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Somatic cell populations in milk: Importance in mammary gland physiology and behaviour during technological processing

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Contents

Abstract

Milk represents a fundamental nutrition resource. Its somatic cell counts (SCC) is one of the most important parameters for interpreting milk hygiene and quality. It includes all types of cells in milk and is therefore an indicator for the activity of the cellular immune defence of the udder and thus of udder health and physiology. As each cell type has its own specific function during the immune response their distribution in milk directly reflects the immunological status of the mammary gland. The aim of the present study was to enlighten the explanatory power of SCC from both, the technological and the physiological point of view, and to enhance it by establishing new methods for differential cell counting.

Although immune cells are essential in mammary gland physiology they need to be eliminated during milk processing. Therein the crucial step for milk cell separation is centrifugation. The effects of technological processes on milk SCC were identified by simulating centrifugation processes in the laboratory. Astonishingly, cells were not only found in the achieved pellet but also in the fat phase. This alludes to an affinity of fat globules towards cell membranes resulting in tearing up the cells towards the top fraction. It was shown that this effect can be partially overcome by elevating relative centrifugal force (RCF), centrifugation time and temperature. The subsequent investigation of two industrial production lines showed that during milk processing the step bactofugation is more effective than the separator. Therefore, arranging the bactofuge in front of the milk separator can enhance the cell separation. For the reverse case, i.e. scientific research, it was shown that the centrifugation setup must be adapted to the investigators' goals, e.g. if working with vital cells is intended, RCF has to be moderate as high values lead to their destruction and death.

To improve the immunological interpretation and thus the explanatory power of the factor SCC, a second parameter, the differential cell count, was established by inventing a staining method to characterize cell populations in milk. The staining method according to Pappenheim, usually used for blood, was modified and adapted to the matrix milk. Microscopically investigations showed a clear contrast in the appearance of all types of immune cells. The procedure was then used for various investigations of milk fractions collected during routine milking and under distinct physiological udder status. Thereby, a clear correlation between the differential cell count and the mammary immunology was observed. Milk from udders presenting very low SCC was identified to possess very high amounts of lymphocytes and accordingly low amounts of macrophages and polymorphonuclear neutrophils (PMN). As the immune response of the mammary gland is mainly formed by the latter cells, a significant immunological deficit therein was concluded. Additionally, the definition of the taken milk fraction proved to be essential when the interpretation of milk

quality or udder status was conducted based on SCC and differential cell count. Thus, even strict foremilk can differ dramatically in cell composition from the cisternal fraction.

In addition to direct cell visualization, mRNA expression levels of various inflammatory factors were investigated in the milk fractions. The achieved results generally supported the interpretation of the differential cell count, as increasing mRNA expression levels of the investigated genes with increasing SCC indicated a higher overall activity of the immune cells. In contrast, the reduced immune response in quarters with very low SCC was underlined by very low mRNA expression levels. Thus, results based on mRNA expression levels clearly reflected the physiological picture derived from the cellular composition.

Flow cytometry was used as another tool for cell differentiation. It was shown that in principal FACS can be adapted to milk cell analysis but it can not be compared directly to microscopic results, as the antibodies did not exclusively bind to one cell type. Diapedesis appeared to be the main problem as the surface of the milk cells was altered and the commonly available antibodies showed obvious cross-reactivity.

Consequently, the achieved results show that the composition of the milk fraction clearly reveals the role of the somatic cells for the immune response in different udder compartments. Especially the differential cell count gives important information as each cell type has its own specific function in the mammary gland. Furthermore, the definition of the sampled milk fraction is necessary for the prediction of the total quarter SCC and the udder health status. The application of the established methods and the detailed consideration of the mentioned parameters provide new insights into mammary gland physiology.

Zusammenfassung

Milch stellt eine der elementarsten Nahrungsquellen dar. Die somatische Zellzahl (SCC) ist einer der wichtigsten Parameter für die Beurteilung von Milchhygiene und -qualität. Diese umfasst alle Zelltypen in der Milch und ist daher ein entscheidender Indikator für die Aktivität der zellulären Immunabwehr des Euters sowie für Eutergesundheit und -physiologie. Da jeder Zelltyp seine eigene spezifische Aufgabe während der Immunantwort übernimmt, kann aus deren Verteilung in der Milch direkt auf den immunologischen Zustand des Drüsengewebes geschlossen werden. Das Ziel der vorliegenden Arbeit lag in der Betrachtung der Aussagekraft der SCC, sowohl aus technologischer als auch aus physiologischer Sicht, sowie deren Verstärkung durch die Etablierung neuer Methoden zur differenzierten Zellzählung.

Obwohl die Immunzellen eine entscheidende Rolle innerhalb der Physiologie der Milchdrüse einnehmen, müssen sie während der Verarbeitung entfernt werden. Der kritische Schritt für die Abtrennung der Milchzellen ist dabei die Zentrifugation. Der Einfluss der technologischen Verarbeitung auf die SCC wurde durch Simulation dieses Zentrifugationsschrittes im Labor analysiert. Erstaunlicherweise befanden sich dabei Zellen nicht nur im erhaltenen Pellet, sondern auch in der Fettphase. Dies lässt auf eine Affinität der Fettkügelchen zur Membran der Immunzellen schließen, welche zu einem Auftrieb der Zellen in die obere Fraktion führt. Es zeigte sich, dass dieses Phänomen durch die Erhöhung der Zentrifugalkraft (RCF), der Zentrifugationszeit sowie -temperatur teilweise überwunden werden kann. Die nachfolgende Untersuchung von zwei industriellen Produktionslinien zeigte, dass im Verlauf der Milchverarbeitung der Schritt der Baktofugation effektiver als der Milchseparator ist. Daher kann eine Platzierung der Baktofuge vor dem Milchseparator zu einer besseren Zellabtrennung führen. Für den umgekehrten Fall der wissenschaftlichen Untersuchung konnte gezeigt werden, dass der Zentrifugationsschritt an die Ziele des Forschers angepasst werden muss. Wenn z.B. Arbeiten an lebenden Zellen angestrebt werden, müssen moderate RCF gewählt werden, da hohe Werte zur Zerstörung der Zellen und deren Tod führen.

Um die immunologische Beurteilung mittels Zellzahl sowie deren Aussagekraft weiter zu verbessern, wurde ein zweiter Parameter, das Zelldifferentialbild, etabliert. Dies erfolgte durch die Entwicklung einer Färbemethode zur Charakterisierung einzelner Zellpopulationen in der Milch. Hierzu wurde die für Blutproben verwendete Pappenheim-Färbung modifiziert und an die Matrix Milch angepasst. Die Untersuchungen zeigten unter dem Mikroskop klare Unterschiede im Erscheinungsbild aller Immunzellen. Die Methode wurde dann für eine Reihe von Untersuchungen an Milchfraktionen verwendet, welche während der Routinemelkung unter bestimmten physiologischen Eutergesundheitszuständen gewonnen wurden. Dabei zeigte sich ein klarer Zusammenhang zwischen der differenzierten Zellzahl und der Immunologie des Eutergewebes. Milch von Eutern mit sehr niedriger Zellzahl verfügte über

einen hohen Anteil an Lymphozyten und dementsprechend geringe Mengen an Makrophagen und polymorphkernigen Neutrophilen (PMN). Da die Immunantwort des Euters hauptsächlich durch letztere Zelltypen reguliert wird, kann hieraus auf eine signifikant verringerte immunologische Aktivität geschlossen werden. Zusätzlich erwies sich die genaue Definition der jeweiligen Milchfraktion als entscheidend, wenn eine Beurteilung der Milchqualität und des Eutergesundheitsstatus auf der Basis von Zellzahl und Zelldifferentialbild erfolgen soll. In diesem Zusammenhang kann sich sogar reines Vorgemelk in seiner Zusammensetzung extrem von Zisternenmilch unterscheiden.

Zusätzlich zur direkten visuellen Zellbestimmung wurden die mRNA-Expressionen verschiedener Entzündungsfaktoren in den einzelnen Milchfraktionen untersucht. Die dabei erhaltenen Ergebnisse untermauerten generell die Schlussfolgerungen des Zelldifferentialbildes, da erhöhte mRNA-Expressionswerte der jeweiligen Gene zusammen mit steigender Zellzahl auf eine höhere Aktivität der Immunzellen hindeuteten. Im Gegenzug unterstrichen sehr niedrige mRNA-Expressionswerte die verminderte Immunantwort in Euterviertеln mit sehr niedriger Zellzahl. Diese Ergebnisse deckten sich klar mit dem aus der Zellzusammensetzung erhaltenen physiologischen Gesamtbild.

Die Durchflusszytometrie wurde als ein weiteres Werkzeug für die Zelldifferenzierung eingesetzt. Es konnte gezeigt werden, dass die FACS-Analytik prinzipiell an Milchzellen angepasst werden kann. Allerdings konnten die Ergebnisse dieser Messungen nicht direkt mit den mikroskopisch erzielten Werten verglichen werden, da die verwendeten Antikörper nicht nur einen Zelltyp erkannten. Diapedese stellte sich als das Hauptproblem heraus, da sich durch sie die Oberfläche der Milchzellen verändert und gebräuchliche Antikörper somit Kreuzreaktionen eingehen.

Letztlich zeigen die erhaltenen Ergebnisse, dass die Zusammensetzung der Milchfraktionen in den unterschiedlichen Euterkompartimenten deutlich die Rolle der somatischen Zellen bei der Immunantwort widerspiegelt. Besonders die differenzierte Zellzahl ermöglicht wichtige Rückschlüsse, da jeder Zelltyp über seine eigene spezifische Funktion bei der Immunantwort verfügt. Weiterhin stellte sich heraus, dass die genaue Definition der gewonnenen Milchfraktion für die Aussagekraft der Gesamtzellzahl sowie die Beurteilung des Eutergesundheitszustandes von entscheidender Bedeutung ist. Die Anwendung der hier eingeführten Methoden sowie die genaue Berücksichtigung der erwähnten Parameter ermöglichen neue, tiefer gehende Einblicke in die Euterimmunologie.

1 Introduction

1.1 Milk

"Milk and honey are the only diets whose sole function in nature is food." Statements like this show the high importance of milk in a very simple way.

Milk has been a food source for humans since the dawn of history. The role of it is to provide nourishment and protection for the mammalian young. Milk is a biological fluid containing a large number of different constituents (Davies *et al.* 1983). Therefore, only an approximate composition of milk is usually given. The major constituents of milk are water, carbohydrates, fat, protein, minerals and vitamins (Schlimme *et al.* 1998). One has to bear in mind that milk is secreted as a complex mixture of these components and a composition of several phases. As an emulsion of fat globules and a suspension of casein micelles all components are suspended in an aqueous phase (Belitz *et al.* 2001). This also accounts for the leukocytes, being the major part of the somatic cells in milk.

1.2 Mammary Gland

The udder is one of the most important physiological and conformational peculiarities of the cow (Akers 2002) due to its ability to produce milk. The mammary gland of the dairy cow consists of four separate compartments each with a teat (Wittke *et al.* 1983). Milk which is synthesized in one gland cannot pass over to any of the other glands.

Within the mammary gland the milk producing unit is the alveolus (Inset a in Fig. 1). It contains a single layer of epithelial secretory cells surrounding a central storage area called the lumen, which is connected to a duct system. The secretory cells are, in turn, surrounded by a layer of myoepithelial cells and blood capillaries. The milk is synthesized in the secretory cells, which are arranged as a single epithelial layer on a membrane in a spherical structure called alveoli. The diameter of each alveolus is about 50-250 µm. Several alveoli together form a lobule (Akers 2002). The milk which is continuously synthesized in the alveolar area is stored in the alveoli, milk ducts, udder, and teat cistern between milkings. 60-80% of the milk is stored in the alveoli and small milk ducts, while the cistern only contains 20-40% (Knight *et al.* 1994; Pfeilsticker *et al.* 1996; Ayadi *et al.* 2003).

The teat consists of a teat cistern and a teat canal. Where the teat cistern and teat canal (Inset b in Fig. 1) meet, folds form the so called Fürstenbergs rosette. The teat canal is surrounded by bundles of smooth muscle fibres. Between milkings the smooth muscles

function to keep the teat canal closed (Paulrud 2005). The teat canal is also provided with keratin or keratin like substances (Hogan *et al.* 1988).

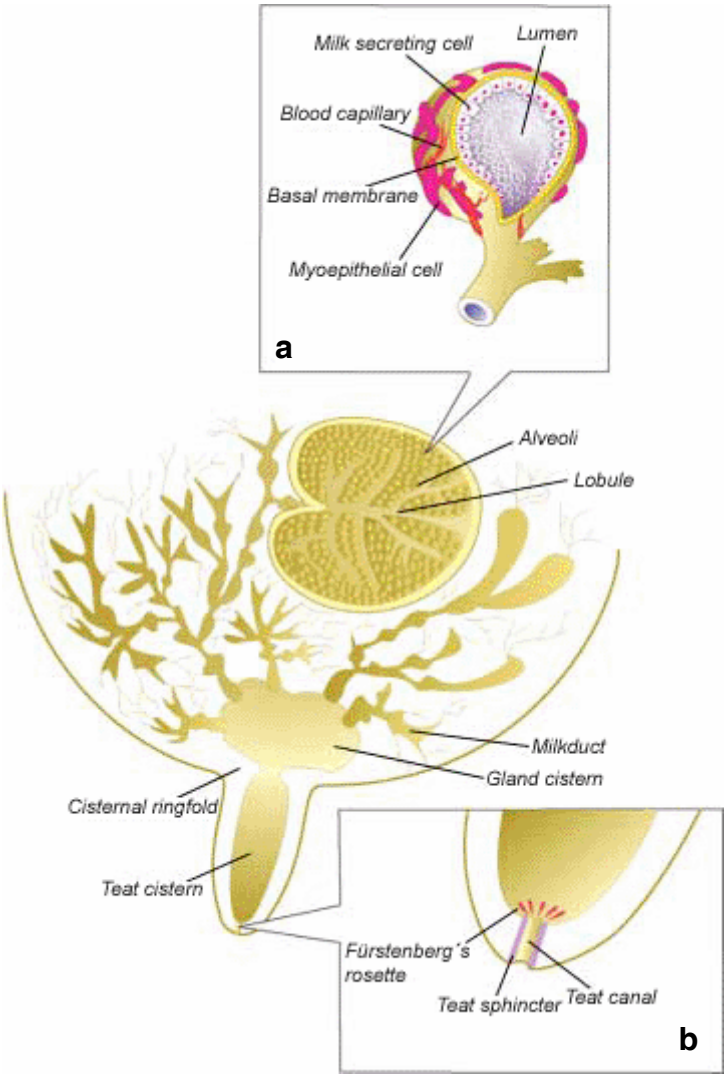

Fig. 1. Anatomy of the bovine mammary gland, illustrating the udder, a detailed structure of an alveolus (a) and the teat (b) (following DeLaval).

Resistance to bacterial invasion of a mammary quarter is in part determined by the structure and function of the teat canal. The normal teat canal has several anatomic features that act as barriers to penetration of bacteria (Zecconi *et al.* 2000). The cells lining the teat canal, for example, produce keratin, a fibrous protein with lipid components acting as barrier to microorganisms involved in mastitis. Probably the major role of this waxy plug is to form a physical barrier preventing the penetration of bacteria (Senft *et al.* 1990). Additionally, some components of the keratin like the lipids have antimicrobial properties (Craven *et al.* 1985; Hogan *et al.* 1988).

1.3 Mastitis

Mastitis is classically defined as inflammation of the mammary gland (Kehrli *et al.* 1994), while inflammation simply means a reaction to injury. Hence, any type of injury of the mammary tissue may be expected to induce an inflammatory response or mastitis (Jain 1979). However, the udder disease usually originates from microbial infection (Bradley 2002). The symptoms of mastitis vary according to factors in the cow and the invading pathogen (Leigh 1999). The complexity is reflected in the numerous causative pathogens and the variety and strength of the physiological responses to these pathogens (Burvenich *et al.* 2003; Bruckmaier *et al.* 2004b).

Virtually the internal environment of a normal mammary gland is sterile, but bacteria are frequently found in mammary glands. However, to induce mastitis, pathogenic bacteria must first penetrate through the teat canal (streak canal) into the interior of the mammary gland. They have to survive the intramammary bacteriostatic substances and then proliferate to significant numbers. Thus, products of bacterial growth and metabolism may irritate the mammary tissue and induce an inflammatory reaction. In reality, the clinical signs of mastitis are an expression of the host defense intended to destroy the invader and to repair the mammary tissue (Jain 1979). Infections of the mammary gland by pathogens result in decreased milk production and compositional changes that vary with the intensity of the infection.

