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Differential Cell Count (DCC) as an Alternative Method to Diagnose Dairy Cow Mastitis

VET05

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1. INTRODUCTION

1.1. Bovine Mastitis

Mastitis is the inflammation of the mammary gland. Such inflammation can be caused by many types of injuries, but most cases are caused by infectious agents and their toxins. Less often, physical trauma or chemical irritants can be sources of mastitis (Bramley et al., 1996).

Clinical mastitis is the most obvious manifestation of inflammation, resulting in secretion abnormalities, that include flakes, clots, or watery appearance. Udder may exhibit heat, swelling, pain and edema (Radostits et al., 2007), and systemic response may be present, including fever, loss of appetite, reduced rumen function, rapid pulse, dehydration, weakness and depression (Bramley et al., 1996).

To the contrary, in the course of subclinical mastitis there are no clinical abnormalities in milk, udder, or systemic signs (Bramley et al., 1996). The condition is therefore undetectable without laboratory examination of milk (Radostits et al., 2007). Subclinical mastitis is 15 to 40 times more prevalent than the clinical form, and, if not recognized and treated, is of long duration, impairing milk production (Philpot and Nickerson, 1991, Radostits et al., 2007)). Subclinical mastitis can be identified either by demonstrating the presence of microorganisms, or by detection of inflammatory changes in milk (Bramley et al., 1996).

1.2. Epidemiology and Economic Implications

Staphylococcus aureus (*S. aureus*) is a contagious pathogen and one of the most widely distributed causative agents of subclinical mastitis (IDF, 2006). Other contagious pathogens include *Streptococcus agalactiae*, *Mycoplasma bovis*, and

Corynebacterium bovis (Radostits et al., 2007). In case of contagious pathogens, the prompt identification of infected animals is crucial to implement measures to avoid spreading of the infection. Nevertheless, the detection of *S. aureus* can be difficult due to the intermittent shedding of the pathogen in milk (Sears et al., 1990), frequently below the detection limit of the bacteriological method (Zecconi et al., 1997), and to the presence of persistent infections without increase of milk cells. As a consequence, the cyto-bacteriological analysis has only a partial reliability, particularly when performed on a single sample (Schroder and Hamann, 2005).

Environmental pathogens, mainly environmental streptococci and coliform bacteria, may enter the mammary gland, typically causing clinical mastitis. Coliform mastitis is usually acute, and rarely results in chronic infections (White et al., 2009). On the other hand, mastitis by environmental streptococci is mild to moderate, and the infection chronicizes more frequently. Environmental mastitis is managed through improved sanitation of cow's environment, better udder hygiene, and adequate fly control (Radostits et al., 2007). 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).

Opportunistic pathogens normally reside on the teat skin. Coagulase-negative staphylococci (CNS) are the most common teat skin opportunistic etiologic agents of mastitis. CNS cause infections by ascending through the teat canal. Mastitis by CNS is mild, and spontaneous cure is common. They are classified as minor pathogens, and no specific control measures are indicated (Radostits et al., 2007).

Bovine mastitis is a major health problem in dairy cattle. Economical losses are mostly associated with decreased production and milk quality, as a consequence of subclinical infections (Philpot and Nickerson, 1991). It is estimated that 75% of the costs of subclinical mastitis are attributable to loss in milk production (Radostits et al., 2007).

Milk composition changes with the increase of inflammatory cells in milk. In the presence of subclinical mastitis, casein content is reduced, while the concentration of low quality whey proteins is increased. Lactose and fat contents are also reduced. Furthermore, enzymes originating from damaged mammary tissue, blood stream or milk somatic cells are increased, further reducing casein content and altering fat composition. Compositional changes in milk due to mastitis affect both quality and yield of cheese. Finally, both pasteurized milk and dairy products obtained from milk with high cellular content present reduced shelf-life, and may exhibit off-flavours, reducing consumer acceptance. (Bramley et al., 1996)

1.3. Mammary Gland Defences

Mastitis occur when pathogens enter the mammary gland via teat canal. For this reason, the teat end is considered the first line of defence against invading pathogens (Sordillo et al., 1997). The teat end comprises sphincter muscles that maintain tight closure between milkings, preventing bacterial penetration. The teat canal is also lined with keratin, a waxy material that hinders the migration of bacteria into the gland cistern (Sordillo and Streicher, 2002). The teat keratin contains esterified and nonesterified fatty-acids, such as mystiric acid, palmitoleic acid, and linoleic acid, that have bacteriostatic properties (Sordillo et al., 1997). In addition, cationic proteins in the teat canal can bind electrostatically to pathogens, alter their cell walls, and render them more

susceptible to osmotic pressure (Sordillo and Streicher, 2002). Finally, in the upper end of the teat canal, mucosal folds named Furstenberg's rosette assist in teat closure, protecting the cistern against ascending pathogens (Barone, 1983).

If bacteria are able to overcome the anatomical defence provided by the teat end, they still must evade the cellular defences of the mammary gland in order to establish disease. In the mammary gland, number and distribution of leukocytes are important for the successful defence against invading pathogens (Leitner et al., 2003). Lymphocytes, macrophages, and neutrophils play an important role in the inflammatory response within the mammary gland (Paape et al., 1979; Sordillo and Nickerson, 1988), determining the severity and duration of intramammary infections (Sordillo and Streicher, 2002). Resident cells which are already in the milk without a preceding infection are the first that come into contact with invading pathogens (Baumert et al., 2009). Shortly after pathogen recognition, additional cells are recruited into milk, resulting in an increase of somatic cell counts (SCC) (Baumert et al., 2009).

Macrophages are active phagocytic cells, capable of ingesting bacteria, cellular debris, and milk components (Sordillo and Nickerson, 1988). In healthy cows, macrophages are the predominant cells in the cisternal milk fraction, which is close to the entrance port of pathogens (Sarikaya et al., 2006). They actively recognize and engulf pathogens, releasing chemotactic and inflammatory mediators, initiating the inflammatory response through rapid recruitment of neutrophils (Paape et al., 2002; Oviedo-Boyso et al., 2007; Rainard & Riollet, 2006).

The main task of neutrophils is to defend against invading bacteria at the beginning of acute inflammatory process (Paape et al., 1979; Oviedo-Boyso et al., 2007). Neutrophils kill ingested bacteria through a respiratory burst that produces

hydroxyl and oxygen radicals (Sordillo and Streicher, 2002). In response to chemotatic stimuli, neutrophils migrate from blood into milk. However, diapedesis utilizes energy reserves that are needed for efficient phagocytosis. Furthermore, upon arrival in the mammary gland, neutrophils ingest fat and casein, further decreasing phagocytosis ability. As a result, large numbers of neutrophils are needed to effectively defend the mammary gland against invading pathogens (Paape et al., 2003). In addition to their phagocytic abilities, neutrophils are also a source of small antibacterial peptides, the defensins, which are able to kill a number of mastitis pathogens (Sordillo et al., 1997).

Lymphocytes regulate the induction and suppression of immune responses (Nickerson, 1989), recognizing antigens through membrane receptors specific for invading pathogens (Sordillo et al., 1997). Becoming sensitized to those antigens, they elicit a memory response to subsequent antigen exposure, through the production of immunoglobulins and lymphokines. Immunoglobulins serve as opsonins promoting phagocytosis, while lymphokines increase leukocyte infiltration and activate phagocyte microbicidal properties (Nickerson, 1989).

Finally, mammary epithelial cells also play a role in mammary gland immunity. In fact, in healthy quarters, they may often be in direct contact with bacteria. Mammary epithelial cells are known to express toll-like receptors, and can produce several soluble factors, including interleukins and tumor necrosis factors (Rainard and Riollet, 2006). It is currently believed that epithelial cells are highly relevant effector cells of the innate immune defence for the cow (Goldammer et el., 2004).

In addition to cellular defences, soluble factors play an important role in mammary immune response. Such factors may be either specific, like immunoglobulins,

or non specific, like lactoferrin, transferrin, lysozyme, lactoperoxidase, the complement system, and cytokines (Sordillo and Streicher, 2002; Rainard and Riollet, 2006).

Immunoglobulins, or antibodies, can originate from blood, or be produced directly in the milk by B-lymphocytes (Rainard and Riollet, 2006). In milk, different types of immunoglobulins can be found. IgA is associated with the fat portion of milk; it agglutinates invading bacteria preventing the spread in the mammary gland (Sordillo and Streicher, 2002). Other antibodies (IgM, IgG1 and IgG2) act as opsonins, enhancing bacterial phagocytosis by neutrophils and macrophages (Sordillo and Streicher, 2002). IgM also allows complement fixation, and is the most abundant opsonic antibody in bovine milk (Rainard and Riollet, 2006).

