australia. February 5, 2012.

X   D. Schwarz, U. S. Diesterbeck, S. König, K. Brügemann, K. Schlez, M. Zschöck, W. Wolter, and C.-P. Czerny. Zelldifferenzierung zur Identifikation von entzündlichen Reaktionen in klinisch gesunden Eutervierteln. Tagung der Arbeitsgruppe Sachverständigenausschuss „Subklinische Mastitis": Herausforderungen der Zukunft in der Mastitisbekämpfung. Grub/Poing, Germany. March 22, 2012.

XI   I. Völkel, D. Schwarz, J. Schüttler, U. Westenhorst, P. Münster, K. Fechner, W. Wemheuer, and C.-P. Czerny. Zytologische und hämatologische Parameter im Verlauf natürlicher Infektionen mit *Mycobacterium avium* ssp. *paratuberculosis* bei Besamungsbullen. 5. Arbeitstagung Mykobakterieninfektionen. Jena, Germany. January 29, 2013.

Curriculum Vitae

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Education

Name of school/college	Subject	Date
Modellschule Obersberg, Bad Hersfeld	Certificate of qualification for universitiy matriculation	08/2001-06/2004
Georg-August-University Göttingen, Göttingen	Bachelor of Science in Agriculture Specialization: Animal Sciences Thesis: Differential cell counts in milk of cows with healthy mammary glands	04/2005-08/2007
Georg-August-University Göttingen, Göttingen	Master of Science in Agriculture Specialization: Animal Sciences Thesis: Flow cytometric differential cell counts in milk of cows with somatic cell counts <50,000 cells/mL	10/2007-07/2009
Georg-August-University Göttingen, Division of Microbiology and Animal Hygiene, Institute of Veterinary Medicine, Department of Animal Sciences, Göttingen	PhD student	since 12/2009

Awards

"Sponsorship award for master thesis" of the journal top agrar. Münster. March 12th, 2009

"Award for the best master thesis 2009" awarded by Gesellschaft der Freunde der Landwirtschaftlichen Fakultät. Göttingen. November 20th, 2009

Acknowledgements

Declaration

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examination body either in its present or a similar form. Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

   Göttingen, ……………………

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   (Name in block capitals)

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

   Göttingen, ……………………

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   (Signature)

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