From the patho-physiological point of view one can distinguish between subclinical and clinical mastitis. In the former no visible changes occur in the appearance of the milk or udder, but milk production and composition is altered. The latter is additionally characterized by abnormal milk and swelling of the udder and can also be accompanied by clinical signs.

1.4 Somatic Cell Counts

Mastitis has various effects on the milk being produced. Whether accompanied by clinical signs or not, an inflammatory reaction caused by infections of the mammary tissue is always associated with an increase in the somatic cell counts (SCC) in milk. Therefore the commonly used tool to monitor udder health and thus milk quality is the SCC (Kitchen 1981; Kehrli *et al.* 1994; O`Brien *et al.* 1999).

The term somatic (i.e. body) alludes to the fact, that the cells are body-derived cells. “Somatic cells” is a term which refers to the leukocytes, i.e. polymorphonuclear neutrophils (PMN), macrophages and lymphocytes. In addition, a small percentage of epithelial cells count to this term (Concha 1986; Burvenich *et al.* 1994; Paape *et al.* 2002). Milk from normal i.e. uninfected

quarters generally contains below 100 x10^3 cells/ml (Hillerton 1999). An elevation of SCC can result in compositional changes in the milk and a reduction of the milk yield. Furthermore, it is an indication for inflammation in the udder. Bacteriologically positive milk samples from infected quarters can even reach a SCC of several million cells/ml. The SCC is not exclusively influenced by intramammary infection. Other non-infectious factors like animal's age, lactation stage, breed etc. have also a moderate impact (McDonald *et al.* 1981a; McDonald *et al.* 1981b; Laevens *et al.* 1997). In addition to the changes of SCC based on the factors mentioned previously there are also alterations during the course of milking, i.e. in different milk fractions (Ontsouka *et al.* 2003; Bruckmaier *et al.* 2004a).

Studies have shown that the severity and duration of mastitis is critically related to the promptness of the leukocyte migratory response and the bactericidal activity of cells at the site of infection (Hill 1981; Grommers *et al.* 1989). If immune cells move rapidly from the blood stream and are able to eliminate bacteria, recruitment of leukocyte ceases and SCC returns to baseline levels.

1.5 Cell Populations

Total number and activity of mammary gland leukocyte populations play early and vital roles in determining the severity and duration of intramammary infections. Resident as well as newly recruited mammary leukocytes consist of several cell types including PMN, macrophages and lymphocytes. These cells mediate both innate and acquired immune response (Concha 1986; Paape *et al.* 2002). The epithelial cells form also a part of the SCC but are of minor importance in milk as they appear in very low counts.

PMN are of myeloid origin and their cytoplasm is filled with granules, while they hold lobulated nuclei (Fig. 2). According to the response of the granules on dyes they can be classified as basophils, eosinophils and neutrophils (Paape *et al.* 2003). These leukocytes contribute to the non-specific immune response and are recruited actively to the site of infection. They are the major cell type found in mammary tissues and secretions during the early stage of the inflammatory process (Jensen *et al.* 1981). While PMN numbers are relatively low in the healthy mammary gland their numbers increase up to 90% of the total SCC during mastitis (Burvenich *et al.* 1994; Paape *et al.* 2002). In response to a variety of inflammatory mediators PMN migrate from the

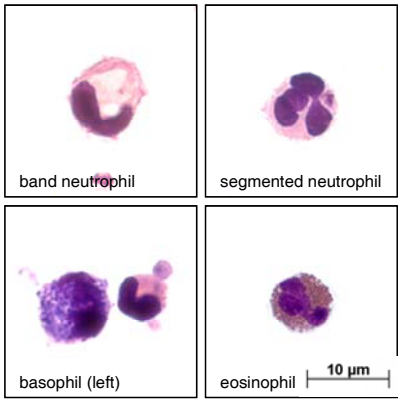

Fig. 2. Stained milk PMN (Sarikaya *et al.* 2001)

blood to the udder in order to phagocytize and kill bacterial pathogens (Paape *et al.* 1979; Craven 1983; Persson *et al.* 1993). PMN exert their bactericidal effect through a respiratory burst.

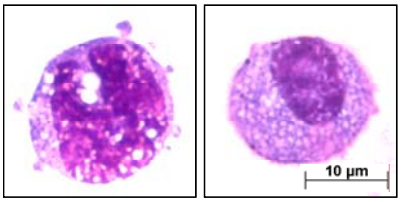

Fig. 3. Stained milk macrophages (Sarikaya *et al.* 2001)

Macrophages are mononucleated cells (Fig. 3) and monocytes in blood are the precursor forms of them. After entering the tissue or milk they mature to macrophages. They represent the dominant cell type in milk of healthy mammary glands. Similar to neutrophils, the non-specific functions of macrophages are to phagocytize bacteria and destroy them with proteases and reactive oxygen. Macrophages are less active than milk neutrophils at phagocytosis and both milk cell types are less efficient than their blood counterparts (Lee *et al.* 1980; Mullan *et al.* 1985). However, macrophage numbers tend to be lower during inflammation. The ability of macrophages to release chemical messengers or chemoattractants that facilitate the migration of neutrophils is believed to be of great importance for the non-specific immune response (Cassatella 1995; Hoeben *et al.* 2000; Wittmann *et al.* 2002). Being activated through pathogens, macrophages release chemo-attractants like cytokines, leukotrienes etc. that mediate the migration of the leukocytes from the blood into the milk.

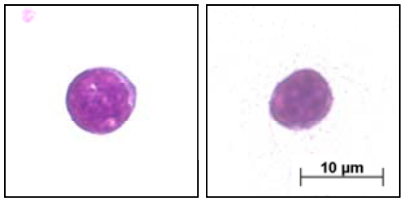

Fig. 4. Stained milk lymphocytes (Sarikaya *et al.* 2001)

Lymphocytes are generally small round cells containing a large nucleus (Fig. 4). They are the only cells of the immune system that are able to recognize antigens through specific membrane receptors. Therefore, lymphocytes represent the specific immunity of the system (Outteridge *et al.* 1981; Taylor *et al.* 1997), building a large number of different populations with distinct functions. Subsets are divided into two main groups, type T and B. The T-cells (T-helper CD4 and T-cytotoxic CD8) are involved in cell mediated immunity. The CD4 cells produce cytokines with regulatory and effector functions. The CD8 cells kill specific target cells or cells containing intracellular bacteria. The primary role of B-cells, including the plasma cells, is to produce antibodies against invading pathogens (Riollet *et al.* 2000). This cell fraction is supposed to play no major role in infections of the mammary gland or to operate rather in the mammary tissue than in milk (Riollet *et al.* 2001).

The epithelial cells of the mammary gland are active secretory cells that are normally subjected to turnover and must be replaced with new cells continually. These displaced cells are partially discharged into the milk and form a small part of the somatic cells there (Lee *et al.* 1980). It has been suggested that the presence of epithelial cells in milk provides a means of evacuating dead cells which have reached the end of their secretory life (Boutinaud *et al.* 2002). In milk they are often described to appear in clumps of several (2 to 15) cells (Buehring 1990).

1.6 Inflammatory Response

An inflammatory response is initiated when bacteria enter the mammary gland and bacterial populations reach a certain threshold concentration. Accordingly, the bacteria get recognized by the mammary epithelium and the leukocytes in the milk. The cells respond by secreting chemotactic and inflammatory mediators. These mediators stimulate the migration of leukocytes, mainly PMN, from the blood into the milk (Persson *et al.* 1993; Riollet *et al.* 2000). While cytokines and lipid mediators are expressed by immune cells, antimicrobial defence proteins like lactoferrin originate mainly from the mammary tissue (Pfaffl *et al.* 2003). Among the leukocytes the cytokine and mediator expressions are much higher in the macrophage than in the PMN population (Wittmann *et al.* 2002).

One important part of the signaling cascade initiated by bacterial influx into the mammary gland is depicted in Figure 5. Herein, the cluster of differentiation (CD) 14 antigen, which is commonly found on monocytes/macrophages, binds lipopolysaccharide (LPS)-protein of gram negative bacteria. This induces the synthesis and secretion of proinflammatory cytokines, e.g. tumor necrosis factor TNF-α or interleukin IL-1β (Wright *et al.* 1990). These cytokines again modulate the chemotactic activity and the expression of adhesion molecules on leukocytes (Shuster *et al.* 1993; Sanchez *et al.* 1994). Furthermore investigations showed that TNF-α and IL-1β stimulate IL-8 secretion (Persson *et al.* 1993), what results in a rapid influx of PMN to the place of infection.

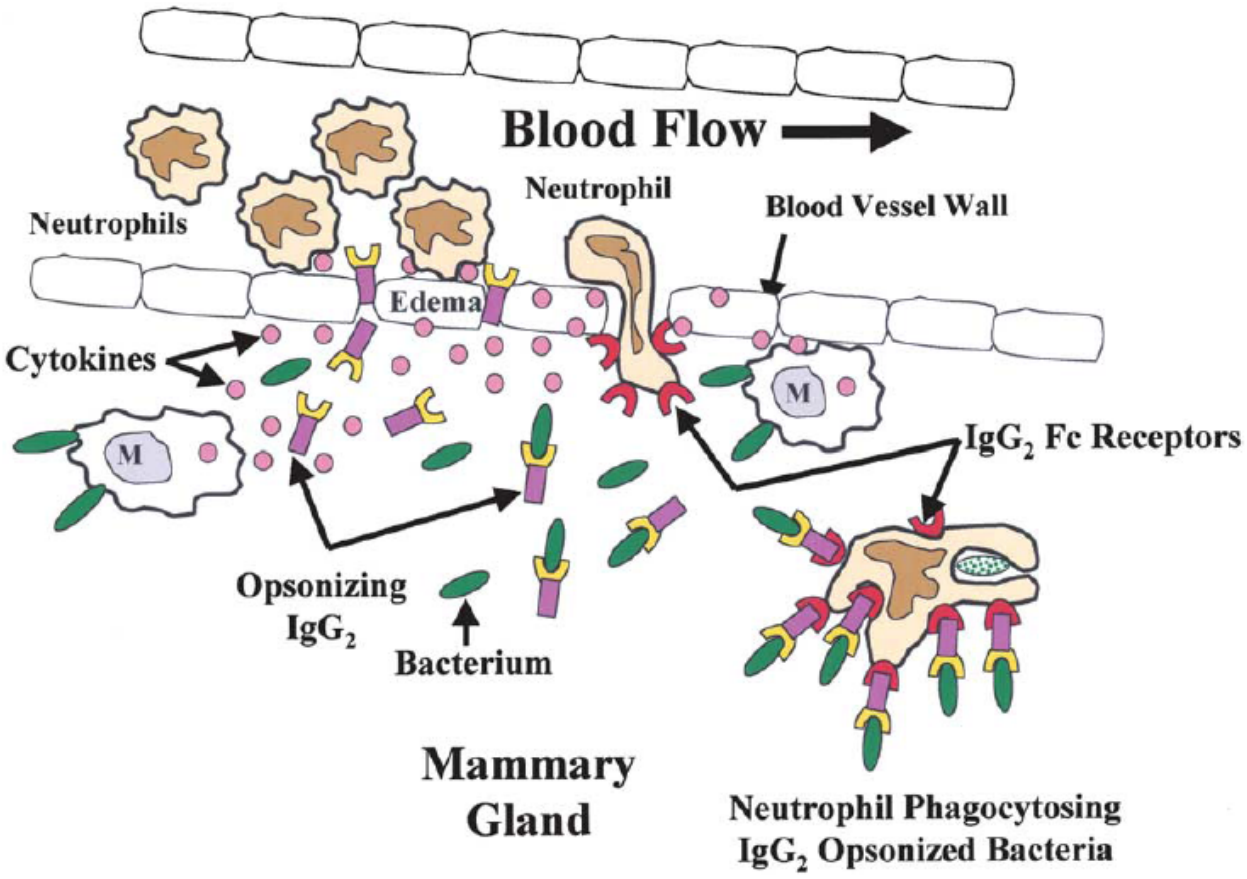

Fig. 5. Schematic diagram of inflammatory response initiation mediated by secretion of chemoattractants by the leukocytes (Burton *et al.* 2003). Blood neutrophils are recruited into infected mammary quarters when tissue macrophages (M), mammary epithelial cells, and blood vessel endothelial cells secrete cytokines (pink dots) after coming in contact with the infecting bacteria (green rods). On the way into the gland, neutrophils become highly activated for phagocytosis and killing.

Additionally to this inflammatory response the mammary gland also possesses humoral defense in terms of soluble factors bearing antimicrobial effects. Lactoferrin, an iron-binding glycoprotein, is known for its bacteriostatic effect for a variety of microorganisms. It inhibits the bacterial growth by depriving them of the iron. Furthermore it adheres to the surface of bacteria and weakens their resistance (Ellison *et al.* 1991; Baveye *et al.* 1999). Lysozyme also belongs to the bactericidal soluble factors. It acts by cleaving peptidoglycans, which constitute the major part of the bacterial cell wall of gram-positive bacteria (Carlsson *et al.* 1989).

1.7 Milk Processing

In modern nutrition milk still gains increasing importance as it builds the basis of a great diversity of dairy products. In this context the parameter SCC represents a very important factor for processing workflows. One central step in milk processing is the centrifugal separation which causes the removal of solid impurities, including somatic cells, from milk prior to pasteurization. This step is crucial as high SCC can effect lipolysis and proteolysis in milk, which lead to off-flavors and reduce the products' shelf-life (Ma *et al.* 2000b; Santos *et al.* 2003). This also counts for the dairy products produced from milk. For example, cheese making is negatively influenced by high SCC through diminished cheese yield and modified cheese composition (Politis *et al.* 1988a; Politis *et al.* 1988b; Klei *et al.* 1998). Therefore it is important to avoid high SCC milk during processing of milk or better to reduce the SCC before production very efficiently.

1.8 Milk SCC in Immunological Context

SCC has a great impact on usability of milk in industrial processes including its successional products. Furthermore, it can hint to the immunological status of the mammary gland. In this context a distinct knowledge of cell populations and subpopulations might lead to a more detailed understanding of immunological interrelations. Thus, interpreting udder health status could be improved by including these parameters.

2 Aim of the Study

For controlling the quality of milk, being one of the fundamental nutrition resources, the most widely used parameter is the somatic cell count (SCC). Its value is routinely determined by established analysis methods. Although the SCC in milk represents one of the most important parameters for the interpretation of udder health the physiological interrelations between immune cell distribution and SCC are still not clearly defined. Additionally, during technological milk processing centrifugation is the fundamental tool for the removal of cells. However, the effect of this step on cell integrity remains to be elucidated.

The aim of the present study was to enlighten the explanatory power of SCC from both, the technological and the physiological point of view, and to enhance it by establishing new methods for differential cell counting. Therefore, the effects of technological processes on milk SCC shall be identified by simulating centrifugation processes in the laboratory. Industrial production lines need to be investigated to optimize the processing of fluid milk. It was a further goal to establish the differential cell count beyond SCC to improve the functional, immunological interpretation of milk cells. This method may be used for various investigations of milk fractions collected during routine milking and under a distinct physiological udder status. The mRNA gene expression levels of various inflammatory factors shall be investigated concomitantly to support the interpretation of the differential cell count in these milk fractions. Additionally, the knowledge about the composition of the milk fraction may hint at a possible role of the somatic cells for the immune response in different compartments of the udder.

The differential cell count in context with SCC and gene expression values may provide new important physiological information as each cell type has its own specific function in the immune response. Together with a definition of the sampled milk fraction they shall clearly improve the prediction of udder health status.

3 Materials and Methods

3.1 Collection of Milk Samples

Farm milk samples

All animals utilized in the study were lactating cows of the German Braunvieh x Brown Swiss, Simmental or Holstein Friesian breed. The milk samples were collected during routine milking times as total quarter milk or fractionized quarter milk samples with a detailed definition of each fraction as described previously (Sarikaya *et al.* 2005; Sarikaya *et al.* 2006b; Sarikaya *et al.* 2006c). A special quarter milking equipment allowed an online separation of the whole quarter milk sample into at least three fractions (Sarikaya *et al.* 2006b; Sarikaya *et al.* 2006c). The groupings of the milk samples were performed on the basis of SCC.

Dairy milk samples

As a further step dairy samples have been investigated. Production lines of interest were milk and whipping cream, analyzing distinct steps of processing in detail (Sarikaya *et al.* 2006a). The parameter of interest was SCC, the initial value in the first step of processing (i.e. tank) was set at 100%. The changes in SCC were calculated according to this value.