Non specific factors act by either killing invading pathogens, or preventing their multiplication. Lactoferrin, for instance, binds free iron ions in milk, preventing the growth of iron-dependent bacteria (Sordillo and Streicher, 2002). Transferrin is also an iron-binding protein, but unlike lactoferrin, is not produced in the mammary gland, originating from blood serum (Rainard and Riollet, 2006). Lysozyme, instead, acts directly on bacterial cells walls, cleaving peptidoglycans of both Gram-positive and Gram-negative bacteria (Sordillo et al., 1997). Lactoperoxidase presents bacteriostatic activities, but its activity is limited since it depends on the presence of both thiocyanate and hydrogen peroxide in milk (Rainard and Riollet, 2006). The complement system is a group of proteins produced mostly by hepatocytes. These proteins are present in both serum and milk, and are implicated in different functions: bacterial lysis, opsonisation, and attraction of phagocytes to the site of complement activation (Sordillo and Streicher, 2002).

Cytokines present immunomodulatory capabilities, and include interleukins (IL), colony-stimulating factors (CSF), interferons (IF), and tumor necrosis factors (TNF). Among IL, IL-2 is the most extensively characterized of all bovine cytokines. It is primarily produced by T-lymphocytes, and enhances mononuclear cell proliferation, as well as cytotoxic and bactericidal activities of lymphocytes (Sordillo and Streicher, 2002). CSF are growth factors; each CSF tends to target a specific cell lineage expanding or activating its functions. IF exhibits a variety of immunomodulatory properties, and stimulates phagocytes increasing both phagocytic and bactericidal abilities (Sordillo et al., 1997). TNF enhances acute phase inflammatory response; it is associated with coliform mastitis, where TNF-α is a major factor that causes endotoxic shock (Sordillo and Streicher, 2002).

1.4. Diagnosis

The presence of subclinical mastitis can be diagnosed by bacteriological analysis, or PCR. Bacteriological analysis can be performed on either individual quarter samples, or composite samples of all four quarters. Culturing of quarter milk samples is more accurate, and more information can be obtained from those samples (Bramley et al., 1996). Bacteriological analysis gives the precise aetiology of infection, and is often necessary to understand specific herd problems. Culturing of all animals in a herd can be indicated when there is a need to identify infected cows for treatment or segregation (Philpot and Nickerson, 1991). Bacteriological analysis, though, is time-consuming and requires experienced personnel. PCR has been proposed as an alternative to bacteriology, as a rapid test (Koskinen et al., 2009) but it is expensive.

Indirect tests for subclinical mastitis measure the level of inflammation, based on the detection of inflammatory changes on the milk sample. Somatic cell counting (SCC)

is an indirect method, and measures the total number of cells in the milk sample. Somatic cells consist of many cell types. Leukocytes (mostly macrophages, neutrophils and lymphocytes) are the largest percentage of cells found in milk, and originate from either blood or mammary parenchyma. Epithelial cells are present in low percentages, and originate from secretory parenchyma.

Other indirect tests include California Mastitis Test (CMT) and the detection of increases in electrical conductivity of milk or in the activity of cell associated enzymes (NAGase). CMT reagent is a detergent that, interacting with cellular DNA, gives a rough estimation of cell content of milk. It requires no other equipment than a plastic paddle, so it can be performed cowside. Electrical conductivity tests evaluate the increased conductivity that derives from a higher concentration of sodium and chloride ions in milk from inflamed quarters. Electrical conductivity tests can be performed by robotic milking systems, giving instant results. The NAGase test measures the levels of N-acetyl-β-D-glucosaminidase, an enzyme associated both with neutrophils and damaged epithelial cells (Radostits et al., 2007).

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 (IDF) recommends the use of both somatic cell count (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. This is supported by Harmon et al. (1994), who shows that quarters with SCC over this threshold present losses in milk production. In addition, Piörälä (2003) states that over 100.000 cells/mL, milk components will differ significantly from the physiological norm.

1.5. Differential Cell Count

Differential cell count (DCC) evidences changes in relative cell proportions. These changes can be used to differentiate healthy glands from inflamed or infected ones. It has been proposed as a valid tool for the identification of inflammatory processes (Rivas et al., 2001). Since low SCC can be found in both healthy and infected quarters, DCC could be used to identify infected quarters despite low SCC. In fact, recent studies (Schwarz et al. 2011a, b) have shown that DCC can reveal inflammatory processes even in milk with 9.000 cells/mL.

In healthy milk, the percentage of each cell type is widely variable. According to some authors, macrophages are the predominant cell type (Lindmark-Mansson et al., 2006; Miller et al., 1991; Östensson, 1993). Others reported that lymphocytes are a major population (Dosogne et al., 2003; Park et al., 1992; Schwarz et al., 2011a, b). Overall, in literature, percentages in healthy milk vary from zero to 80% for macrophages, 1.5% to 60% for lymphocytes, and 3% to 95% for neutrophils (Schröder and Hamann, 2005). Those different results could be attributed to the different criteria applied for the definition of healthy quarters (Schröder and Hamann, 2005, Schwarz et al., 2011b). In fact, Lindmark-Mansson et al. (2006) and Miller et al. (1991) considered quarters with no bacteriological growth as healthy, regardless of SCC. On the contrary, others proposed both bacteriological results and SCC as criteria to define a gland as healthy or infected. Namely, Park et al. (1992) established a cut-off of 500.000 cells/mL, Dosogne et al. (2003), 200.000 cells/mL, and Östensson (1993) and Schwarz et al. (2011a, b) considered 100.000 cells/mL.

In the acute phase of infection, neutrophils are the predominant cell type in milk (Leitner et al. 2000; Östensson, 1993), with values that can account to more than 90%

of the total mammary leukocyte population (Sordillo and Streicher, 2002). Chronic mastitis caused by *Streptococcus dysgalactiae* presents leukocyte population patterns similar to those of acute infections (Leitner et al. 2000). Instead, in chronic mastitis caused by *S. aureus* and coagulase-negative Staphylococci (CNS), neutrophils percentage can be as low as that in uninfected quarters, while macrophage percentage is higher (Leitner et al. 2000).

Moreover, cell percentages can vary dependently of the milk fraction sampled, since cisternal milk showed lower neutrophils percentage when compared to alveolar milk (Sarikaya et al., 2005). In addition to the milk fraction, parity number should be taken in to account (Dosogne et al., 2003). Dosogne et al. (2003) also reported the effect of days in milk on cell percentages, showing that lymphocytes decreased while neutrophils and macrophages increased in the course of lactation.

Differential cell count can be performed using different methods. Microscopic DCC is a simple and cost-effective method. It can be performed by either direct smear or using a cytospin centrifuge (Dulin et al., 1982). Dulin et al. (1982) found no significant difference between DCC obtained with both methods. However, while both techniques equally allow good adhesion to slides, cytospin concentrates cells in a smaller area, rendering slide reading faster, and is particularly useful for low SCC milk samples (Dulin et al., 1982).

The first report of DCC using a flow cytofluorometric technique was made in 1986 (Hageltorn and Saad, 1986). Flow cytometric identification of milk leukocytes based on side and forward scatter is difficult because phagocytosis of milk components alters both size and granularity of cells. Therefore most DCC flow cytometric techniques for milk are based on DNA labeling, or on marked monoclonal antibodies

against CD molecules. The first technique reported involved incubation of milk with hypotonic phosphate buffer containing supravital acridine orange, and was later modified by Redelman et al. (1988). The modified technique stained cells in isotonic buffer with 5(6)-carboxy-4'-5'-dimethylfluorescein diacetate, and allowed the detection of degenerating neutrophils and activated macrophages that were previously lost presumably due to hypotonic conditions (Redelman et al., 1988). Further DNA stains were since then applied, namely SYBR green 1 and propidium iodide (Pillai et al., 2001), or SYTO 13 (Dosogne et al., 2003).

The use of marked antibodies for flow cytometric analysis of milk cells by targeting cell surface antigens may provide additional information on subpopulations. Park et al. (1992) studied mononuclear subpopulations, and found that the largest subpopulation of lymphocytes in milk was represented by T lymphocytes, followed by B lymphocytes. Leitner et al. (2000) considered B lymphocytes to be negligible.