3.2 Milk Constituents

Milk samples were analysed for fat, protein and lactose in every total quarter milk sample or quarter milk fraction in an accredited milk laboratory (Milchprüfring Bayern e.V., Wolnzach, Germany) using the the MilkoScan 4500 analyser (Foss, Hillerød, Denmark). Potentiometric measurements with ion selective electrodes (model 9811 and 9617BN, pH/Ise Meter 720 Aplus, Orion Research, USA) were performed directly in milk for determination of sodium (Na) and chloride (Cl). Electrical conductivity (EC) was measured in milk using the LDM 130 electrode from WTW (Weilheim, Germany).

3.3 Effect of Centrifugation

The three parameters of centrifugation are relative centrifugal force (RCF), centrifugation time and temperature. The effect of centrifugation on milk at different RCF, centrifugation times and temperatures on the distribution of somatic cells (SC) in the evolving phases fat, skim milk and cell pellet was investigated systematically. Altered parameters for RCF were 200, 850, 1850 and 3300 x*g*, for centrifugation times 15, 20 and 30 min, and for centrifugation temperatures 4,

25, 38 and 55°C (Sarikaya *et al.* 2006a). The detection of the somatic cell content in each phase is described in detail in Sarikaya *et al.* 2006a.

3.4 SCC and Cell Staining Methods

For determination of SCC milk samples were preserved and analysed in a Fossomatic cell counter (Foss, 3400 Hillerød, Denmark) or directly with a DCC (DeLaval cell counter, Tumba, Sweden).

The somatic cells in milk were separated by different centrifugation and washing steps. Afterwards a viability test was performed by the exclusion method with trypane blue and cells were counted in a Neubauer chamber. Differential cell count was investigated under a light microscope using a modified panoptic staining according to Pappenheim, which has formerly been established for haematological analysis (Pappenheim 1912). This staining method was optimized and established for the purpose of identifying milk SCC. Detailed separation, staining and counting procedures have been described in Sarikaya et al. 2004.

3.5 Total RNA Extraction and Oligonucleotide Primers

Total RNA extraction was carried out on milk cells separated as described recently (Sarikaya *et al.* 2006c). For RNA isolation TriPure (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturers' recommendations. To quantify the amount of total RNA optical density (OD) was measured at three different dilutions at 260 nm, corrected by the 320 nm background absorption. RNA integrity was verified by an OD_{260nm}/OD_{280nm} absorption ratio of >1.7.

The different primers of every housekeeping and target gene were synthesized commercially (MWG Biotech, Ebersberg, Germany) using already published bovine specific primer sequences (Wittmann *et al.* 2002; Schmitz *et al.* 2004). All primer information is given in Table 1.

Table 1. Sequence of PCR primers (forward: for; reverse: rev), accession number and PCR product length

Primer	Sequence (5`→ 3`)	Accession No.	Length (bp)
UbQfor UbQ rev	AGA TCC AGG ATA AGG AAG GCA T GCT CCA CCT CCA GGG TGA T	Z18245	198
GAPDH for GAPDH rev	GTC TTC ACT ACC ATG GAG AAG TCA TGG ATG ACC TTG GCC AG	U85042	197
TNF-α for TNF-α rev	TAA CAA GCC GGT AGC CCA CG GCA AGG GCT CTT GAT GGC AGA	AF011926	256
IL-1β for IL-1β rev	TTC TCT CCA GCC AAC CTT CAT T ATC TGC AGC TGG ATG TTT CCA T	M37211	198
COX-2 for COX-2 rev	TCT TCC TCC TGT GCC TGA T CTG AGT ATC TTT GAC TGT GG	AF031698	358
Lf for Lf rev	GGC CTT TGC CTT GGA ATG TAT C ATT TAG CCA CAG CTC CCT GGA G	AB046664	338
Lz for Lz rev	GAG ACC AAA GCA CTG ATT ATG GGA TCC ATG CCA CCC ATG CTC TAA	U25810	195

3.6 Quantification by real-time RT-PCR

Quantitative analysis of PCR products was carried out on the RotorGene 3000 (Corbett Research, Sydney, Australia) via one-step qRT-PCR. Extracted mRNA solutions with a defined concentration were mixed with the reaction components of Master Mix (SuperScript™ III Platinum SYBR® Green One-Step qRT-PCR Kit, Invitrogen, Germany) including 2x SYBR® Green, SYBR® Green One-Step Enzyme Mix, forward primer and reverse primer.

Crossing point (CP) values were achieved by RotorGene software (Version 5.0). A normalization of the target genes with an endogenous standard was performed. Therefore the expression levels of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitin (UbQ) were measured. The relative mRNA levels were calculated by normalization of the CP of the target gene to the mean CP of the two housekeeping genes UbQ and GAPDH.

3.7 FACS Analysis

For investigating the distribution of the somatic cell populations in milk a flow cytometric analysis method (FACS Calibur, Becton Dickinson, Germany) was established additionally. First of all the evaluation contained an optimization of staining protocols and measurement parameters. The optimized parameters were concentration of cell suspension and antibody, incubation time and temperature of cells with specific antibody, unspecific binding of second antibody, indirect staining protocol. After completing the fundamental investigations every antibody was tested by comparing the FACS data with microscopic results of the differential cell count (Pappenheim staining, Sarikaya *et al.* 2004).

The separated cells (see 3.3) were counted in a Neubauer chamber and diluted to a final concentration of 10^7 cells per ml cell suspension. Afterwards 100 µl of cell suspension was incubated with the cell specific monoclonal antibodies according to protocols for direct or indirect staining. An antibody concentration of 15 µg/ml was used and 50 µl antibody solution was added to the cell suspension. Incubation time was always 30 min at room temperature. For elimination of unbound antibodies samples were centrifuged and the labeled cells in the pellet were resuspended in PBS. Table 2 summarizes the information about the bovine specific first antibodies and the second antibodies for labeling.

Table 2. Antibodies for differentiation of bovine somatic cells by means of FACS-Analysis. The antibodies marked with the asterisk present already with FITC-labeled antibodies, i.e. using a direct staining method. All other antibodies were FITC-labeled with the second antibody according to their isotype, i.e. using an indirect staining method.

Description	Specifity	Host	Name	Isotype	Company
CD 45* *(leukocytes)*	Bovine	Mouse	MCA832F	IgG1	Serotec
CD 14 (*Macrophages/Monocytes)*	Bovine	Mouse	BAQ151A	IgG1	NatuTec
CD 4 *(T-helper-lmyphocytes)*	Bovine	Mouse	IL- A11	IgG2a	NatuTec
CD 8 *(T-cytotoxic-lmyphocytes)*	Bovine	Mouse	CACT80C	IgG1	NatuTec
CD 21 *(B-lymphocytes)*	Bovine	Mouse	GB25A	IgG1	NatuTec
PMN	Bovine	Mouse	MM20A	IgG1	NatuTec
Macrophages/Monocytes*	Bovine	Mouse	MCA874F	IgG1	Serotec
B and activated T cells	Bovine	Mouse	MCA899	IgM	Serotec
FITC - Marker	Mouse	Goat	STAR86F	IgM	Serotec
FITC - Marker	Mouse	Rat	11- 4011	IgG	NatuTec

4 Results and Discussion

4.1 Effect of Centrifugation on SCC

Centrifugation is the common method for removal of SC from milk. Besides cell separation it also divides the fat phase from the skim milk, building the basic components of milk processing. Here, detailed investigations on the effect of this technological process on SCC from different milk samples were performed to evaluate possible changes originating from centrifugation.

4.1.1 Farm Milk Samples

Using total quarter milk samples the effect of RCF, centrifugation time and temperature on the distribution of SC in the evolving phases fat, skim milk and cell pellet was evaluated.

Increasing RCF values led to higher amounts of separated cells in the pellet. Surprisingly, the highest RCF values showed decreasing amounts. To achieve an optimal separation the RCF had to be adapted to a slightly lower level (Sarikaya *et al.* 2006a). The viability of the separated cells decreased with higher RCF. The distribution of SC in the three evolving phases after centrifugation showed that most of the cells remained in the fat. Their amount was partially reduced by increasing RCF. Results indicate the importance of optimized RCF values for improving the efficiency of SCC separation out of milk. The optimum of RCF hints towards a disruption of SC above certain values causing cell fragments floating in the supernatant.

The effect of the parameters time and temperature on the distribution of cells in the three evolving phases is summarized in Figure 6. Generally, increasing centrifugation time led to fewer cells in the fat, more cells in the pellet and unchanged levels in the skim milk. With increasing temperatures cells left the fat phase and appeared mainly in the skim milk and also in the pellet.

The main targets of SC during separation are the fat phase and the cell pellet. The skim milk plays a minor role. This implies a link between SC and fat globules as both present a membrane. In accordance to former works (Russell *et al.* 1977; Lee *et al.* 1980), a certain affinity of the SC membrane towards fat globule membrane is obvious. As the fat globules show a dominate amount, they might tear up the cells towards the top fraction during the centrifugal separation (Ma *et al.* 2000a). The affinity between the SC and the fat could partially be overcome by increased RCF, centrifugation time and temperature (Fig. 6).

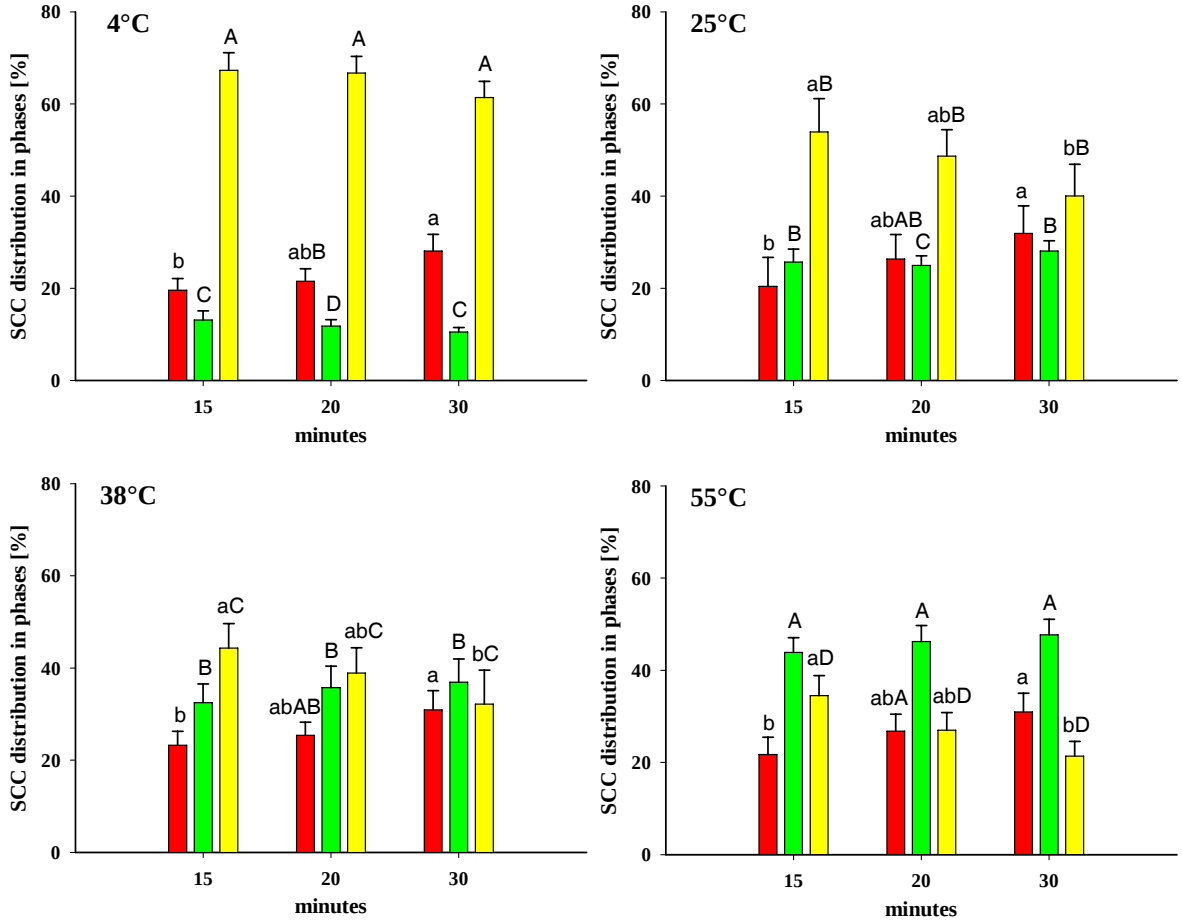

Fig. 6. Distribution of SCC in the three phases cell pellet, skim milk and fat depending on centrifugation time and at 4 different temperatures. Letters represent significances ($P<0.05$). a, b: Means without common letters within phase and temperature differ significantly between times. A, B, C, D: Means without common letters within phase and time differ significantly between temperatures.

The above mentioned results show that the centrifugation setup must be adapted to the investigators goals, as often work with milk includes the separation or extraction of the target component (Boutet *et al.* 2004; Prgomet *et al.* 2005). For instance, if working with alive SC is intended, RCF has to be moderate as high values lead to death and destruction of SC. If a maximum of SC separation is intended, e.g. to extract a certain protein, the centrifugation time and temperature have to be elevated additional to the RCF.

4.1.2 Dairy Milk Samples

Dairy production lines include different centrifugation steps. To assess their efficiency samples from distinct production steps were collected and subjected to SC determination. In this context the production lines ´milk` and ´whipping cream` were chosen (Sarikaya *et al.* 2006a).

In the production line milk a continuous decrease of SCC during processing was observed. The final product contained only a marginal amount of the initial SCC found in raw milk (Fig. 7a). The milk after separator contained still ~50% and the cream ~30% of the initial SCC. In the flow chart of the dairy production line the skimming step is performed before the bactofuge separation. But the most efficient separation of SC is obtained during bactofugation. It reduced the SCC value of the skim milk after separator to ~5% (milk after bactofuge). According to these results, it should be considered to place the bactofugation in front of the skimming step. Bactofugation of the total tank milk will obviously separate more cells from the milk especially from the included fat phase.

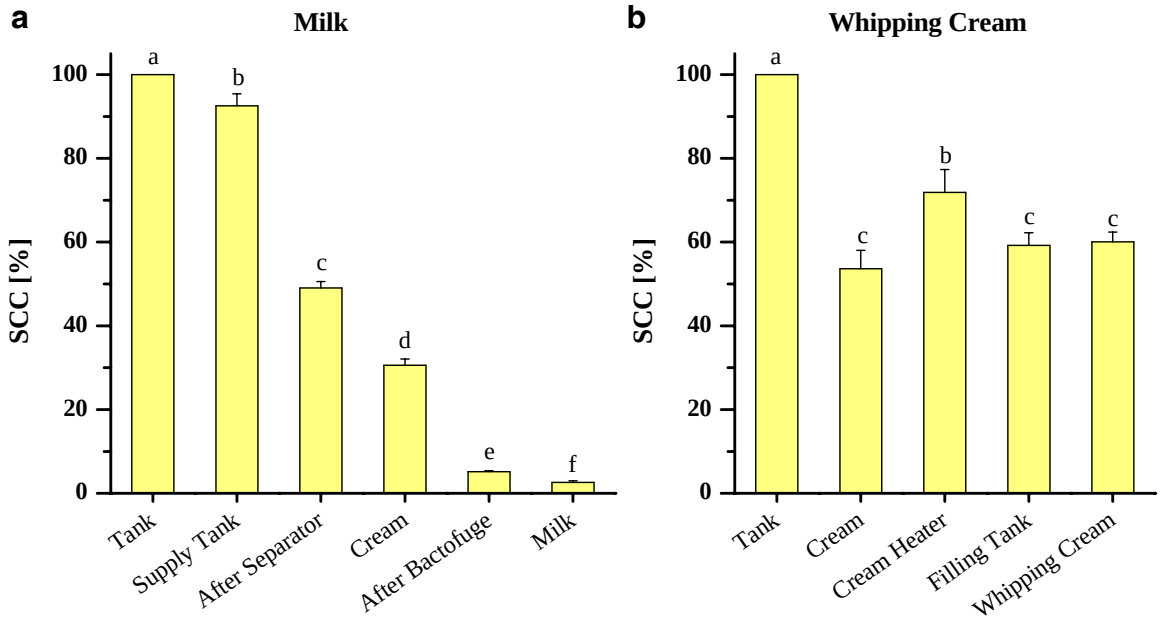

Fig. 7. Changes in SCC during distinct steps of processing in the two production lines milk (a) and whipping cream (b). SCC in the first step of processing was set 100%. a, b, c, d, e, f: Means without common letters within production line differ significantly ($P<0.05$) between steps of processing.

Due to the observations that a not negligible amount of SC remained in the fat phase, additional investigations were carried out on the production line whipping cream (Fig. 7b). The steps investigated in this production line showed a correlation between the amount of SC and the respective fat content. A decrease of SC from raw (tank) milk to cream was only detected during skimming/creaming. As no further technological separation is performed, the SC shows

no decreasing tendencies in the further steps. A temporary increase in the amount of SC in the cream heater is caused due to a marginally decreased water content. In this context the fat content also shows a temporary increase at this processing step (Sarikaya *et al.* 2006a).

Interpreting the results from an industrial point of view implies that one of the critical points in the processing is the skimming step. It is known that SCC can affect the products shelf-life and lead to off – flavours (Ma *et al.* 2000b; Santos *et al.* 2003). Therefore it is very important to remove the SC from the milk. The bactofugation is definitely more effective than the milk separator. Placing the bactofuge in front of the milk separator will enhance the SC separation.

4.2 Modified Pappenheim Staining Method

As each cell type has its own specific function in the immune response of the mammary gland the differential cell count of milk SC can be a useful tool in research. Therefore, a staining method for milk SC was developed and validated (Sarikaya *et al.* 2004) by modifying the panoptic staining method according to Pappenheim. This method allows the differentiation of milk SC populations under the light microscope (Fig. 8) and provides additional information on the immunological status of the mammary gland.

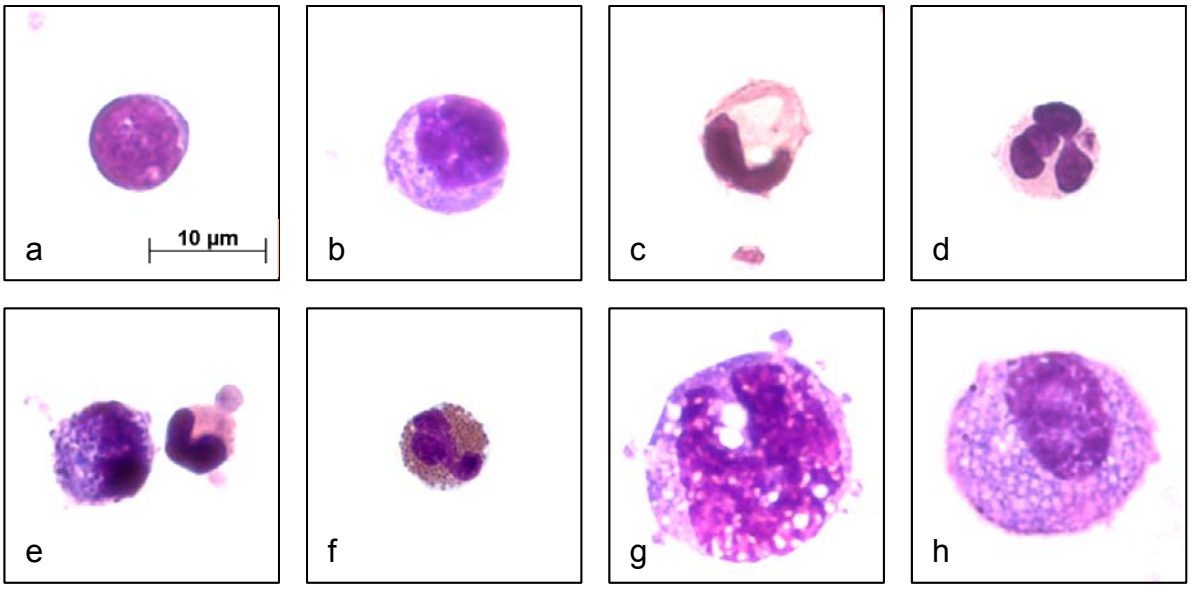

Fig. 8. Photograph of immune cells in milk (modified Pappenheim method); a. small, b. large lymphocyte; c. band, d. segmented PMN; e. basophil (left); f. eosinophil; g-h. macrophages; magnification 1000-fold

As the immune cells separated from milk appeared to be very sensitive against laboratory processing, the staining had to be modified to achieve a gentle procedure avoiding disruption

of the cells. Figure 8 shows examples that demonstrate a clear contrast in appearance for each cell type. Thus, this method builds the basis for successful differentiation of SC and interpretation of mammary gland immunological status.

4.3 Quarter Milk Fractions

During routine milking the composition of total milk and bulk milk is of interest for classifying milk quality. Here, the variation of milk composition during the course of milking was investigated in detail. First, the progression of SCC in quarter milk samples during milking were determined emphasizing on foremilk and cisternal milk fractions. Second, milk constituents, distribution of cell populations, and mRNA expression levels of inflammatory factors were additionally analyzed, to create an immunological picture of the udder status.

4.3.1 SCC in Quarter Milk Samples (Practical Approach)

A new cell counting technology (DeLaval Cell Counter) allows smallest sample sizes of less than 100 µl. Here, this technique was used to study sampled milk fractions and to predict udder health status in the context total quarter milk SCC (Sarikaya *et al.* 2006b).

The changes of SCC were investigated in different fractions of milk with special emphasis on foremilk and cisternal milk. Therefore, in a first approach (Trial 1) quarter milk samples were defined as strict foremilk (F), cisternal milk (C), first 400 g alveolar milk (A1) and the remaining alveolar milk (A2). Thereby, to obtain strict foremilk (i.e. free of cisternal or alveolar milk) milking was performed without any udder preparation to avoid milk ejection and mixing of milk fractions (Bruckmaier *et al.* 1996; Bruckmaier *et al.* 2001). In a second approach (Trial 2) foremilk was investigated in detail. The six foremilk fractions (F1 – F6) consisted of one hand-stripped milk jet each. The remaining cisternal milk (RM) included also the entire alveolar milk.

As different total SCC values account for specific udder health status, milk samples of each quarter were assigned to four somatic cell score groups shown in Table 3.

Table 3. Somatic cell score grouping of quarter milk samples according to their total quarter SCC.

Group		1	2	3	4
SCC [x 10^3/mL]	**Trial 1**	< 20	20 – 50	50 – 100	> 100
	Trial 2	< 50	50 – 100	100 – 350	> 350

Figure 9 shows the results of Trial 1. The decline in the first three fractions F, C and A1 was enormous in milk with high total quarter SCC (Group 4). The changes during milking in low (Group 2) or very low (Group 1) SCC milk were marginal. Significant differences between somatic cell score groups were only seen in fractions F and C. This characteristic was not observed in the later alveolar fractions A1 and A2. These results indicate the importance of defining the respective sampled milk fraction for interpreting SCC.

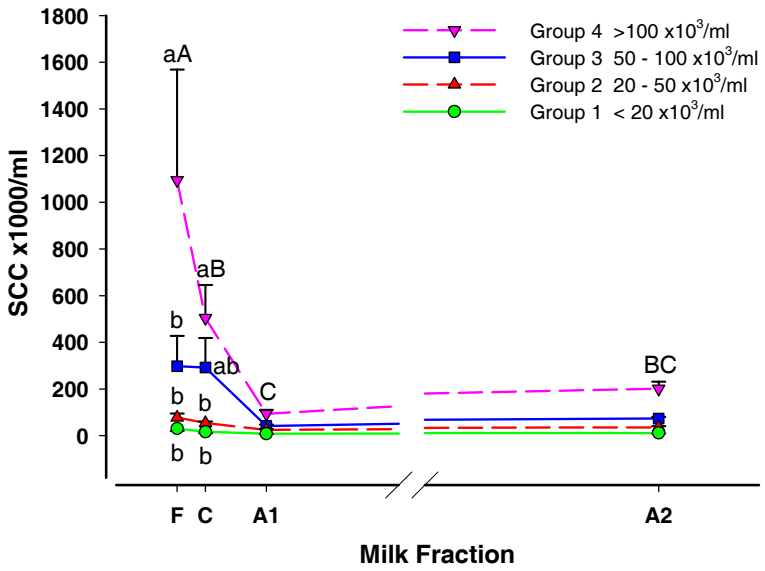

Fig. 9. Changes in SCC in the defined milk fractions strict foremilk (F), cisternal milk (C), first 400g alveolar milk (A1), and the remaining alveolar milk (A2) obtained via fractionized milking; additionally assigned to four different somatic cell score groups according to their total quarter SCC. Letters present significances ($P<0.05$). a, b: Means without common letters within milk fraction differ significantly between groups. A, B, C: Means without common letters within group differ significantly between milk fractions.

In Trial 2 the foremilk fractions were investigation in more detail (Fig. 10). High SCC milk (Group 4) showed a significant decline in SCC even in the foremilk fractions F1 to F6. Despite the fact that these foremilk fractions present only 0.1-0.2 % of the total milk amount, the SCC therein was two- to threefold higher than the SCC in total quarter milk. SCC in F1 through F6 of group 4 increased significantly in comparison to the respective fraction in SCC groups 1, 2 and 3. Surprisingly, this difference could not be observed in remaining cisternal plus alveolar milk (RM). In the context of RM, Bruckmaier et al. (2004) already investigated the effect of milk ejection on the sensitivity of mastitis indicators like physiochemical factors and somatic cells. They could show striking indicator differences between high and low SCC quarters only before milk ejection.

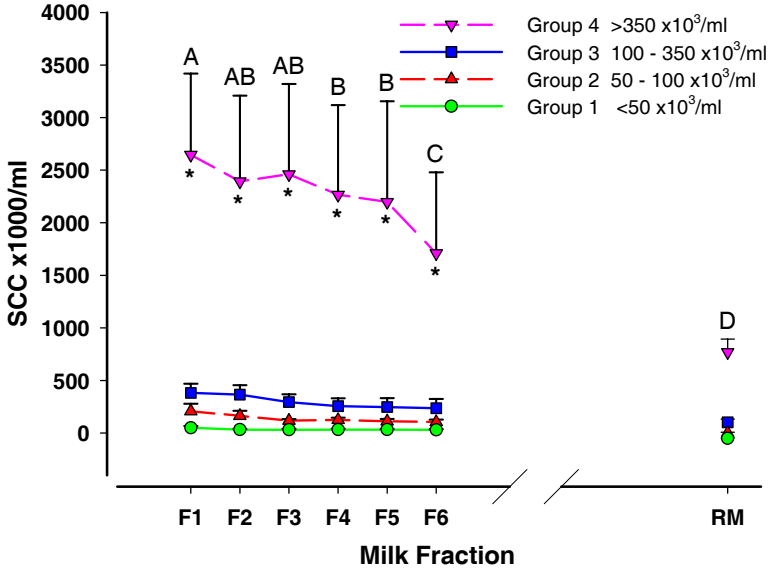

Fig. 10. Changes in SCC in the defined milk fractions F1 to F6 consisting of one hand-stripped milk jet each and RM (remaining cisternal plus alveolar milk) additionally assigned to four different somatic cell score groups according to their total quarter SCC. Letters and asterisks present significances (P<0.05). *: Means with asterisk within milk fraction differ significantly between groups. A, B, C: Means without common letters within group differ significantly between milk fractions.

Generally, the results from trial 2 supported the above mentioned findings of trial 1. Concluding, in quarters with high SCC the sampled milk fraction has a crucial influence on the measured SCC value. It must be considered that even SCC in strict foremilk can dramatically differ from that in the total cisternal fraction. Consequently, these results demand the definition of the milk fraction being taken in practice if interpretation of the milk quality or the udder status is conducted based on SCC.

4.3.2 Differential Milk Composition (Immunological Approach)

To reach a more differentiated picture of milk compositional changes during milking the milk samples were collected in fractions and investigated for a broad set of parameters (Sarikaya *et al.* 2006c). These included main nutritive milk constituents, the distribution of cell populations and mRNA expression levels of inflammatory factors (TNF-α, IL-1β, COX-2, lactoferrin, lysozyme). Hereby, quarter milk samples were defined as cisternal (C), first 400 g of alveolar (A1), and remaining alveolar milk (A2) during the course of milking. Additionally, quarter milk samples were assigned to one of four somatic cell score groups according to their total SCC (Table 4).

Table 4. Somatic cell score grouping of quarter milk samples according to total quarter SCC.

Group	1	2	3	4
SCC [x 10^3/mL]	< 12	12 – 100	100 – 350	> 350

Nutritive Milk Constituents

The milk constituents fat, protein, lactose and the milk electrolytes sodium and chloride as well as the electrical conductivity (EC) were investigated in each fraction of milking. A clear influence of the milk fraction and/or the total quarter SCC was shown regarding those parameters (Table 5).

Table 5. Tendencies of changes in milk constituents depending on milk fraction (C, A1, A2) and total quarter SCC (Group 1, 2, 3, 4). For details and significances see (Sarikaya *et al.* 2006c).

Milk Constituents	**Milk Fraction**	**Total Quarter SCC**
Fat	C < A1 << A2	$1 = 2 \leq 3 \leq 4$
Protein	C = A1 = A2	1 = 2 = 3 < 4
Lactose	$C \leq A2 \leq A1$	$1 = 2 \geq 3 > 4$
Sodium	C > A1 = A2	1 < 2 = 3 < 4
Chloride	C > A1 = A2	$1 \leq 2 = 3 \leq 4$
EC	C > A1 = A2	$1 \leq 2 \leq 3 < 4$

Results show that the content of fat increased significantly during the course of milking as well as with elevated SCC. This change throughout the fractions can be explained by the lower density of the fat globules and the successional ascending force in the udder. Furthermore, a possible adhesion of the globule membranes to the alveolar lumina may support this phenomenon. Hence, fractions with the highest fat content appear at the end of milking. Fat content and SCC correlated significantly in fractions C and A1 of all four SCC groups. The elevated fat content may be a consequence of reduced lactose synthesis, as lactose concentrations show the opposite tendency compared to fat content with increasing SCC (Bruckmaier *et al.* 2004a). As lactose defines the milk volume originating from osmolar effects, the slight fat concentration change may be a secondary effect.

During the course of milking only minor changes of lactose concentrations were determined in each SCC group. The concentration of sodium and chloride must be considered in context

with the parameter lactose, as the combination of these is responsible for isoosmolarity. The concentrations of sodium and chloride differed only marginally up to a SCC of 350 x10^3 cells/ml (Group 1-3), but a significant increase was seen in group 4, presenting a distinct increased level of SCC (> 350 x10^3 cells/ml). This can be explained by the circumstance that only during high leukocyte diapedesis the tight junctions are leaky enough to permit elevated movement of ions from blood into milk (Nguyen *et al.* 1998; Bruckmaier *et al.* 2004b). Within one fraction, only the electrolytes in the cisternal milk showed a direct correlation with SCC. After milk ejection, the sensitivity of ion measurements is reduced due to the mixture of alveolar and cisternal milk (Bruckmaier *et al.* 2004b). The observed decrease of sodium and chloride during the course of milking in all four SCC groups can be assigned to this phenomenon.

Electrical conductivity comprises all ions dissolved in milk, mainly sodium and chloride. Therefore, EC follows the same trends as the electrolytes. Furthermore, the decline of EC in successive milk fractions is affected by the increase of fat at the end of milking because fat modulates the EC measurement (Woolford *et al.* 1998).

SCC and Leukocyte Populations

The combination of SCC and differential cell count offers a more precise immunological interpretation of the milk samples. Results show that SCC in fractions C and A2 were significantly higher than in fraction A1 (Sarikaya *et al.* 2006c). This agrees with previous findings (Woolford *et al.* 1998; Ontsouka *et al.* 2003) and underlines the importance of defining the milk fraction if udder health monitoring and judgment of milk quality is based on the parameter SCC.

The distribution of cell populations depended on the SCC as well as on the milk fraction (Fig. 11). Macrophages were the predominant cell type in group 2 and decreased with elevating SCC. It is generally accepted that macrophages present the major cell fraction in healthy quarters (Lee *et al.* 1980; Paape *et al.* 2002). Furthermore, the highest content of macrophages was always observed in fraction C and decreased during milking. As fraction C is located at the main point of entry for pathogens, the teat, macrophages are the first cells acting after contact with pathogens (Sordillo *et al.* 2002). They initiate the inflammatory response by releasing chemoattractants, which is necessary to eliminate invading pathogens. These chemoattractants cause a rapid influx of PMN into the milk. This is the most effective mechanism against invading pathogens, as these two cell populations represent the phagocytic cells of the mammary gland (Burvenich *et al.* 2003; Paape *et al.* 2003). This signaling cascade explains why PMN were determined as the major cell fraction at elevated SCC.