Recently, most authors seem to prefer cytometric analysis, for its higher accuracy. Leitner et al. (2000) encountered a high correlation between the two methods for neutrophils and lymphocytes, and a lower one for macrophages and epithelial cells, probably due to the difficulty in the differentiation between these cell populations with light microscopy. Both techniques seem to be applicable for milk DCC, and method choice depends on the aim of the study.

2. AIM OF THE STUDY

A successful control of contagious mastitis is based on prompt identification of infected quarters. In fact, most control programs for *S. aureus* involve the immediate segregation or culling of infected animals to prevent transmission of the contagious pathogens during milkings. SCC has been applied as an indicator of infection, since most infected quarters show an increase in cell counts. Nevertheless, in some cases infections may be present in quarters with low and very low SCC, allowing such animals to remain as reservoir in the herd. Identification of infected quarters through bacteriological analysis or PCR is more accurate, since the detection of *S. aureus* in milk is considered the gold standard for the diagnosis of *S. aureus* mastitis. On the other hand, false-negative results are often found as consequence of *S. aureus* intermittent shedding pattern, and repeated samplings are necessary to ensure a reliable diagnosis.

DCC has been proposed as a diagnostic method for the identification of subclinical mastitis. Such method is particularly useful in low SCC quarters, where the deviation from a healthy milk cellular profile is indicative of an inflammatory process.

The study had two aims: first, to identify the DCC variable which is best indicative of udder health. Such variable must be applicable to milk samples independently of cow parity and lactation stage. The second aim was to evaluate the applicability of the variable in the identification of infected quarters. The use of DCC as a diagnostic method is possible only in the presence of short-term stability of mammary immune response. Therefore, the variations in milk cellular profiles need to be investigated to assess the applicability of DCC for diagnostic purposes. The final goal was to find a cutoff value for the variable to be used for diagnostic purposes in the control of *S. aureus* mastitis.

3. MATERIALS AND METHODS

3.1. Trial 1

3.1.1. Animals

Three herds in Lombardy region, Italy, were enrolled in the study. The selected herds were participating in a voluntary control program for contagious or environmental pathogens. All the three herds were free stalls and housed Holstein-Frisian cows which were milked twice daily in a milking parlour. Herd A, with 120 animals, was characterised by the absence of contagious microorganisms. Herd B, with 180 animals, had 20% prevalence of *S. aureus* mastitis, while in herd C, with 180 animals, approximate prevalence of *S. aureus* mammary infections was 50%.

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

3.1.2. Milk Sampling

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

3.1.3. Somatic Cell Count and Bacteriological Analysis

All samples were submitted to bacteriological analysis, which was performed as previously described (Oliver et al., 2004). Briefly, an aliquot of 10µl of each sample was spread onto blood-agar plates (5% bovine blood; Oxoid, UK), and incubated at 37°C. Plates were evaluated after 24 and 48h, and colonies of growth were isolated.

Large and haemolytic colonies, that were catalase and coagulase positive, were identified as *S. aureus* and thereafter confirmed by API ID32 Staph (Biomerieux, F). 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, USA).

3.1.4. Differential Cell Count

Slides were prepared following Dulin et al. (1982), with slight modifications. Briefly, for each sample, a milk aliquot of 4mL was diluted with 10mL of phosphate buffered saline (PBS) with 0,5% EDTA (PBS-EDTA). Samples were then centrifuged at 125 x *g* for 15 minutes and cell pellets were resuspended in PBS-EDTA. Cell suspensions were centrifuged on a cytocentrifuge (Cytospin, Thermo Scientific, USA) at 20 x *g* for 5 min. Slides were air-dried and stained with May Grunwald-Giemsa stain.

Each slide was evaluated by light microscopy and 100-200 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.

3.1.5. 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 detection of 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), and included culture-positive quarters with SCC >100,000 cells/mL.

The impact of the four 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:

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

where:

y_{ijklmn}	=	observation for the individual cell population 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* parity of cow l
DIM_k	=	fixed effect of the k-*th* 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

e_{ijklmn} = random residual effect

For a second analysis, udder quarters were classified into two different categories healthy and diseased. Healthy udder quarters were assigned a score of 0 and comprised the group N, whereas diseased udder quarters (including groups LM, UM, and M) were assigned a score of 1. Because the defined disease was treated as a binary trait, a logistic model was applied to assess the impact 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:

$$\text{logit}(\pi_{rstu}) = \log\left[\frac{\pi_{rstu}}{1-\pi_{rstu}}\right] = \eta_{rst} = \varphi + \gamma_r + \lambda_s + \tau_t + \upsilon_u + \varphi_v + b_1 Y_{rstu} \quad [2]$$

where

π_{rstu} = 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

b_1 = linear regression of the disease on the value of the individual cell population

3.2. Trial 2

3.2.1. Animals

The herd enrolled in the study was located in Lombardy region, Italy, and was certified free of paratuberculosis, bovine viral diarrhoea and infectious bovine rhinotracheitis; it also had no history of contagious pathogens in the last 10 years. The herd consisted of 50 lactating Holstein-Frisian dairy cows, that were housed in free stalls and milked twice daily in a milking parlour.

Overall 8 cows were selected, based on low SCC and two negative results of bacteriological analysis on the week before samplings. Out of them, 3 cows were primiparous, 4 were in the second or third lactation, and 1 had calved four times. Two animals were in early lactation (83 to 111 d), 3 were in midlactation (144 to 172 d), and 3 in late lactation (233 to 357 d).

3.2.2. Milk and Blood Sampling

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 250mL milk was aseptically collected from each quarter in sterile plastic tubes (Falcon; BD Biosciences, Franklin Lakes NJ, USA).

Blood samples (10mL) were collected by tail venipuncture into commercial EDTA-containing evacuated tubes (Vacutainer; BD Biosciences, San Jose CA, USA). Samples were kept under refrigeration until arrival to laboratory facilities

3.2.3. Somatic Cell Count and Bacteriological Analysis

All samples were submitted to bacteriological analysis and somatic cells counting as described in the first trial.

3.2.4. Differential Cell Count

Differential cell counts were performed on blood samples and on all quarter milk samples by cytometric method. Milk cells were isolated according to the protocol described by Koess and Hamann (2008), with modified centrifugation conditions. Briefly, 200mL milk was centrifuged at 250 *g* for 30 min at 4°C. Cream layer and supernatant were discarded, and cell pellet was washed twice in 30mL phosphate-buffered saline (PBS). Blood leukocytes, instead, were collected by centrifugation at 500 *g* for 10 min, after erythrocyte lysis with Cell Lysis Solution (Promega #A7933, Madison, WI, USA).

Cell pellets were resuspended in 500µL of RPMI with 10% foetal calf serum, and cells were counted in a haemocytometer; finally, cell concentration was adjusted to 2 x 10^{6} cells/mL. Aliquots of 100µL of each sample were incubated with FITC and RPE marked antibodies (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 3-18h and analysed using a FACScalibur flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA, USA). Eight thousand events were acquired per sample. Data were further analysed using Cyflogic v. 1.2.1 free software.

CD molecule	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

Table 1: List of antibodies used. All antibodies were purchased from Ab Serotec, Oxford, UK. Fluorescein isothiocyanate (FITC), R-phycoerythrin (PE)

Percentages of PMNL, lymphocytes and macrophages were calculated. In addition, to increase discrimination power of DCC, logarithmic PMNL/lymphocytes ratio (log PMNL/Lym) and logarithmic phagocytes/lymphocytes ratio (log Phag/Lym) were calculated as described in the first trial (Pilla et al., 2012).

3.2.5. Statistical Analysis

For analyzing the impact of fixed effects, random effects, and covariates on DCC in milk and blood, linear mixed models were applied. The statistical model was defined as follows:

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

where y_{ijklm} = DCC of cow l, μ = overall mean effect, $parity_i$ = fixed effect of parity i (first parity or higher lactation no.), DIM_j = fixed effect for classes of days in milk j (early, middle, or late), quarter = fixed effect of udder quarter k, cow = random effect of cow l, day = consecutive no. of measurement within cow from day 1 to day 5 (sampling no.), α_1 = linear regression of sampling no. on DCC, DIM* $\alpha_1 day_{ijkl}$ = interaction between DIM and sampling no., 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 SAS-procedure 'proc mixed'.

For the determination of the cutoff value for log PMNL/Lym in milk, quarters samples with SCC under 10^5 cells/mL and negative bacteriological results were considered healthy, and were attributed a score of 0. All other samples were considered diseased, and attributed a score of 1. Log PMNL/Lym values and attributed scores were then tested with receiver operator characteristics analysis using SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL), and a cutoff value was chosen to maximize both sensitivity and specificity.

3.3. Trial 3

3.3.1. Animals

The herd enrolled in the study was located in Lombardy region, Italy, and was participating in a voluntary control program for contagious pathogens. The herd consisted of 180 lactating Holstein-Frisian dairy cows, that were housed in free stalls and milked twice daily in a milking parlor. The herd presented a history of high prevalence of *S. aureus* (approximately 50% prevalence at the beginning of the control program); recently, mammary infections by *Prothoteca zopfii* were also detected.

Overall 16 cows were randomly selected from the last milking group, which included animals previously diagnosed as infected by *S. aureus* or *P. zopfii*, and other animals before culling. Out of them, 9 cows were primiparous and 7 pluriparous.

3.3.2 Milk Sampling

Quarter milk samples for bacteriological analysis were collected at three consecutive milkings, following procedures described in the first trial. At the second milking, an additional 200mL quarter milk was sampled for DCC analysis. Samples were kept under refrigeration until arrival to laboratory facilities.

3.3.3. Somatic Cell Count and Bacteriological Analysis

All samples were submitted to bacteriological analysis and somatic cells counting as described in the first trial. Suspected *S. aureus* isolates were further confirmed by real-time PCR for the presence of the *nuc* and *Sa442* genes as described elsewhere (Brakstad et al., 1992; Martineau et al., 1998). Somatic cells were counted on a Bentley Somacount 150 (Bentley Instruments, Chaska MN, USA).

3.3.4. Differential Cell Count

Differential cell counts were performed on quarter milk samples as described in the previous trial.

3.3.5. Statistical Analysis

Quarter milk samples were classified as diseased or healthy in basis of SCC and bacteriological results. Quarters with SCC under 10^5 cells/mL and negative bacteriological results were considered healthy, and were attributed a score of 0. All other samples were considered diseased, and attributed a score of 1. Such classification was considered as gold standard.

The cutoff value defined in the previous trial was then applied to calculated log PMNL/Lym. All quarters with values under the cutoff were considered healthy, and attributed a score of 0. All samples with values over the cutoff were considered

diseased, and attributed a score of 1. Sensitivity and specificity of the method under field conditions were then calculated.

4. RESULTS

4.1. Trial 1

4.1.1 Somatic Cell Count 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 one showed a major pathogen, *Escherichia coli (E. coli)*. Also, CNS were detected in 5 samples, *Enterococcus faecalis (E. faecalis)* in 4, and *Streptococcus dysgalactiae (S. dysgalactiae)* in one sample. In herd B, 57 out of 78 quarters tested (73.1%) were bacteriologically negative, while 13 (16.6%) were positive for *S. aureus*. Four samples showed the presence of CNS, and further 4 samples were considered contaminated. Finally, in herd C, 25 out of 40 quarters tested (62.5%) were bacteriologically negative, while 10 (25%) were positive for *S. aureus*. In 3 samples CNS were detected, and *E. faecalis* in one sample.

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

Group	No. Quarters	Bacteriological result	Bacteria isolated	No. Quarters	SCC (× 1,000 cells/mL)	SCC mean ± SEM (× 1,000 cells/mL)
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	1367.80 ± 840.58

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

4.1.2. Differential Cell Count

Differential cell count of all quarter foremilk samples analyzed were determined by microscopic differentiation of 100 to 200 cells into lymphocytes, macrophages and PMNL. Beside the percentages of individual cell populations, the following variables were also considered: phagocytes (Phag, combining the percentages of macrophages and PMNL); logarithmic PMNL/lymphocyte-ratio (log PMNL/Lym); logarithmic phagocytes/lymphocyte-ratio (log Phag/Lym, involving the three cell populations).

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%), while proportions of PMNL ranged between 0 and 96% with a mean of 55.8% (SD 25.8%).

Cell population or variable	Farm					
	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

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

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 herd A, B and C, respectively. Mean percentages of lymphocytes showed similar values in the 3 herds (29% in herd 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), while herd C showed a much higher mean value (42.6%). Mean percentages of phagocytes were similar in all herds (71% in herd A, 79.9% in B and 74.4% in C), while the mean values of both log PMNL/Lym and log Phag/Lym were higher in herd B, in comparison to those in herd 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 the farm, and macrophages were further significantly ($P < 0.01$) influenced by days in milk. None of the individual cell populations was influenced by quarter position or by lactation number (Table 3). The variables Phag, log Phag/Lym, and log PMNL/Lym were significantly ($P < 0.01$) influenced by the farm but not by quarter position, lactation number, or days in milk (Table 3). Overall, a significant ($P < 0.0001$) effect of the 4 health groups was demonstrated on percentages of lymphocytes and PMNL and in all combined variables, but not on macrophages (Table 3).

Cell population or variable	Effect				
	Group	Quarter position	Lactation number	Days in milk	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

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. Analyzed factors were group (N, LM, UM, M), 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-c).

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). Also, the value in group LM was significantly ($P < 0.05$) higher in than 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 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).

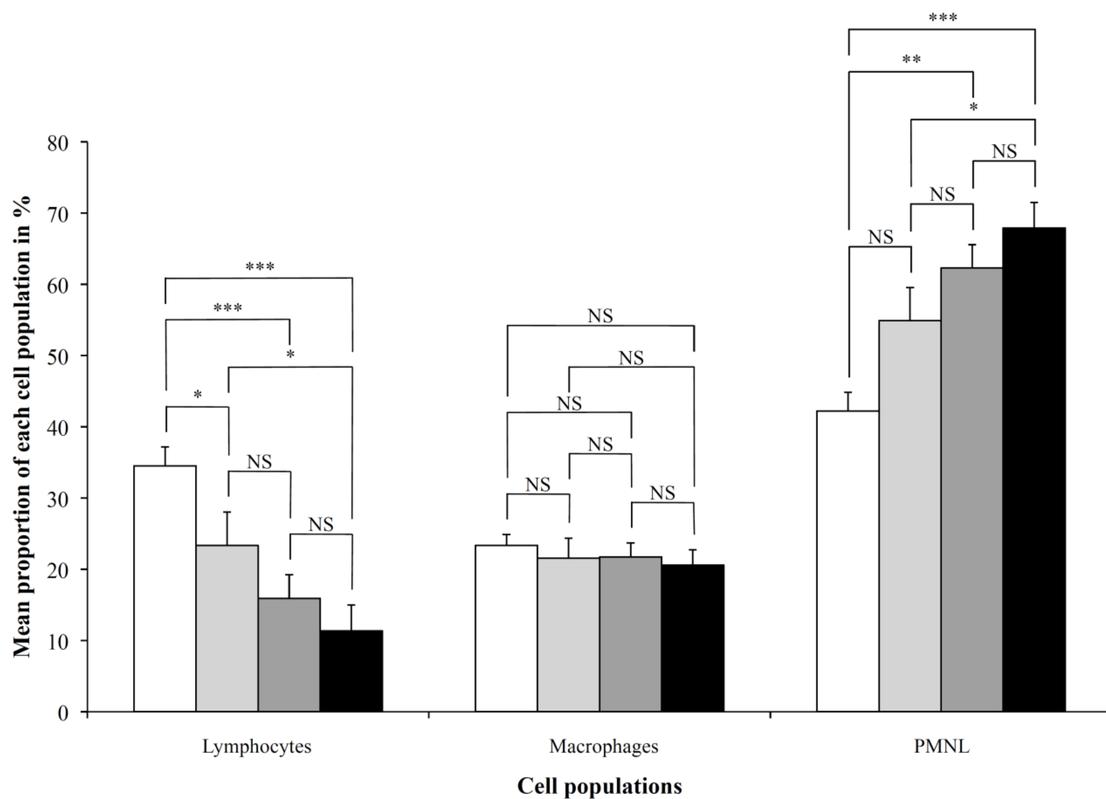

Figure 1. Comparison of mean percentages of lymphocytes, macrophages, and PMNL in milk of udder quarters with different health status. All 188 udder quarters analyzed were classified into groups N (empty bars), LM (light gray bars), UM (dark gray bars), and M (black bars). Data are expressed as mean ± 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 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, group LM value was significantly ($P < 0.05$) lower than group M.