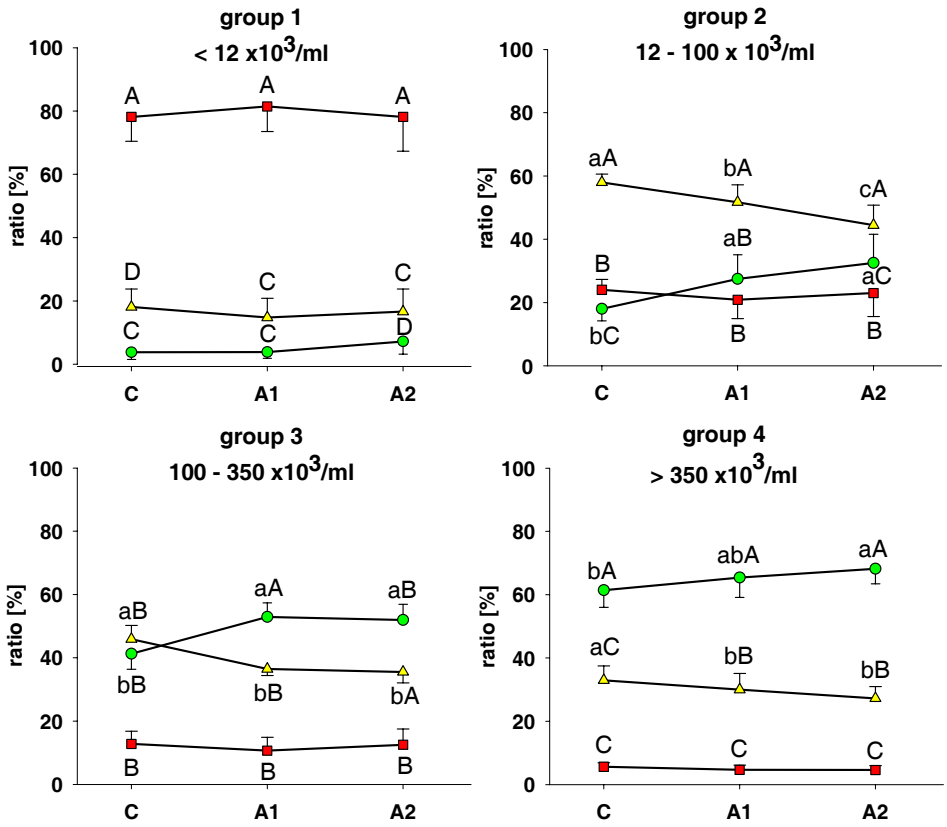

Fig. 11. Distribution of cell populations (■ lymphocytes, ▲ macrophages, ● PMN) according to milk fractions (C, A1, A2) and somatic cell score groups (1, 2, 3, 4). Letters present significances (P<0.05). a, b, c: Means without common letters within group and cell population differ significantly between milk fractions. A, B, C: Means without common letters within milk fraction and cell population differ significantly between groups.

The lymphocytes form only a small percentage of the cells in SCC groups 2, 3, and 4, and decreased with elevated SCC. Surprisingly, lymphocytes were the predominant cell type in SCC group 1 (< 12 x10^3 cells/ml) with up to 80% of the total. The percentage of lymphocytes did not change during milking. Lymphocytes have been identified as the specific immune defence of the mammary gland (Taylor *et al.* 1997). In this context, it is suggested that this cell type does not play a major role in infections of the mammary gland and that it operates in the mammary tissue rather than in milk itself (Riollet *et al.* 2001). The defense mechanism of the udder is mainly related to the innate immunity mediated by macrophages and PMN. The surprisingly low content of macrophages and PMN and the contrasting high content of lymphocytes in quarters with SCC < 12 x10^3/ml (Group 1) might thus cause a reduced immune response to invading pathogens. This is supported by previous investigations indicating that a very low SCC increased the risk of establishing infection with major udder pathogens (Schukken *et al.* 1989; Schukken *et al.* 1999). Furthermore, very low SCC was

associated with a higher risk of a severe infection with pathogens (Sol *et al.* 2000; Suriyasathaporn *et al.* 2000).

mRNA Expression of Inflammatory Factors

The results presented here already show a clear coherence between differential cell count and mammary gland immunology. During mammary infection, nonspecific immunological responses are the predominant defenses. To enlighten defense activities in more detail, immunomediators were investigated during milking and subject to SCC by determining their mRNA expression levels.

Five immunologically important humoral factors that are known to be involved in the natural defense mechanisms of the mammary gland against invading pathogens (Kawai *et al.* 1999; Schmitz *et al.* 2004; Prgomet *et al.* 2005) were selected (Table 6).

TNF-α and IL-1β are important proinflammatory cytokines and therefore play a major role in the defense against mastitis (Blum *et al.* 2000; Riollet *et al.* 2000). TNF-α is one of the cytokines mediating the acute phase response. In the present study, the rise of TNF-α mRNA expression occurred concomitantly with the increase of SCC, which supports previous findings. IL-1β showed the same expression pattern. It is known, that these two cytokines stimulate IL-8 secretion (Persson *et al.* 1993), an important mediator of PMN migration. The results show, that influx of PMN into the mammary gland affects the progress of the infection.

mRNA gene expression level was affected marginally during the course of milking. Compared to TNF-α and IL-1β, an increase of Lactoferrin (Lf) was detected with increasing SCC levels (Table 6). This is consistent with literature, as Lf increases in bovine milk during clinical mastitis (Kawai *et al.* 1999). It is produced mainly by stimulated PMN within the immune cells (Prgomet *et al.* 2005), which were detected in higher amounts with increasing SCC (Figure 11). A significant increase of lysozyme (Lz) mRNA expression was also observable, showing peak values in SCC group 4 (>350 $x10^3$ cells/ml). These increasing expression levels of Lz with SCC level indicate a possible relevance of Lz in the mammary gland immune defense due to its bacteriostatic effects on udder pathogens (Carlsson *et al.* 1989). The increase of COX-2 mRNA gene expression from SCC groups 1 to 4 shows an elevated capacity of prostaglandin synthesis with increasing SCC.

Table 6. Tendencies of changes in mRNA expression of inflammatory factors depending on milk fraction (C, A1, A2) and total quarter SCC (Group 1, 2, 3, 4). For details and significances see (Sarikaya *et al.* 2006c).

	Effect of	
	Milk Fraction	**Total Quarter SCC**
TNF- α	No effect[1]	1 < 2 = 3 < 4
IL- 1β	No effect[1]	1 < 2 = 3 < 4
Cox- 2	No effect[1]	1 < 2 = 3 < 4
Lactoferrin	No effect[1]	1 < 2 = 3 < 4
Lysozyme	No effect[1]	2 = 3 < 4[2]

[1] except for SCC group 3: C = A1 < A2

[2] SCC group 1 not investigated due to technical reasons

The results show that most of the investigated factors have significantly higher expression levels with increasing SCC. Here, higher levels of mRNA expressions occurred without any experimental induction of mastitis. For example, other studies used LPS injection (Schmitz *et al.* 2004). This means that here natural stimuli like pathogens cause the effect of up-regulating all inflammatory factors. Thereby, changes in mRNA expression of the housekeeping genes ubiquitin and glyceraldehyde-3-phosphate dehydrogenase did not occur. Thus, the observed changes in mRNA of those chemoattractants represent specific responses of the mammary gland to stimuli that correlate with increasing SCC levels.

4.4 FACS Analysis

Flow cytometry is a powerful tool for the measurement of antigen expressions on the surface of cell populations in blood. These clusters of differentiation (CD) can be detected by specific antibodies labeled with fluorophores. Here, a FACS method was developed to detect the different cell populations in milk. This included the testing of various antibodies. Further, the staining protocol was optimized and each antibody was tested for its binding capacity.

A histogram provides information about the amount of cells with the specific fluorescence intensity. This graph enables to detect labeling efficiency of cells with the respective antibody, i.e. whether the staining of the cells worked. Figure 12a shows an unstained sample (negative control) with no fluorescence, a sample containing only one cell population shows a single fluorescence peak (b), and a sample containing different cell populations shows a small

fluorescence peak at higher intensity (c, right) and a second peak with no fluorescence intensity (c, left). This demonstrates that stained cells can be clearly distinguished from unstained cells. Those preliminary experiments were carried out for each antibody before analyzing results in detail.

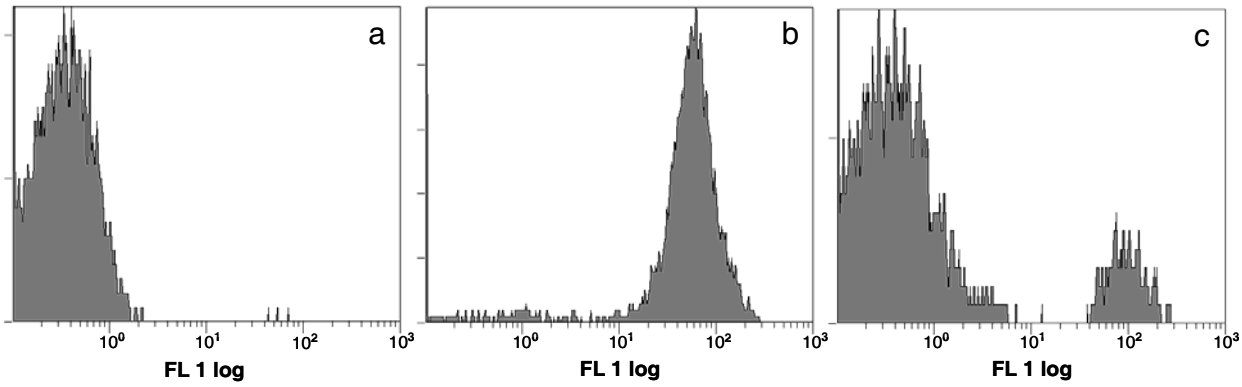

Fig. 12. Typical histogram recorded by FACS of an unstained sample (a) with no fluorescence, a sample containing only the cell population to be stained (b) with one specific fluorescence peak, a sample containing some of the cells to be stained(c) with a small fluorescence peak higher intensity (right) and a bigger peak with no fluorescence intensity (left).

It is commonly known that bovine cells present a different expression pattern of CD clusters than human cells. The present investigations and results additionally show that even bovine milk cells present altered CD patterns than bovine blood cells. Although antibodies were chosen based on literature, discrepancies occurred between differential cell counts acquired with flow cytometric analysis and microscopic counting after staining (Sarikaya *et al.* 2004). The main difficulty was the unintended cross-reactivity of several cell specific antibodies with other cell types. Besides the discrepancies of microscopic and flow cytometric results, the cross-reactivity phenomenon was displayed by detection of multiple clouds in the plots, although only one was expected (Fig. 13).

To explain those results it was suggested that the cells` surface undergoes an unknown change during diapedesis from the blood into the milk. The diapedesis might modify the CD clusters on the surface, which form the target domain for the specific antibodies. This problematic occurs as antibodies are always established for the matrix bovine blood, but never for milk.

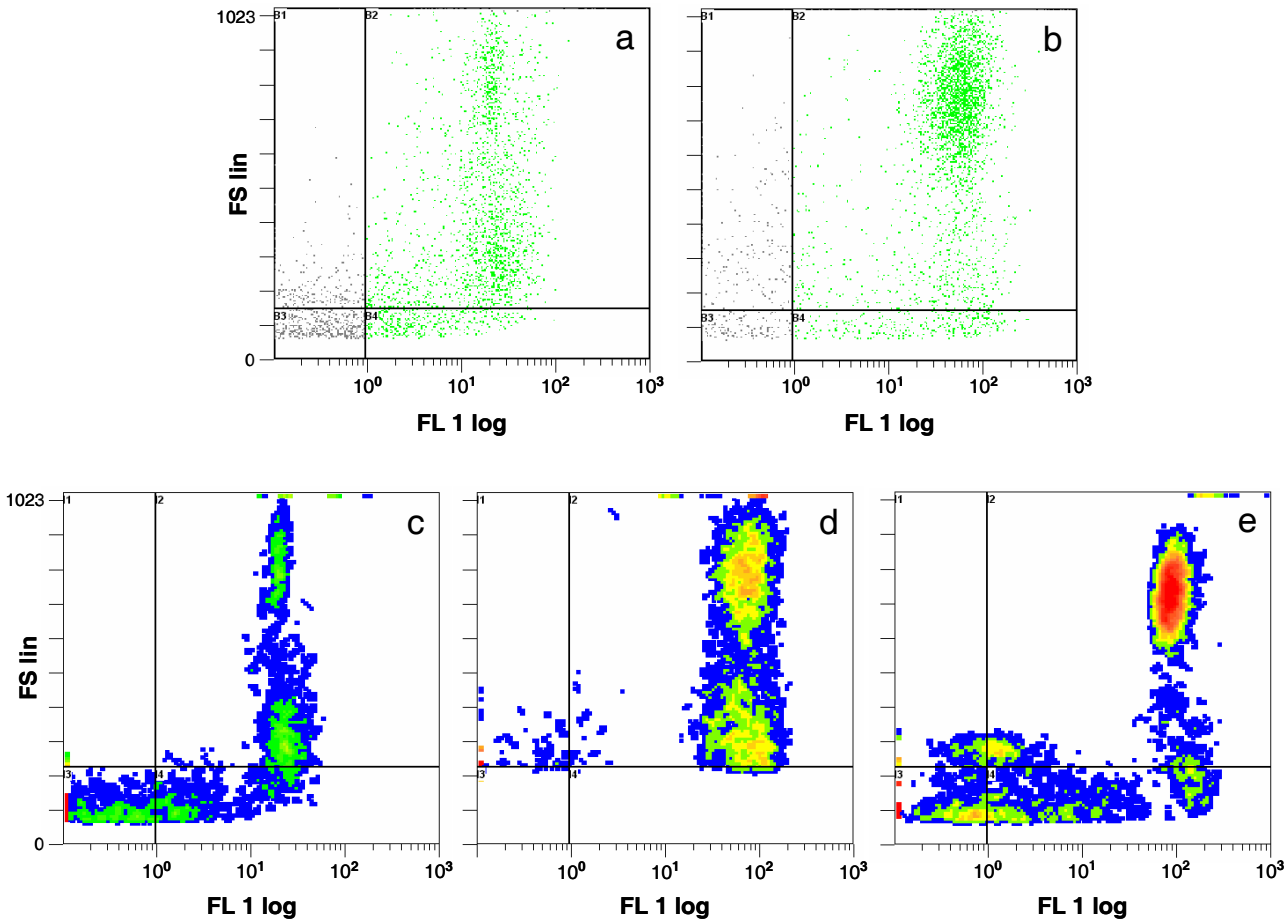

Fig. 13: Typical dot plots and density plots recorded by FACS. Dot plots (a, b) and density plots (c, d, e) present multiple event clouds of cell populations although one cell population cloud is expected.

The flow cytometer used here did not possess a cell sorter. This auxiliary device offers the possibility to extract the detected cell population. They can afterwards be subjected to a microscopic investigation. This additional information might help to confirm the suggestions mentioned above.

The results of the flow cytometric cell differentiation cannot be compared directly to those gained by microscopy, as the antibodies used do not bind exclusively one cell type. The selection of the monoclonal antibodies is of great importance and currently also a problem, i.e. not all antibodies available in the bovine system can be used to stain milk cells. The binding behaviour of the antibodies differs for cells isolated from blood and from milk. The initial experiments presented here show the potential of applying FACS analysis to milk samples. To overcome the specificity problem of the antibodies work has to be continued to transform the system applied originally in blood into a milk cell differentiation tool.

5 Conclusion

In the present study the importance of the somatic cells and their populations in mammary gland physiology as well as their behaviour during technological processing were investigated. Industrial cell separation was optimized by scaling down the steps of processing to laboratory size. Knowledge achieved during centrifugation under different parameters helped to improve cell extraction from milk for scientific research. Further, respective steps during industrial milk processing were enlightened and the dairy industry was encouraged to place the bactofuge before the milk separator to enhance cell separation.

A new method was developed to differentiate milk cell populations under the light microscope, which enables to achieve additional information on the immunological status of the mammary gland. Using this method, quarter milk samples were investigated during fractionized milking and depending on udder health status. Thereby a clear correlation was shown between the differential cell count and the mammary immunology. Astonishingly, udders with very low SCC were identified to possess very high amounts of lymphocytes and accordingly low amounts of macrophages and PMN. As the immune response of the mammary gland is mainly formed by the latter cells a significant deficit in immune responsiveness was detected in this case. Further, the definition of the milk fraction appeared to be very important for the interpretation of results based on SCC and differential cell count. It must be considered that even strict foremilk can dramatically differ in cell composition from the cisternal fraction. mRNA expression analysis of various meaningful inflammatory factors supported the above mentioned findings regarding mammary gland immunology. Increasing mRNA expression levels of the investigated genes with increasing SCC indicated a higher overall activity of the immune cells and the reduced immune response in quarters with very low SCC was underlined by very low mRNA expression levels.