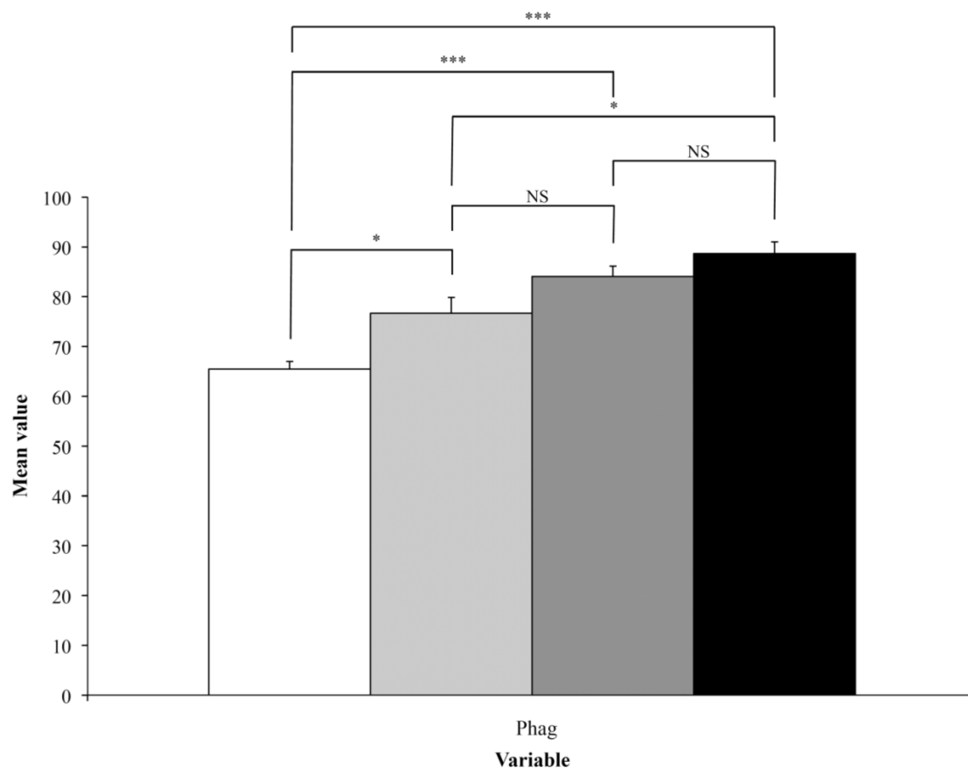

Figure 2. Comparison of mean values of phagocytes (macrophages and PMNL) in milk of udder quarters with different health status. All 188 udder quarters analyzed were classified into groups N (empty bars), LM (light gray bars), UM (dark gray bars), and M (black bars). Data are expressed as mean ± SEM. *** $P < 0.001$; * $P < 0.05$; NS = $P > 0.05$.

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); also the mean value in group LM was lower in comparison to group M ($P < 0.05$, Figure 3).

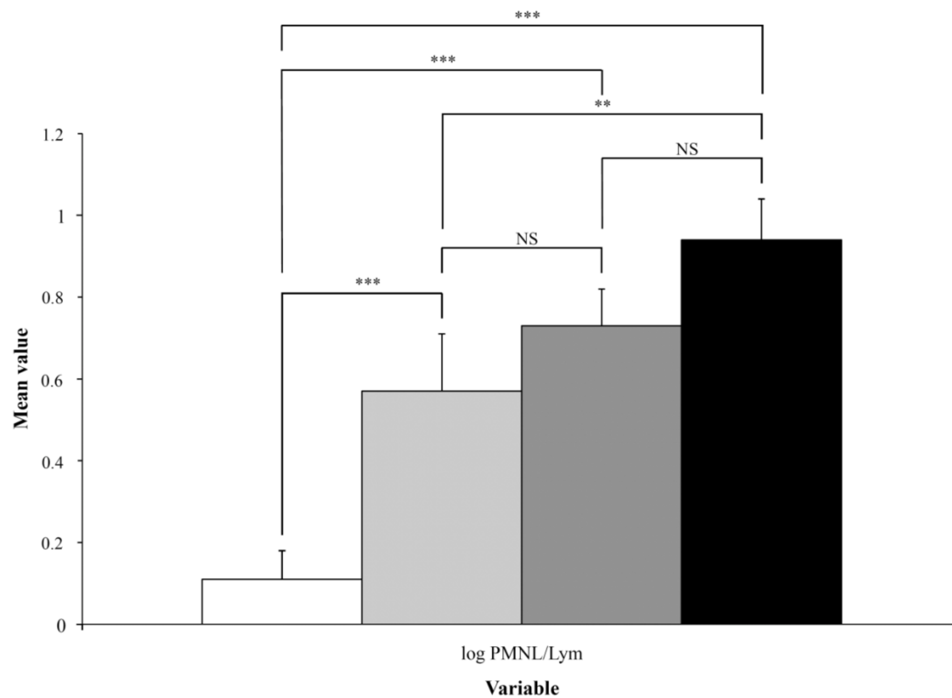

Figure 3. Comparison of mean values of the variable log PMNL/Lym (logarithmic polymorphonuclear neutrophilic leukocytes/lymphocytes ratio) in milk of udder quarters with different health status. All 188 udder quarters analyzed were classified into groups N (empty bars), LM (light gray bars), UM (dark gray bars), and M (black bars). Data are expressed as mean ± SEM. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS = $P > 0.05$.

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 significant ($P < 0.05$) higher mean value than group LM (Figure 4).

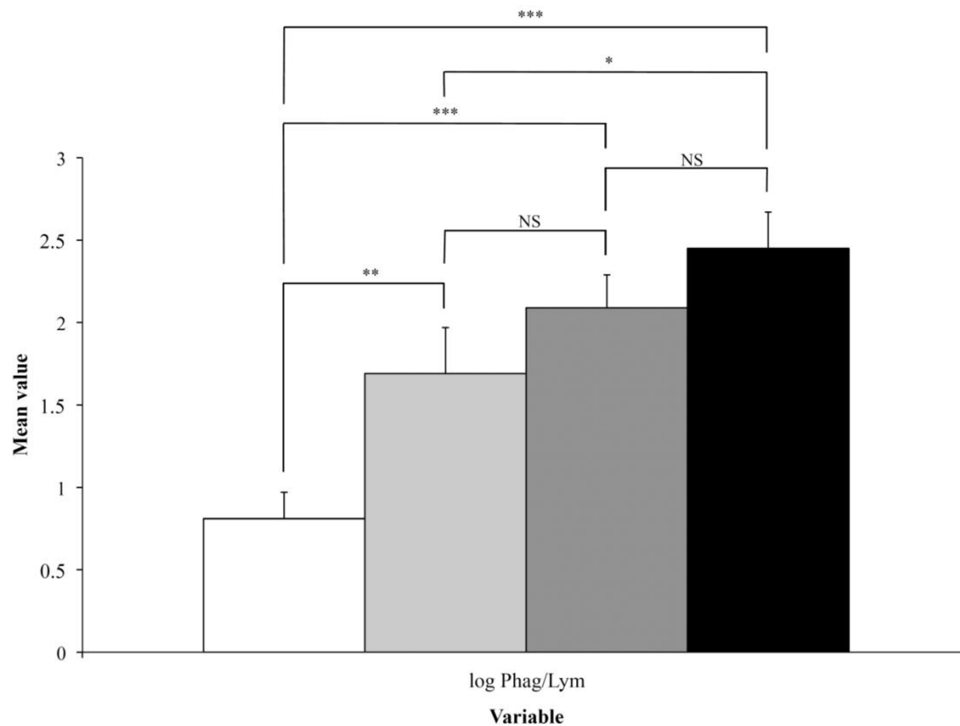

Figure 4. Comparison of mean values of the variable log Phag/Lym (logarithmic phagocytes/lymphocytes ratio) in milk of udder quarters with different health status. All 188 udder quarters analyzed were classified into groups N (empty bars), LM (light gray bars), UM (dark gray bars), and M (black bars). Data are expressed as mean ± SEM. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS = $P > 0.05$.

The possibility to differentiate between healthy and diseased udder quarters was further evaluated using individual cell populations as well as the variables combining different cell populations. For this purpose, quarters were split into two groups, and Fisher-values were calculated. The first group (N) included all healthy quarters (n = 96), while groups LM, UM, and M were combined in the group of diseased mammary quarters (D, n = 92). Out of the 3 individual cell populations, lymphocytes were the best one to differentiate between group N and D (Table 4). While macrophages showed an

F-value of 1.65 and did not differ significantly between group N and D, the F-value of PMNL of 15.54 was clearly 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 at a level of P < 0.0001.

Marker	*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

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.

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

4.2. Trial 2

4.2.1. Somatic Cell Count and Bacteriological Analysis

From 159 quarter samples analyzed, mean SCC was 77.770 cells/mL (SD 185.510). Thirty-four samples had SCC over 100.000 cells/mL, with a maximum value of 1.512.000 cells/mL. Only 4 samples were bacteriologically positive. Of those, coagulase-negative Staphylococci (CNS) were isolated from 3 samples, and in one sample *Streptococcus* sp. was isolated. Data from positive quarters was excluded from the statistical analysis.

4.2.2. Differential Cell Count

Overall (n = 155 samples), the proportion of PMNL ranged between 2.2 and 93.5%, with a mean of 43.1% (SD 23.5%), that of lymphocytes ranged between 1.3 and 96.4% with a mean of 30.1% (SD 19.4%), and proportions of macrophages ranged between 1.4 and 69.6% with a mean of 26.9% (SD 15.7%). Log PMNL/Lym ranged between -1.63 and 1.86, with a mean value of 0.22 (SD 0.62), and log Phag/Lym values ranged between -1.43 and 1.89, with a mean value of 0.48 (SD 0.53).

Results of variance analysis in milk are summarized in Table 5. Sampling day showed no significant effect on the percentages of individual cell populations. P-value associated with PMNL percentage was 0.7847, with lymphocytes was 0.9797, and with macrophages was 0.6805. Associating individual cell populations into ratios produced similar results, with P-values of 0.8894 for log PMNL/Lym and 0.8127 for log Phag/Lym. Even though differences correlated to lactation stage were seen for all variables, no statistic significance could be found, with associated P-values ranging

from 0.7319 to 0.8891. No significant influence was found for either parity or quarter position.

Cell population or variable	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

Table 5. Probability values (*P*-values) for testing significance of fixed effects of 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 the linear regression of sampling day (days 1-5) on the percentage of individual cell populations as well as combinations of cell populations in milk

Variance analysis of blood samples showed similar results (Table 6). Neither sampling day nor parity or lactation stage showed significant effect on the percentages of individual cell populations and ratios.

Cell population or variable	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

Table 6. Probability values (*P*-values) for testing significance of fixed effects of parity (1 or 2, and 3 or 4), lactation stage (early, mid or late lactation), and the linear regression of sampling day (days 1-5) on the percentage of individual cell populations as well as combinations of cell populations in blood

Figure 5 shows least square mean values in blood for individual cell populations in each sampling day, corrected for quarter position and parity, and separated for lactation stage. While no significance could be attributed to lactation stage, samples from early stages of lactation presented lower values of lymphocytes and higher values of macrophages and PMNL than mid and late lactation.

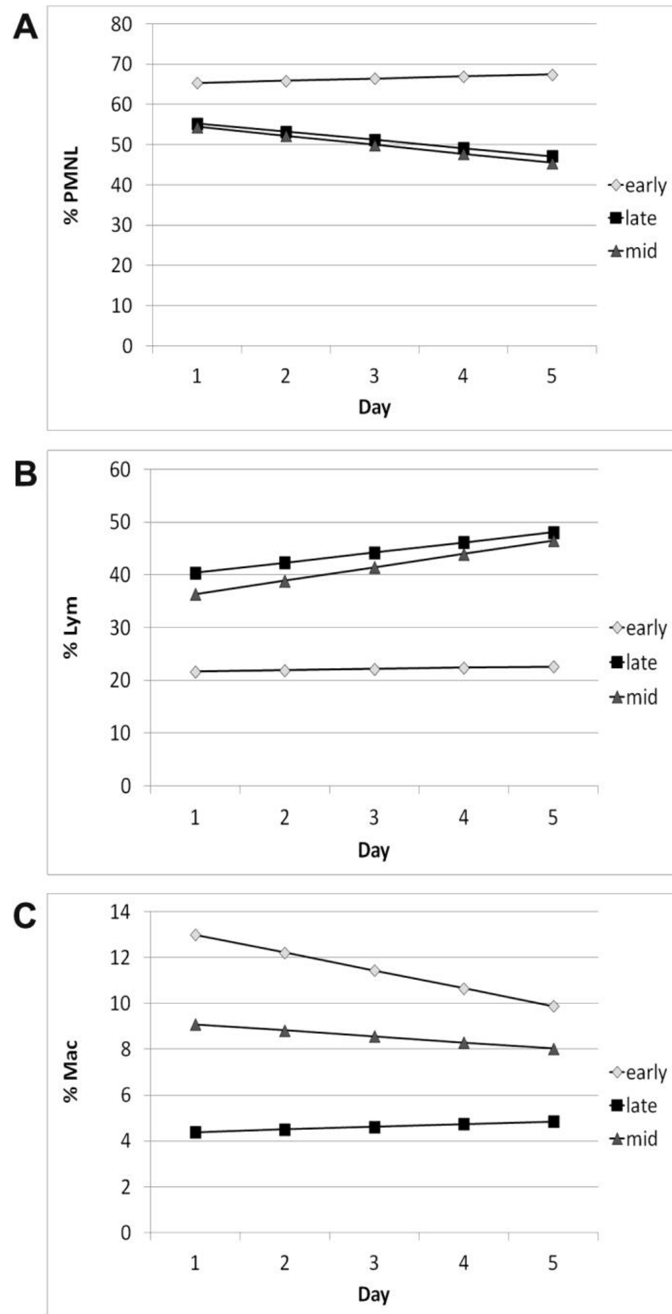

Figure 5. Least square means of PMNL percentages (A), lymphocyte percentages (B), and macrophage percentages (C) in blood for each sampling day, separated by lactation stage (early, mid or late).

Similarly, in milk, lymphocyte percentages were lower in early lactation, and macrophage percentages were lower in late lactation (Figure 6). These trends on the course of lactation are better shown in Figure 8a. Figure 7 shows least square mean values in milk for log PMNL/Lym and log Phag/Lym in each sampling day, corrected for quarter position and parity, and separated for lactation stage. Both ratios presented higher values on early lactation, showing an increasing trend on the following days, but no significance could be found. Least square mean values with associated errors for both ratios are shown in Figure 8b.

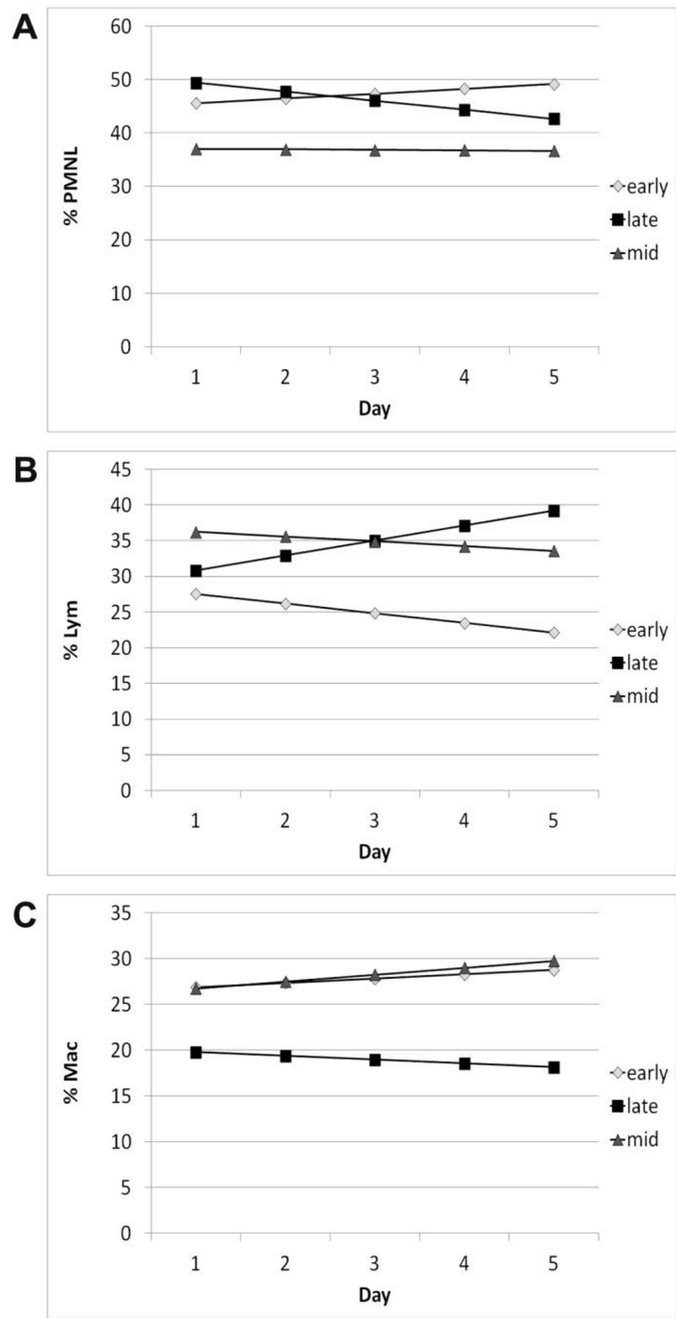

Figure 6. Least square means of PMNL percentages (A), lymphocyte percentages (B), and macrophage percentages (C) in milk for each sampling day, separated by lactation stage (early, mid or late).