Additionally to the classical histological approaches milk samples were subjected to FACS analysis. This modern technique is widely used for blood analysis in humans as well as animals. This tool offers a fast and objective routine analytics. It was shown that in principal FACS can be adapted to milk cell analysis. Thereby, diapedesis appeared to be the main problem as the surface of the milk cells is altered and the common antibodies show cross-reactivity. The development of new specific antibodies may help to overcome this problem and render FACS analysis a very useful tool in future. Thus, if the differential cell count in milk samples will be established in routine milk analytics, the immunological interpretation of milk samples would reach higher levels.

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Abbreviations

CD	cluster of differentiation
Cl	cloride
COX-2	cyclooxygenase-2
CP	crossing point
EC	electrical conductivity
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IL-1β	interleukin-1beta
Lf	lactoferrin
LPS	lipopolysaccharide
Lz	lysozyme
mRNA	messenger RNA
Na	sodium
OD	optical density
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PMN	polymorphonuclear neutrophils
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RCF	relative centrifugal force
RM	remaining cisternal plus alveolar milk
RNA	ribonucleic acid
SCC	somatic cell counts
SC	somatic cells
TNF-α	tumor necrosis factor-alpha
UbQ	ubiquitin

Scientific Communication

Publications

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Sarikaya, H., M. Atzkern, C. Werner-Misof and R. M. Bruckmaier. “Verteilung der Zellpopulationen in Milch”. Milchkonferenz Deutsche Gesellschaft für Milchwissenschaft, Osnabrück (18. – 19.09.2003)

Sarikaya, H. and R. M. Bruckmaier. “Ein Blick in die Zellbiologie der Milch”. Weihenstephaner Milchwirtschaftliche Herbsttagung, Freising (09. – 10.10.2003)

Sarikaya, H. and R. M. Bruckmaier. “Milchzellpopulationen in verschiedenen Gemelksfraktionen”. Fortbildungsseminar Physiologie und Biotechnologie der Milchabgabe, TU München, Freising-Weihenstephan (02.- 03.09.2004)

Posters and Abstracts

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Sarikaya, H. and R.M. Bruckmaier. Poster: “Distribution of cell populations in the milk of dairy cows at different somatic cell count levels”. EAAP/ASAS Workshop `Biology of Lactation in Farm Animals`, Bled, Slovenia, (09. – 10.09.2004)

Sarikaya, H. and R.M. Bruckmaier. Poster: “Importance of the sampled milk fraction for the prediction of total quarter milk SCC”. 4th International IDF Mastitis Conference `Diagnosis of mastitis & indicators for milk quality`, Maastricht, The Netherlands, p. 872 (12. – 15.06.2005)

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Appendix

Appendix I. Sarikaya, H., C. Prgomet, M. W. Pfaffl and R. M. Bruckmaier. 2004. Differentiation of leukocytes in bovine milk. Milchwissenschaft. 59: 586-589.

Appendix II. Sarikaya, H., G. Schlamberger, H. H. D. Meyer and R. M. Bruckmaier. 2006. Leukocyte populations and mRNA expression of inflammatory factors in quarter milk fractions at different somatic cell score levels in dairy cows. Journal of Dairy Science. 89 (7): 2479-2486.

Appendix III. Sarikaya, H. and R. M. Bruckmaier. 2006. Importance of the sampled milk fraction for the prediction of total quarter SCC. Journal of Dairy Science. 89 (11): 4246-4250.

Appendix IV. Sarikaya, H. and R. M. Bruckmaier. 2006. The effect of centrifugation on somatic cell content and distribution in milk. International Journal of Dairy Science. (submitted)

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Differentiation of leukocytes in bovine milk

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The somatic cell count (SCC) in milk is used as an indicator of udder health status. Elevated SCC are generally considered as an indication of mastitis. In addition, the differential cell count of milk somatic cells can be a useful tool in research because each cell type has its own specific function in the immune response.

The aim of this study was to develop and validate a staining method for milk somatic cells. Therefore the panoptic staining method according to Pappenheim was adapted for the differentiation of milk somatic cell populations. Quarter composite milk samples from 28 German Braunvieh x Brown Swiss cows were separated into 3 groups based on their SCC levels. Group 1 consisted of 12 samples with a mean of 4.57 ± 0.10, group 2 of 8 samples at a mean of 5.39 ± 0.06 and group 3 of 8 samples at a mean of 6.15 ± 0.07 $\log_{10}$ cells/ml, respectively. Results showed a distribution of lymphocytes, macrophages and neutrophils of 20.9, 45.6 and 33.5% in group 1, of 11.4, 25.1 and 63.5% in group 2 and of 3.3, 9.5 and 87.2% in group 3, respectively. In conclusion, the ratio of lymphocytes and macrophages decreases whereas the ratio of neutrophils increases with rising SCC.

Differenzierung von Leukozyten in Kuhmilch

Die somatische Zellzahl der Milch wird als Indikator für den Eutergesundheitsstatus verwendet. Erhöhte Zellzahlen werden im Allgemeinen als Anzeichen für Mastitis betrachtet. Zusätzlich kann die Zusammensetzung der Populationen der somatischen Zellen der Milch eine weitergehende Information liefern, da jeder Zelltyp eine spezifische Funktion in der Immunantwort der Milchdrüse hat.

Ziel dieser Studie war die Entwicklung und Validierung einer Färbemethode für die somatischen Zellen der Milch. Daher wurde die panoptische Färbemethode nach Pappenheim für die Differenzierung von somatischen Zellpopulationen aus der Milch optimiert. Die Viertelgemelke von 28 Deutschen Braunvieh x Brown Swiss Kühen wurden hinsichtlich ihrer Zellzahlniveaus in 3 Gruppen eingeteilt. Gruppe 1 bestand aus 12 Proben mit einem Mittelwert von 4.57 ± 0.10, Gruppe 2 aus 8 Proben mit einem Mittelwert von 5.39 ± 0.06 und Gruppe 3 aus 8 Proben mit einem Mittelwert von 6.15 ± 0.07 $\log_{10}$ Zellen/ml. Die Ergebnisse zeigten eine Verteilung von Lymphozyten, Makrophagen und Neutrophilen von 20.9, 45.6 bzw. 33.5% in Gruppe1, von 11.4, 25.1 bzw. 63.5% in Gruppe 2 und von 3.3, 9.5 bzw. 87.2% in Gruppe 3. Somit konnte gezeigt werden, dass der Anteil von Lymphozyten und Makrophagen mit steigender Zellzahl abnimmt wohingegen der Anteil der Neutrophilen zunimmt.

06 Somatic cells (differentiation in bovine milk) **06 Somatische Zellen** (Differenzierung in Kuhmilch)

1. Introduction

The mammary gland immune response to invading pathogens is predominantly based on cellular reactions mediated mainly via macrophages, neutrophils and lymphocytes (2, 12). While milk from healthy, i.e. noninfected glands is supposed to represent macrophages as the major cell fraction, the percentage of neutrophils increases dramatically in secretions from infected glands in response to inflammation (4, 12, 16). Neutrophils and macrophages are the functional phagocytes of the mammary gland (11) and phagocytic active in milk (6).

Macrophages play a central role as alarming cells after the invasion of microorganisms, shown as increased expression of cytokines and inflammatory mediators (1, 17). The synthesis of chemoattractants initiates a rapid influx of neutrophils into the gland (3). Thus both cell populations together have microbicidal activity, whereby neutrophils dominate (10, 15). Specific immune response to bacteria is mediated by lymphocytes that recognize antigens through membrane receptors and produce antibodies against invading pathogens (14). This condition is primarily intended to be used for vaccination to enhance the neutrophil recruitment in cases

cination to enhance the neutrophil recruitment in cases of mastitis by the localized antigen-specific lymphoid population (15).

The commonly used parameter for udder health is the somatic cell counts (SCC), however this parameter does not consider any changes of distribution of cell populations. An elevated value is being associated with mastitis. SCC is liable to fluctuations according to the stage and period of lactation even without any infection of the mammary gland (5, 8, 9). The somatic cells consist of lymphocytes, macrophages and neutrophils originating from the bloodstream and of epithelial cells from the tissue. Previous studies using electron microscopy for analysing the SCC show a predominant percentage of immune cells whereas epithelial cells were rarely found (6).

Besides the number of cells also the distribution of cell populations depend markedly on the physiological status of the mammary gland (2, 8, 9). The purpose of the present study was to establish and validate a staining method for differential somatic cell count in secretions to present a potential new technique for precise evaluation of immunological activity of the mammary gland.

2. Materials and methods

2.1 Animals and milking

Twenty-eight cows of the German Braunvieh x Brown Swiss breed were used. Nine animals were in their first, 9 in their second, 5 in their third and 5 in their fourth lactation. Eleven animals were in early (33–127 d), 8 in mid (139–216 d) and 9 in late lactational stage (228–322 d), respectively.

Milking was performed twice daily at 5.00 and 16.00. Quarter composite milk samples (QMS) were collected during morning milking and SCC was measured with Fossomatic™ (FOSS Analytical A/S, Hillerød, Denmark). QMS were classified according to the SCC level into 3 groups: SCC < 100,000/ml, SCC 100,000–400,000/ml and SCC > 400,000/ml. To verify stability of milk cells samples were processed within 2–3 h after milking.

2.2 Cell isolation and viability test

All QMS were gently mixed and 50 ml were centrifuged for 30 min and 1500 x g at 4 °C in conical tubes. The fat layer on top was removed, the supernatant (skim milk) was discarded and the cell pellet was resuspended in 5 ml of ice-cold phosphate buffered saline (PBS, pH 7.5). After refilling to the original volume the suspension was centrifuged for 15 min and 460 x g at 4 °C for washing. The received cell pellet was resuspended in PBS up to 5 ml according to the estimated size of the pellet. The viability of the cells was investigated by the exclusion method with the dye trypan blue (7). Thereby viable cells appear shining white under the microscope while dead cells are blue.

2.3 Panoptic cell staining

Cell differentiation was performed under the light microscope using a panoptic staining method according to Pappenheim, which has formerly been established for haematological analysis (13). Briefly, 25 µl of cell suspension was smeared gently on a slide in wavy lines. After air drying, slides were first stained for 3 min with undiluted and then for 1 min with 1:2 diluted May-Gruenwald solution (Sigma-Aldrich, Steinheim, Germany). As this solution contains methanol, no previous fixation of cells was needed. Slides were further stained for 14 min with a 3% Giemsa solution (Sigma-Aldrich, Steinheim, Germany). Afterwards slides were washed gently with Sörensen buffer (0.01 M, pH 7). To achieve durably stained cells the slides were covered after drying with Eukitt® (Plano GmbH, Wetzlar, Germany).

2.4 Differential cell counting

Two hundred cells were counted under a light microscope with x 1000-fold magnification using immersion oil. Photographs of the detected cell types are shown in Fig. 1. For evaluation cells were classified into lymphocytes, macrophages and neutrophils according to their morphology and diameter. Epithelial cells, eosinophils and basophils appeared in a neglectable number and were not considered for differential cell count calculation.

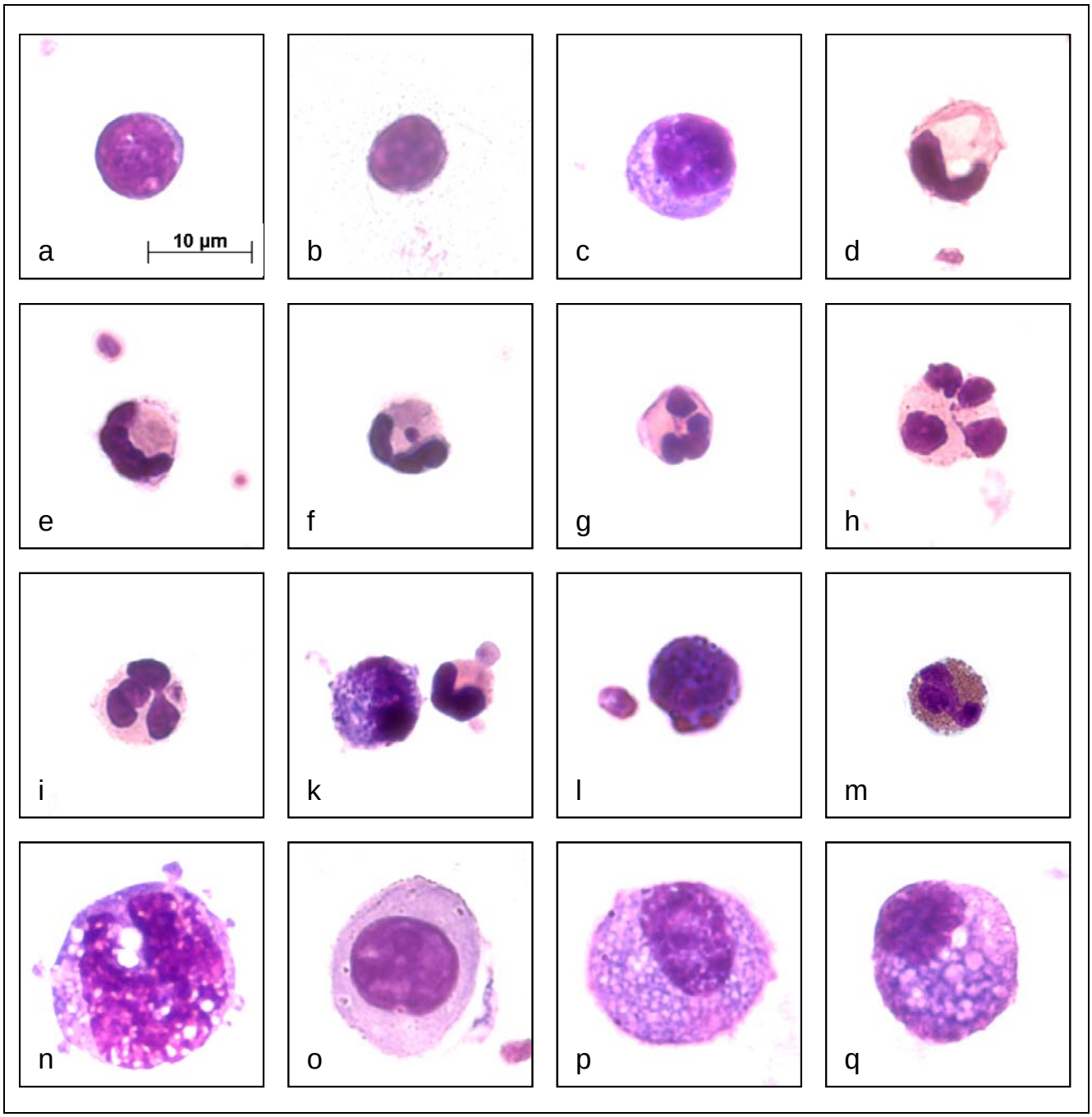

Fig. 1: Photographs of cells in bovine milk stained according to method of Pappenheim; a-b = small lymphocytes; c = large lymphocyte; d–f = band neutrophils; g–i = segmented neutrophils; k = basophil (left) and band (right) neutrophil; l = basophil; m = eosinophil; n–q = macrophages; magnification x 1000-fold

Table1: Cell distribution quarter milk samples of different SCC levels (mean ± SEM)			
	Group 1 SCC < 100,000/ml n = 12	Group 2 SCC 100,000– 400,000/ml n = 8	Group3 SCC > 400,000/ml n = 8
SCC (log_{10} cells/ml)	4.57 ± 0.10	5.39 ± 0.06	6.15 ± 0.07
Lymphocytes [%]	20.9 ± 1.2[a]	11.4 ± 1.4[b]	3.3 ± 0.3[c]
Macrophages [%]	45.6 ± 1.9[a]	25.1 ± 3.5[b]	9.5 ± 1.9[c]
Neutrophils [%]	33.5 ± 1.7[c]	63.5 ± 3.8[b]	87.2 ± 1.8[a]
[a,b,c] Means within line are significantly different ($p<0.05$)			

Lymphocytes are characterized by a round nucleus of blue-red colour and a grey-blue cytoplasm. The volume of the latter distinguishes this cell type into small and large lymphocytes. Thus cell size varies between 8–16 µm. Band neutrophils are circular and present a light-grey to pink cytoplasm. The nucleus in blue-red colour is bent and oblong. These characteristics serve as differentiation parameters against the segmented neutrophils. According to the rule of filament these cells are counted as the latter cell type as soon as the nucleus is threadlike and constricted. The diameter of neutrophils varies from 10 to 15 µm. The subpopulations basophils and eosinophils can be easily distinguished. Their densely packed granules appear dark blue and orange-red, respectively. The macrophages show a diameter of 15–25 µm and are the largest cells in milk. Their shape is diverse with a bluish-grey cytoplasm. Vacuoles and phagocytized fat globules are observed frequently (see Fig. 1).