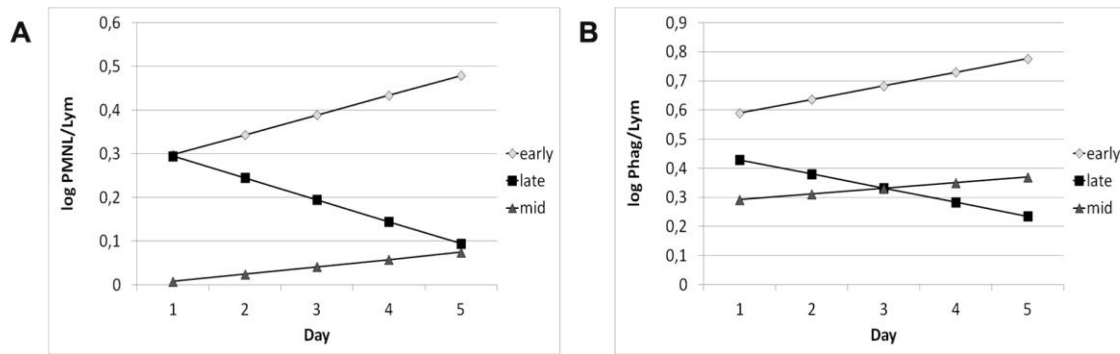

Figure 7. Least square means of logarithmic PMNL/Lymphocyte ratio (A), and logarithmic Phagocyte/Lymphocyte ratio (B), in milk, for each sampling day, separated by lactation stage (early, mid or late).

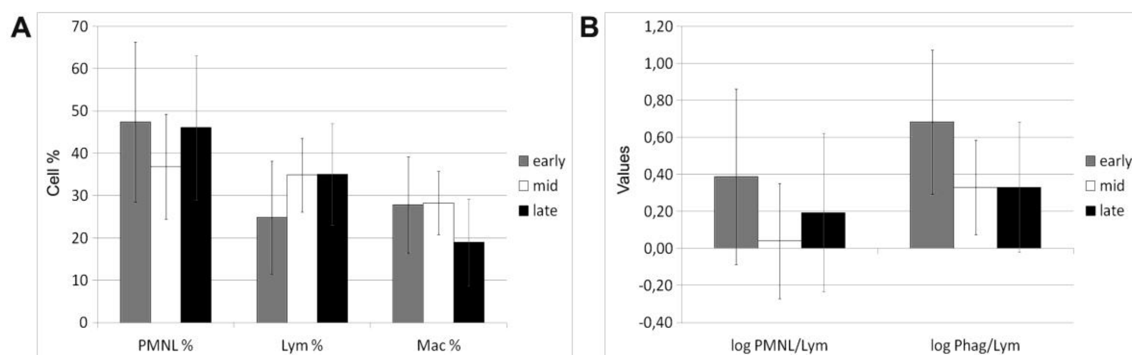

Figure 8. Least square means and standard error of PMNL, lymphocyte and macrophage percentages (A); of logarithmic PMNL/Lymphocyte ratio and logarithmic Phagocyte/Lymphocyte ratio (B), in milk , separated by lactation stage (early, mid or late).

Similarly, Figure 9a shows different individual cell populations for two parity groups, showing that animal that calved 3 or 4 times, when compared with primiparous or secondiparous cows, present lower lymphocyte and higher macrophage percentages,

but no significance could be found. Those animals also presented higher values, but not significant, for both ratios considered (Figure 9b).

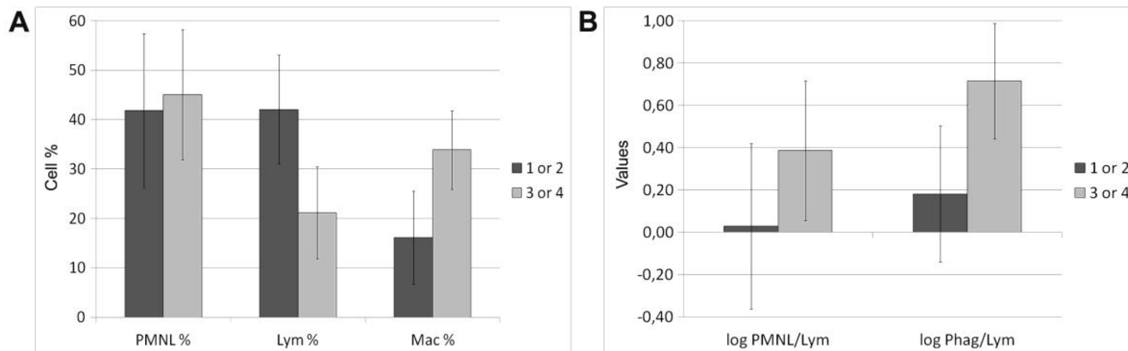

Figure 9. Least square means and standard error of PMNL, lymphocyte and macrophage percentages (A); of logarithmic PMNL/Lymphocyte ratio and logarithmic Phagocyte/Lymphocyte ratio (B), in milk, separated by parity number. Dark grey bars represent animals that calved once or twice, light grey bars represent animals that calved three or four times.

For the cutoff determination, the area under the curve given by the ROC analysis was 0.775. The chosen cutoff was 0.495, resulting in a sensitivity of 73.3%, and a specificity of 73.6%.

4.3. Trial 3

4.3.1. Somatic Cell Count and Bacteriological Analysis

From 192 samples analyzed, mean SCC was 543.230 cells/mL (SD 816.730). One hundred two samples were bacteriologically positive. Of those, *S. aureus* was isolated

from 71 samples, 12 samples were positive for *Prototheca* sp., CNS were isolated in 11 samples, *Enterococcus faecalis* in 7, and *Serratia* sp. in 1.

Out of 64 quarters, 27 were considered as healthy. Thirty-one quarters were considered as diseased in basis of isolation of *S. aureus* or at least 10^3 UFC/mL of *Prototheca* sp. in one or more samples. Four quarters were considered as diseased in basis of isolation of at least 10^3 UFC/mL of other pathogens in all three samples. Two quarters were considered as diseased in basis of mean SCC over 100.000 cells/mL.

4.3.2. Differential Cell Count

Overall (n = 64 samples), the proportion of PMNL ranged between 8.1 and 93.3%, with a mean of 56.4% (SD 25.1%), that of lymphocytes ranged between 0.9 and 85.9% with a mean of 30.3% (SD 23.9%), and proportions of macrophages ranged between 2.3 and 45.4% with a mean of 13.2% (SD 7.4%). Log PMNL/Lym ranged between -1.02 and 2.00, with a mean value of 0.38 (SD 0.68).

When the cutoff was applied on the data from the third trial, among 64 quarters tested, 28 were correctly classified as positive, and 24 as negative. 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.

5. DISCUSSION

The diagnosis of intramammary infections is mostly based on SCC and bacteriological analysis. SCC is low in healthy mammary quarters, but also in the very first 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 to identify changes in the relative cell populations before the rising of total cell number in the course of 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).

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. (2000) reported a high correlation between the two methods for PMNL and lymphocytes, but a lower correlation for macrophages and epithelial cells. This result may 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 account to percentages as low as 1-3% (Sarikaya et al., 2004, Schwarz et al, 2011a). Different results were reported by Miller et al., (1991) and Leitner et al., (2000), who recorded higher values (10-19% or 44%, respectively). However, according to Schwarz et al., (2011a), percentages above 10% should be critically discussed. In fact, Miller et al. (1991) evaluated milk samples only from primiparous cows in early lactation, while Leitner et al. (2000b) estimated epithelial cells percentages using a non-

specific method. Therefore, the potential misclassification of epithelial cells could represent only a minor error that probably does not affect the result.