2.5 Statistical evaluation

The effect of groups on the distribution of cell populations was calculated by 1-way ANOVA and Bonferroni`s t-test. Level of significance was determined at $p < 0.05$.

3. Results and discussion

The 28 investigated samples were divided into 3 groups according to their SCC. Group 1 consisted of 12 samples at a mean SCC of 4.57 ± 0.10 log_{10} cells/ml, which is assigned with healthy quarters and the macrophages as dominant cell type (4, 6). As expected due to comparable literature the predominant cell type in group 1 was the macrophages followed by neutrophils and lymphocytes. Group 2 with a mean SCC of 5.39 ± 0.06 log_{10} cells/ml ($n = 8$) showed a shift towards the population of neutrophils. The percentage of lymphocytes and macrophages decreased. The distribution of the cell population in group 3 ($n = 8$) was dominated by neutrophils. At a mean SCC of 6.15 ± 0.07 log_{10} cells/ml lymphocytes and macrophages played a minor role (see Table 1). According to several studies the cell population of neutrophils in group 2 and 3 increased with rising SCC levels (4, 6, 8, 9). The viability of the separated cells, determined by trypan blue staining, ranged from 94 to 98%.

4. Conclusions

The used technique allows cell differentiation in milk samples and hence additional information on the immunological status of the mammary gland can be achieved. To preserve cellular morphological features it is necessary to remove the milk fat completely before preparation of smears. Cell diameter and morphology are an important tool in identification of cell populations.

Our results show that the content of macrophages decreased markedly and that of lymphocytes slightly while the content of neutrophils increased with increasing SCC. The distribution of milk cell populations corresponded with previous findings based on other methods. Therefore, it can be assumed that the cell differentiation based on Pappenheim staining provides reliable results at all SCC levels.

The application of this procedure provides additional information about the distribution of cell types for the understanding of the udder health status. It is conceivable that not only the quantity of cells but also their functionality should be taken into account.

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Leukocyte Populations and mRNA Expression of Inflammatory Factors in Quarter Milk Fractions at Different Somatic Cell Score Levels in Dairy Cows

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ABSTRACT

The effect of somatic cell count (SCC) and milk fraction on milk composition, distribution of cell populations, and mRNA expression of various inflammatory parameters was studied. Therefore, quarter milk samples were defined as cisternal (C), first 400 g of alveolar (A1), and remaining alveolar milk (A2) during the course of milking. Quarters were assigned to 4 groups according to their total SCC: 1) $<12 \times 10^3$/mL, 2) 12 to 100×10^3/mL, 3) 100 to 350×10^3/mL, and 4) $>350 \times 10^3$/mL. Milk constituents of interest were SCC, fat, protein, lactose sodium, and chloride ions as well as electrical conductivity. Cell populations were classified into lymphocytes, macrophages, and neutrophils (PMN). The mRNA expression of the inflammatory factors tumor necrosis factor-α, interleukin-1β, cyclooxygenase-2, lactoferrin, and lysozyme was measured via real-time, quantitative reverse transcription PCR. Somatic cell count decreased from highest levels in C to lowest levels in A1 and increased thereafter to A2 in all groups. Fat content increased from C to A2 and with increasing SCC level. Lactose decreased with increasing SCC level but remained unchanged during milking. Concentrations of sodium and chloride, and electrical conductivity increased with increasing SCC but were higher in C than in A1 and A2. Protein was not affected by milk fraction or SCC level. The distribution of leukocytes was dramatically influenced by milk fraction and SCC. Lymphocytes were the dominating cell population in group 1, but the proportion of lymphocytes was low in groups 2, 3, and 4. Macrophage proportion was highest in group 2 and decreased in groups 3 and 4, whereas that of PMN increased from group 2 to 4. The content of macrophages decreased during milking in all SCC groups whereas that of PMN increased. The proportion of lymphocytes was not affected by milk fraction. The mRNA expression of all inflammatory factors showed an increase with increasing SCC but minor changes occurred during milking. In conclusion, milk fraction and SCC level have a crucial influence on the distribution of leukocyte populations and several milk constituents. The surprisingly high content of lymphocytes and concomitantly low mRNA expression of inflammatory factors in quarters with SCC $<12 \times 10^3$/mL indicates a different and possibly reduced readiness of the immune system to respond to invading pathogens.

Key words: milk fraction, leukocyte, mRNA expression

INTRODUCTION

Microbiological and SCC testing in milk are the most sensitive methods for measurement of infection of bovine mammary glands. Somatic cell count presents a fast and reliable analytical tool. It is related to the immunological status of the udder and increases in response to an inflammatory stimulus like bacterial infection (O'Brien et al., 1999; Leitner et al., 2000). Therefore, SCC is a widely used indicator for udder health and milk quality.

Somatic cell count varies somewhat according to milking frequency, lactational stage, age, and nutrition (Dohoo et al., 1984; Kelly et al., 2000). Somatic cell count measurement includes all types of cells in milk; the number and the distribution of lymphocytes, macrophages, PMNL, and epithelial cells depend on the immunological status of the mammary gland (Kehrli and Schuster, 1994; Kelly et al., 2000). In milk from healthy udders, macrophages represent the major cell fraction (Burvenich et al., 1994; Paape et al., 2002; Sarikaya et al., 2004), and release chemoattractants such as tumor necrosis factor alpha (**TNF-α**) and interleukin-1β (**IL-1β**) after contact with a pathogen (Hoeben et al., 2000; Wittmann et al., 2002). This stimulus causes a rapid immigration of PMNL into the milk (Jensen and Eberhardt, 1981; Sordillo and Streicher, 2002). Therefore, in mastitic milk, PMNL become the predominant cell fraction (Kehrli and Schuster, 1994; Paape et al., 2002).

In addition to the changes of SCC and cell populations based on immunological status, there are also alterations in milk constituents during the course of milking;

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that is, in different milk fractions (Ontsouka et al., 2003; Bruckmaier et al., 2004a). Because milk ejection is a continuous process during the course of milking (Bruckmaier et al., 1994), it can be hypothesized that there are also changes in cell populations in different milk fractions. Immunomediators support the defense mechanism of the mammary gland by exerting potent chemotactic effects on leukocytes; they also enhance phagocytotic activity (Persson et al., 1993; Sanchez et al., 1994). The mediators of most importance are cytokines such as TNF-α and IL-1β as well as lipid derivatives such as leukotrienes and prostaglandins. Bacteriostatic proteins such as lactoferrin (**Lf**) and lysozyme (**Lz**) have been shown to increase during mastitis (Hagiwara et al., 2003; Schmitz et al., 2004).

The present study aimed to assess the hypothesis that in different udder compartments such as the teat/cisternal area and alveolar tissue, different cell types have specific importance and are therefore present in variable distribution. Quarter milk samples were classified according to their SCC levels to investigate the influence of the immunological status on cell distribution and mRNA abundance. To achieve a detailed overview, the mRNA expression levels of various inflammatory factors in milk cells and concentration of milk constituents were studied concomitantly. Because the immunological status is considered crucial for the current SCC level, grouping of quarters was performed based on SCC without regard to the bacteriological status.

MATERIALS AND METHODS

Animals and Husbandry

In experiment 1, 29 dairy cows (15 Simmental, 7 Brown Swiss, and 7 Holstein-Friesian) in their first to seventh lactations were used. Seven animals were in an early stage of lactation (13 to 94 d), 12 were in mid lactation (107 to 198 d), and 10 were in a late stage of lactation (216 to 377 d).

Experiment 2 included 33 animals (8 Simmental, 20 Brown Swiss, and 5 Holstein-Friesian) in their first to fifth lactations. Nine cows were in an early stage of lactation (10 to 96 d), 7 were in mid lactation (117 to 204 d), and 17 were in a late lactational stage (235 to 533 d).

The average milk production on the day of investigation was 23 kg/cow. Cows were kept in a loose-housing barn, and milked twice daily at 0500 and 1600 h.

Experimental Design

Both studies included fractionized milking during routine milking times with a special quarter milking equipment. This device allowed an online separation of the whole quarter milk sample into 3 fractions: the cisternal milk (**C**), first 400 g of alveolar milk (**A1**), and the remaining alveolar milk (**A2**). To obtain a C fraction free of alveolar milk, milking was performed without any udder preparation to avoid milk ejection (Bruckmaier and Blum, 1996). According to Bruckmaier and Hilger (2001), no milk ejection is expected in the first 50 s after the start of milking. Therefore, all milk removed during the first 50 s was classified as C. All collected samples were immediately stored at 4°C and transferred to further processing.

SCC and Milk Composition

Somatic cell counts of all milk samples in experiments 1 and 2 were measured with a DeLaval cell counter (Tumba, Sweden). The DeLaval cell counter was particularly suitable because it requires a minimum sample size of only 60 μL (Sarikaya and Bruckmaier, 2005). Milk samples were assigned to 1 of 4 groups according to their total quarter SCC: 1) $<12 \times 10^3$/mL, 2) 12 to 100 $\times 10^3$/mL, 3) 100 to 350 $\times 10^3$/mL, and 4) $>350 \times 10^3$/mL.

The milk samples of experiment 1 were analyzed for fat, protein, and lactose in every fraction by an accredited milk laboratory (Milchprüfring Bayern e.V., Wolnzach, Germany) using the MilkoScan 4500 analyzer (Foss, Hillerød, Denmark). Potentiometric measurement using ion-selective electrodes (models 9811 and 9617BN, pH/Ise Meter 720 Aplus, Orion Research, Boston, MA) was performed directly in milk for sodium and chloride. Electrical conductivity (**EC**) was measured in milk using the LDM 130 electrode from WTW (Weilheim, Germany).

Milk Cell Isolation

Within 30 min after sampling, the somatic cells of each fraction were isolated for further investigations. Isolation was performed by centrifugation as described by Sarikaya et al. (2004). The cells were washed, resuspended in PBS (pH 7.5), and kept on ice during all procedures.

Cell Populations

Differential cell counting in experiment 1 was performed by using light microscopy and a modified Pappenheim staining (Sarikaya et al., 2004); 200 cells were counted and the populations were calculated as percentages of the total. Leukocytes were defined as lymphocytes, macrophages, or PMNL.

RNA Extraction

Total RNA of milk cells was isolated using TriPure (Roche Diagnostics, Mannheim, Germany) according to

Table 1. Sequence of PCR primers, accession number, and PCR product length

Primer		Sequence (5′→3′)	Accession no.	Length (bp)
Ubiquitin	Forward	AGA TCC AGG ATA AGG AAG GCA T	Z18245	198
	Reverse	GCT CCA CCT CCA GGG TGA T		
Glyceraldehyde-3-phosphate dehydrogenase	Forward	GTC TTC ACT ACC ATG GAG AAG	U85042	197
	Reverse	TCA TGG ATG ACC TTG GCC AG		
Tumor necrosis factor-α	Forward	TAA CAA GCC GGT AGC CCA CG	AF011926	256
	Reverse	GCA AGG GCT CTT GAT GGC AGA		
IL-1β	Forward	TTC TCT CCA GCC AAC CTT CAT T	M37211	198
	Reverse	ATC TGC AGC TGG ATG TTT CCA T		
Cycloxygenase-2	Forward	TCT TCC TCC TGT GCC TGA T	AF031698	358
	Reverse	CTG AGT ATC TTT GAC TGT GG		
Lactoferrin	Forward	GGC CTT TGC CTT GGA ATG TAT C	AB046664	338
	Reverse	ATT TAG CCA CAG CTC CCT GGA G		
Lysozyme	Forward	GAG ACC AAA GCA CTG ATT ATG GGA	U25810	195
	Reverse	TCC ATG CCA CCC ATG CTC TAA		

the manufacturers recommendations. To quantify the amount of total RNA, optical density was measured at 3 different dilutions at 260 nm and corrected by the 320 nm background absorption. Integrity of RNA was verified by the OD_{260nm}/OD_{280nm} absorption ratio being >1.7.

Oligonucleotide Primers

Primers for the housekeeping and target genes were synthesized commercially (MWG Biotech, Ebersberg, Germany) using previously published bovine-specific primer sequences (Wittmann et al., 2002; Schmitz et al., 2004). Primer information is listed in Table 1.

Quantification by Real-Time, Reverse Transcription-PCR

Quantitative analysis of PCR products was carried out on the RotorGene 3000 (Corbett Research, Sydney, Australia) via one-step quantitative reverse transcription-PCR. In brief, 3.8 μL of extracted mRNA solutions with a concentration of 15 ng/μL were used. Additional reaction components were 6.2 μL of Master Mix (SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit, Invitrogen, Kaulsruhe, Germany) including 5 μL of 2× SYBR Green Reaction Mix, 0.2 μL of SYBR Green One-Step Enzyme Mix, 0.5 μL (10 p*M*) of forward primer, and 0.5 μL (10 p*M*) of reverse primer.

Crossing point values were achieved by RotorGene software version 5.0. A normalisation of the target genes with an endogenous standard was performed. Therefore, the expression levels of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase and ubiquitin were measured. The relative mRNA levels were calculated by normalization of the crossing point of the target gene to the mean crossing point of the 2 housekeeping genes.

Statistical Analyses

Data are presented as means ± standard error of means (SEM). Differences between itemized fractions and SCC groups were tested for significance ($P < 0.05$) by ANOVA using the MIXED models procedure of SAS (SAS Institute, Inc., Cary, NC). The MIXED model included the animal and the milk fraction as class variables. The animal was the repeated term during the course of milking. The compound-symmetry matrix structure was used. Statistical significance between fractions was tested by least significance difference test.

RESULTS

Milk Cells

All investigated milk samples were assigned to 1 of 4 groups according to their total quarter milk SCC. Mean SCC in groups 1, 2, 3, and 4 were 9 ± 1, 39 ± 10, 215 ± 27, and 2,460 ± 1,172 × 10^3/mL, respectively, in experiment 1, and 9 ± 2, 48 ± 6, 186 ± 26, and 1,046 ± 442 × 10^3/mL, respectively, in experiment 2. Somatic cell count decreased in all groups from C to A1 and increased in A2. Somatic cell counts of all quarters were 1.2 × 10^6 ± 5.4 × 10^5 cells/mL in C, 4.9 × 10^5 ± 2.6 × 10^5 cells/mL in A1, and 9.3 × 10^5 ± 5.2 × 10^5 cells/mL in A2. The SCC of all fractions in groups 1, 2, 3, and 4 are shown in Table 2.