The study consisted of three consecutive trials. The aim of the first one was to detect one or more parameters, which could easily identify diseased mammary quarters, independently of the prevalent pathogen in the herd, using light microscopy DCC. Light microscopy DCC was chosen as a more suitable method applicable to routine analysis: it is cost effective, and could potentially be automatized using a slide scanner and computer imaging software.

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 control programs for mastitis. Therefore, the second trial aimed to evaluate whether the results of DCC in healthy quarters are consistent on following days, using both individual cell population, and the two ratios identified in the first trial as capable of increasing discrimination power of DCC. A further aim of the study was to establish a cutoff value for log PMNL:Lym, which was the ratio that best identified healthy and diseased quarters (Pilla et al., 2012).

Cytometric analysis is a more precise technique than light microscopy, evaluating higher numbers of cells per sample (Koess and Hamann, 2008). Therefore, it is more suitable for the identification of small variations in DCC. For this reason, cytometric analysis was the selected method for this trial.

Finally, the reliability of the defined cutoff value for log PMNL/Lym in the correct classification of mammary quarters as healthy or diseased was blind tested under field conditions in the third trial. For that purpose, a different herd, with lower health

and hygiene status, was selected. Such herd presented with a history of infections by both *S. aureus* and the algae *Prototheca* sp.; the animals were randomly selected among the last milking group, where both infected and uninfected animals were kept. Including animals from such different epidemiological situation would ensure that the cutoff value determined in nearly ideal conditions could be applied in more realistic field situations, where it would be most needed.

For the first trial, three dairy herds were selected with different prevalence of intramammary infections, which were also caused by different aetiological agents. Indeed, causative agents of mastitis in herd A were environmental pathogens, but contagious bacteria in the other two herds. Also, *S. aureus* isolates from herd B and C demonstrated a low or high diffusiveness, respectively. The results of DCC showed statistically significant differences among the three dairy herds considered, as expected and in accordance to what previously reported (Schwarz et al., 2011b). Herd A and B showed relative higher values of PMNL, followed by lymphocytes and macrophages, while in herd C, macrophages were the main population, followed by PMNL and lymphocytes. These data suggest that most infections in herds A and B were acute infections, with high increase of PMNL, while herd C probably had a considerable prevalence of chronic infections, which led to an increase of macrophage percentages, as previously demonstrated (Sladek and Rysanek, 2009).

No correlation was found between quarter position or lactation number and cell populations, in accordance to what described by Schwarz et al. (2011a, b). Dosogne et al. (2003) reported the effect of days in milk on DCC in the milk, showing that lymphocytes decreased while PMNL and macrophages increased in the course of lactation. To the contrary, our data indicated that only macrophages were influenced by

days in milk. Such disagreement could be related to the different method used, since 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, since macrophages were the only population that was not significantly influenced by health groups.

Even though two recent publications (Schwarz et al., 2011a, b) indicated that inflammatory profiles can be found in quarters with SCC <100 x 10^3 cells/mL, we decided to follow current recommendations of DVG (2002), considering all quarters with SCC >100 x 10^3 cells/mL as diseased, regardless of bacteriological status. Such decision enabled the 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 above this level, the milk components differ significantly from the physiological norm. Interestingly, 15 milk samples in group N (normal secreting, <100.000 cells/mL and bacteriologically negative) showed marked inflammatory profiles, with PMNL percentages higher than 80%; in particular, one quarter showed a SCC value of 1,000 cells/mL and 91.3% PMNL. Many factors can explain such a high percentage of PMNL in these quarters, including chemical, mechanical, or physical injury. Furthermore, any of these quarters could be a false negative bacteriological results, for different reasons: intermittent shedding of pathogens, shedding of amounts lower than the detection limit of the method applied, or presence of antimicrobials in milk (Sears et al., 1990; Zecconi et al., 1997). It is also possible that inflammatory response was so effective in these quarters, that most bacteria were phagocytosed and killed, or survived only intracellularly by active invasion of cells, or by survival inside phagosomes (Newbould and Neave, 1965; Hill et

al., 1978). Also, cellular profiles were similar in group UM (unspecific mastitis, >100.000 cells/mL and bacteriologically negative) and M (mastitis, >100.000 cells/mL and bacteriologically positive), with no significant difference, showing the presence of an inflammatory process, as indicated by SCC increase and high PMNL percentage, even in the absence of microorganisms in UM quarters. It could be hypothesized that UM samples were false negatives to bacteriological analysis.

Among the three cell populations, PMNL are known to strongly increase in the course of infections, 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 could be considered as 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 group N and LM (latent mastitis, <100.000 cells/mL and bacteriologically positive). Macrophage percentage was very similar in the four groups, because macrophages are associated to the late phase of infection (Leitner et al., 2000; Sladek and Rysanek, 2009), and are expected to increase in chronic infections, which were presumably in low number in the cows object of this study. Lymphocytes, instead, were the only individual cell population showing statistically significant different percentages between healthy quarters (N) and all diseased groups considered (LM, M and UM). Lymphocytes play an important role in the initiation of immune response of the mammary gland. They are mainly T cells, which function is to remove old and damaged secretory cells, thus decreasing the susceptibility of the mammary gland to infections, while suppressor T-lymphocytes modulate the immune response (Sordillo et al., 1997).

In addition, combinations of cell populations were evaluated to increase the discrimination power of DCC, which means that a higher percentage of quarters would be correctly classified. Combining PMNL and macrophages into phagocytes (Phag) increased F-values from 1.65 and 15.54, respectively, to 32.64. Combining PMNL and lymphocytes (Lym) into logarithmic (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, including all three cell populations, led to an F-value of 45.90. All combinations of individual cell populations considered showed statistically significant differences between group N (healthy quarters) and D (diseased quarters), but the best F-value was related to the ratio log PMNL/Lym. Presumably, the explanation for this result could be that log PMNL/Lym is the only variable that involves both cell populations statistically influenced by health groups, but not macrophages.

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

SCC fluctuations were observed in all but 3 quarters during the follow-up period, but no significant DCC variation could be recorded. Even though 4 samples were bacteriologically positive, no bacteria could be detected in the following samples, thus infections were considered as transient. Therefore, while positive milk samples were excluded from the analysis, animals were still considered as healthy, and not excluded from the study. DCC variations could be evidenced in some quarters, that were not

correlated with SCC variations (data not shown). Despite that, statistically significant differences could not be found among sampling days.

Fluctuations of individual cell populations in milk were observed in the different stages of lactation, but the differences were not significant, in accordance with our results from the first trial (Pilla et al., 2012). In contrast, Dosogne et al. (2003) reported higher lymphocyte and lower macrophage values at the beginning of lactation.

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

The results obtained in the third trial showed very high specificity and good sensitivity for the log PMNL/Lym cutoff previously determined. Nine quarters were misclassified as negatives. Among them, 2 had been considered as diseased based only on high SCC, 4 were positive for *S. aureus*, 2 for *Prototheca* sp., and 1 for CNS.

When the total number of cells (SCC) was considered along with log PMNL/Lym, only one quarter was missclassified, 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 *S. aureus* was detected in low counts (10^2 UFC/mL) only in the first and second sampling. Since the animal had two additional *S. aureus* infected quarters shedding high numbers of bacteria, we can speculate that bacteriological positivity of this 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 not always correlated with intramammary infections (Zecconi et al., 1994).

Therefore, the use of DCC and, more specifically, of log PMNL/Lym along with SCC could enable excellent quarter classification, comparable with cytobacteriological analysis of quarter milk samples.

6. CONCLUSION

In the present study differential cell counts were evaluated as an alternative method for mastitis diagnosis. While somatic cell counting is a worldwide accepted method to identify inflammatory processes in mammary quarters, it provides only limited information, since it evaluates only the total number of milk cells, but not its composition. Differential cell counting, instead, can detect changes in the relative cell populations in milk without rising of total cell numbers, identifying inflammatory processes in quarters otherwise considered as healthy. Such information could be particularly useful when control programs for contagious milk pathogens are being applied.

A new parameter, log PMNL/Lym, was found to best indicate the presence of mastitis. No influence of parity, lactation stage or quarter position could be found on DCC results. Information on the consistency of this parameter was obtained considering healthy mammary quarters in order to establish test-retest reliability. Sampling day showed no effect on log PMNL/Lym, therefore a cutoff value to identify healthy or diseased quarters could be determined. Such value of 0.495, was then tested under field conditions, showing that excellent results were obtained combining both DCC and SCC, with very high sensitivity and specificity. In conclusion, the use of DCC and SCC together could represent an excellent diagnostic method to identify inflammatory processes in the mammary gland, avoiding bacteriological analysis.

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