Milk Constituents

As shown in Table 2, the concentration of fat increased ($P < 0.05$) from C to A1 and A2 during milking for all quarter milk samples. This difference could be seen in SCC groups 1 to 4 from C to A1, and from C to A2. An increase ($P < 0.05$) from A1 to A2 was only observed in groups 1, 2, and 4. The concentration of protein showed

Table 2. Composition of quarter milk samples (QMS) within different milk fractions and SCC groups

Composition	Fraction[1]	SCC group[2] 1 (n = 6)	2 (n = 8)	3 (n = 7)	4 (n = 8)	All QMS (mean)[3]
SCC, log10/mL	C	3.84 ± 0.13	4.69 ± 0.15	5.32 ± 0.22	6.15 ± 0.24	5.16 ± 0.18
	A1	3.43 ± 0.04	4.24 ± 0.12	4.91 ± 0.13	5.77 ± 0.24	4.75 ± 0.17
	A2	4.01 ± 0.08	4.51 ± 0.10	5.34 ± 0.03	6.15 ± 0.18	5.14 ± 0.16
Fat,%	C	1.3 ± 0.2^{cB}	1.4 ± 0.3^{cB}	2.2 ± 0.4^{bAB}	2.4 ± 0.4^{cA}	1.9 ± 02^{c}
	A1	2.2 ± 0.3^{bB}	2.1 ± 0.3^{bB}	2.8 ± 0.3^{bAB}	3.0 ± 0.4^{bA}	2.6 ± 0.2^{b}
	A2	4.7 ± 0.6^{a}	4.4 ± 0.3^{a}	4.7 ± 0.1^{a}	4.7 ± 0.3^{a}	4.6 ± 0.1^{a}
Protein, %	C	3.2 ± 0.1^{B}	3.4 ± 0.1^{AB}	3.4 ± 0.1^{AB}	3.7 ± 0.1^{A}	3.5 ± 0.1
	A1	3.2 ± 0.1^{B}	3.5 ± 0.1^{B}	3.4 ± 0.1^{B}	3.8 ± 0.1^{A}	3.5 ± 0.1
	A2	3.1 ± 0.1^{B}	3.4 ± 0.1^{B}	3.4 ± 0.1^{B}	3.8 ± 0.1^{A}	3.5 ± 0.1
Lactose, %	C	5.0 ± 0.1^{A}	4.8 ± 0.2^{AB}	4.6 ± 0.1^{BC}	4.4 ± 0.2^{C}	4.7 ± 0.1^{b}
	A1	5.0 ± 0.1^{A}	5.0 ± 0.1^{A}	4.9 ± 0.1^{AB}	4.7 ± 0.1 ^{B}	4.9 ± 0.1^{a}
	A2	5.0 ± 0.1^{A}	4.9 ± 0.1^{A}	4.8 ± 0.1^{AB}	4.5 ± 0.1^{B}	4.8 ± 0.1^{ab}
Sodium, mmol/L	C	9.6 ± 3.1^{C}	21.8 ± 6.7^{aB}	18.3 ± 2.1^{aBC}	37.2 ± 7.0^{aA}	23.7 ± 3.3^{a}
	A1	7.6 ± 1.9	13.7 ± 4.2^{b}	12.2 ± 1.7^{b}	20.2 ± 3.5^{b}	14.3 ± 1.8^{b}
	A2	7.0 ± 2.2^{B}	11.5 ± 2.2^{bB}	11.8 ± 1.3^{bB}	23.5 ± 5.1^{bA}	14.5 ± 2.0^{b}
Chloride, mmol/L	C	37.4 ± 4.8^{BC}	56.5 ± 10.5^{aB}	41.3 ± 3.2^{aB}	85.8 ± 25.2^{aA}	58.4 ± 8.5^{a}
	A1	32.9 ± 3.7	37.5 ± 4.0^{b}	30.2 ± 1.8^{b}	47.9 ± 8.5^{b}	38.0 ± 3.0^{b}
	A2	34.3 ± 4.4^{B}	38.6 ± 3.7^{bAB}	32.4 ± 2.2^{abAB}	60.2 ± 12.3^{ba}	42.8 ± 4.3^{b}
Electrical conductivity, mS/cm	C	41.6 ± 0.4^{aB}	46.9 ± 2.2^{aB}	46.9 ± 0.9^{aB}	53.7 ± 4.1^{aA}	48.1 ± 1.5^{a}
	A1	40.4 ± 0.5^{b}	41.9 ± 0.8^{b}	42.7 ± 1.7^{b}	43.8 ± 0.9^{b}	42.5 ± 0.6^{b}
	A2	35.7 ± 3.3^{bC}	40.4 ± 0.6^{bBc}	41.7 ± 1.0^{bAB}	45.2 ± 1.6^{bA}	41.5 ± 0.9^{b}

^{a,b}Means without common index within SCC group and composition parameter differ significantly ($P < 0.05$) between milk fractions.
$^{A–C}$Means without common index within milk fraction and composition parameter differ significantly ($P < 0.05$) between SCC groups.
[1]Fractions: C = cisternal; A1 = first 400 g of alveolar milk; A2 = remainder of alveolar milk.
[2]SCC groups: 1 = <12 × 10^3/mL; 2 = 12 to 100 × 10^3/mL; 3 = 100 to 350 × 10^3/mL; and 4 = >350 × 10^3/mL.
[3]Mean of all QMS.

minor changes during milking in all SCC groups. Within fraction in different SCC groups, the protein content was always lowest in group 1. It increased ($P < 0.05$) for C between groups 1 and 4, and for A1 and A2 among groups 1, 2, 3, and 4. Lactose levels increased ($P < 0.05$) from C to A1 and decreased numerically from A1 to A2. Group 1 maintained constant lactose contents throughout milking and showed the highest concentrations compared with SCC groups 2, 3, and 4.

Milk Electrolytes and EC

As demonstrated in Table 2, sodium concentrations decreased ($P < 0.05$) from C to fractions A1 and A2. The changes ($P < 0.05$) between the SCC groups showed lower sodium levels in C and A fractions in groups 1, 2, and 3 compared with group 4. The content of chloride presented the same trends and significances as sodium during milking for all groups. Group 4 had ($P < 0.05$) higher levels in fractions C and A2 compared with groups 1, 2, and 3. Electrical conductivity decreased ($P < 0.05$) from the cisternal to alveolar fractions. Likewise, SCC group 4 showed greater EC ($P < 0.05$) compared with groups 1, 2, and 3.

Cell Populations

The lymphocytes comprised the predominant cell population in SCC group 1, whereas the content of macrophages and PMNL was low. In contrast, the proportion of lymphocytes was low ($P < 0.05$) in all fractions of groups 2, 3, and 4. Macrophages were highest in group 2, and decreased ($P < 0.05$) in groups 1, 3, and 4. In contrast, the ratio of PMNL was low in group 2 and was elevated ($P < 0.05$) with increasing SCC in groups 3 and 4.

The course of milking showed minor changes in lymphocyte concentrations in all SCC groups. The content of macrophages decreased ($P < 0.05$) during the course of milking in all groups, showing highest levels in the C fraction of group 2. An increase ($P < 0.05$) in the proportion of PMNL during the course of milking was observed in all groups. The cell distributions of the 4 groups are presented in Figure 1.

mRNA Expression of Inflammatory Factors

The cytokines TNF-α and IL-1β showed higher mRNA expression levels ($P < 0.05$) with increasing SCC, except in groups 2 and 3, which showed similar values (Figure 2). Similar to TNF-α, the expression of cyclooxygenase-2 (**COX-2**) mRNA increased ($P < 0.05$) to its highest levels in SCC group 4; expression of Lf also increased from group 1 to 4. Group 1 could not be analyzed for Lz expression because of technical reasons. Therefore, only SCC groups 2, 3, and 4 were investigated for this gene. The effect of SCC on Lz gene expression was positively

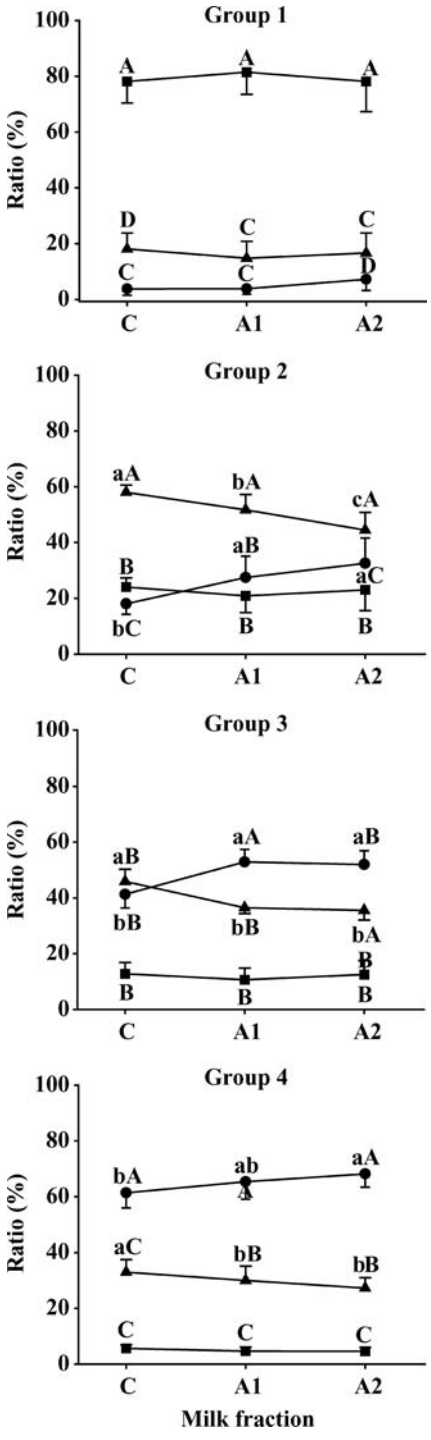

Figure 1. Distribution of cell populations (● = PMNL, ■ = lymphocytes, ▲ = macrophages) subject to milk fractions (C = cisternal; A1 = first 400 g of alveolar milk; A2 = remaining alveolar milk) and SCC groups 1 to 4 (1 = <12 × 10^3/mL; 2 = 12 to 100 × 10^3/mL; 3 = 100 to 350 × 10^3/mL; and 4 = >350 × 10^3/mL). [a–c]Means without common index within SCC group and cell population differ significantly ($P < 0.05$) between milk fractions; [A–C]Means without common index within milk fraction and cell population differ significantly ($P < 0.05$) between SCC groups.

correlated; that is, higher SCC led to higher mRNA expression ($P < 0.05$).

There were no significant differences in expression of cytokine (TNF-α and IL-1β) mRNA during the course of milking among the 4 SCC groups (Figure 2). Only SCC group 3 differed throughout milking by an increased expression level ($P < 0.05$) in fraction A2. Expression of COX-2, Lf, and Lz showed minor changes during milking. The mRNA expression levels for COX-2, Lf, and Lz are shown in Table 3.

DISCUSSION

Our results demonstrate changes in milk composition, cell population, and mRNA expression of different inflammatory parameters based on 2 important factors—SCC and milk fraction. The collected milk fractions of a single quarter were defined as cisternal, first 400 g of alveolar milk, and remaining alveolar milk.

All investigated milk fractions of each quarter were assigned to groups (1 to 4) according to their total quarter SCC. In the present study, SCC in C and A2 fractions were significantly higher than SCC in the A1 fraction. This result agrees with previous findings (Woolford et al., 1998; Ontsouka et al., 2003). It shows the importance of defining the milk fraction used if SCC is used for udder health monitoring and milk quality.

The content of fat increased significantly during the course of milking as well as with elevated SCC. The change throughout the fractions could be explained by the lower density of the fat globules and the ascending force in the udder. Furthermore, a possible adhesion of the globule membranes to the alveolar lumina could support this phenomenon. Therefore, fractions with the highest fat content are removed at the end of milking. The increasing fat content in correlation with the increasing SCC was remarkable in fractions C and A1 of all 4 SCC groups. This elevated fat content could be a consequence of reduced lactose synthesis. Lactose concentrations show the opposite tendency to fat with increasing SCC (Bruckmaier et al., 2004a). Because lactose defines the milk volume, the slight fat concentration change could be a secondary effect. The course of milking showed minor changes to lactose concentrations in each SCC group.

The concentration of sodium and chloride must be considered in context with lactose, because the combination of these parameters are responsible for the osmolar equilibrium. The contents of sodium and chloride showed minor changes between the SCC groups 1, 2 and 3, and a significant increase in group 4, which presents a distinct increased level of SCC. This might be explained by the circumstance that only during high leukocyte diapedesis are the tight junctions leaky enough (Nguyen and Nev-

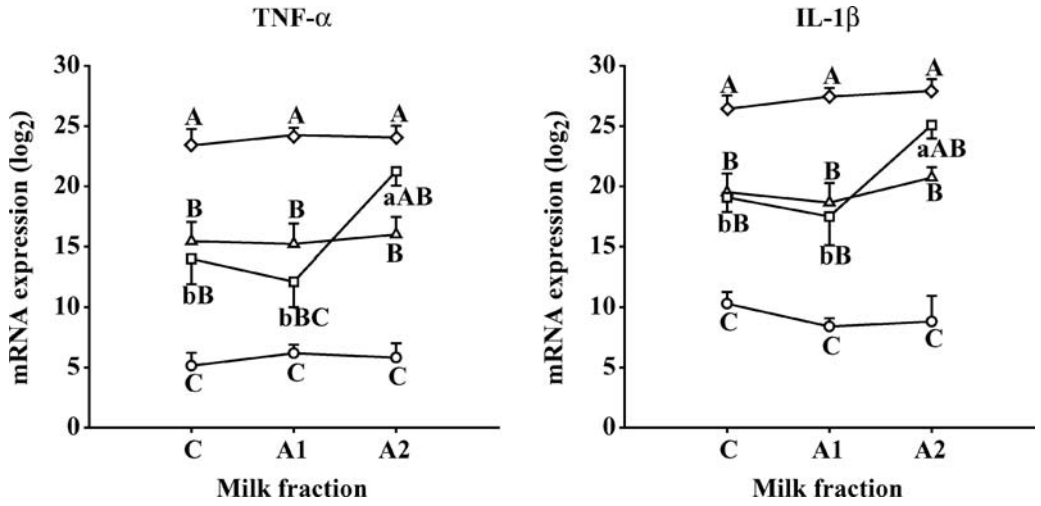

Figure 2. Relative mRNA expression levels of tumor necrosis factor-α and IL-1β in milk fractions (C = cisternal; A1 = first 400 g of alveolar milk; A2 = remaining alveolar milk) in the different SCC groups (○ = group 1: <12 × 10^3/mL; Δ = group 2: 12 to 100 × 10^3/mL; □ = group 3: 100 to 350 × 10^3/mL; ◇ = group 4: >350 × 10^3/mL). [a,b]Means without common index within group and target gene differ significantly ($P < 0.05$) between milk fractions; [A–C]Means without common index within milk fraction and target gene differ significantly ($P < 0.05$) between groups.

ille, 1998; Bruckmaier et al., 2004b) to permit elevated movement of ions from blood into milk. Within a fraction, only the electrolytes in cisternal milk showed a positive correlation with SCC. After milk ejection, the sensitivity of ion measurements was reduced due to the mixture of alveolar and cisternal milk (Bruckmaier et al., 2004b). This resulted in a significant decrease of sodium and chloride during the course of milking in all 4 SCC groups. Electrical conductivity is determined by the ions dissolved in milk (mainly sodium and chloride). Therefore, EC follows the same trends as the electrolytes. The decline of EC in successive milk fractions can be affected by the increase of fat at the end of milking because fat modulates the EC measurement (Woolford et al., 1998).

The distribution of cell populations showed a dependency on the SCC as well as on the milk fraction. Macrophages were the predominant cell type in group 2 and decreased with elevating SCC. It is generally assumed that macrophages present the major cell fraction in healthy quarters (Lee et al., 1980; Paape et al., 2002). The content of macrophages was always highest in the C fraction, and thus decreased during milking. Because this fraction is located at the main point of entry of pathogens (the teat; Sordillo and Streicher, 2002), macrophages can react first after contact with pathogens. They initiate the inflammatory response necessary to eliminate invading pathogens by releasing chemoattractants. These chemoattractants cause a rapid influx of PMNL into the milk. Therefore, the PMNL became the major cell fraction with elevated SCC. This is the most effective mechanism against invading pathogens (Burvenich et al., 2003; Paape et al., 2003) because these 2 cell populations represent the phagocytic cells of the mammary gland. The lymphocytes comprised only a small percentage of the cells in SCC groups 2, 3, and 4, and decreased with elevated SCC. Most surprisingly, lymphocytes were the predominant cell type in SCC group 1 comprising up to 80% of the total. The ratio of lymphocytes did not change during milking. Lymphocytes present the specific immunity of the mammary gland (Taylor et al., 1997). It is suggested that this cell fraction does not play a major role in infections of the mammary gland or that they operate in the mammary tissue rather than in milk (Riollet et al., 2001). The defense mechanism is mainly related to the innate immunity mediated by macrophages and PMNL. Furthermore, very low SCC could be associated with a higher risk of a severe infection with pathogens (Sol et al., 2000; Suriyasathaporn et al., 2000). The surprisingly low content of macrophages and PMNL and the high content of lymphocytes in quarters with SCC <12 × 10^3/mL might cause a reduced immune response to invading pathogens. This is supported by previous investigations indicating that a very low SCC increased the risk of establishing infection with a major udder pathogen (Schukken et al., 1989; Schukken et al., 1999).

During mammary infection, nonspecific responses are the predominant defenses. For our investigations, 5 soluble immunologically important factors that are known to be involved in the natural defense mechanisms of the mammary gland against invading pathogens were selected (Schmitz et al., 2004; Prgomet et al., 2005; Ka-

Table 3. Relative mRNA expression of cyclooxygenase-2, lactoferrin, and lysozyme within different milk fractions and SCC groups

mRNA expression (log_2)	Fraction[1]	SCC group[2] 1 (n = 4)	2 (n = 15)	3 (n = 6)	4 (n = 8)
Cycloxygenase-2	C	11.4± 5.2^{B}	12.8 ± 1.5^{B}	11.4 ± 2.3^{bB}	20.7 ± 1.3^{A}
	A1	6.5 ± 3.6^{C}	12.5 ± 1.6^{B}	8.8 ± 2.5^{bBC}	22.5 ± 0.7^{A}
	A2	5.5 ± 2.0^{C}	14.4 ± 1.3^{B}	21.7 ± 1.6^{aA}	21.9 ± 1.6^{A}
Lactoferrin	C	0.8 ± 0.7^{C}	16.3 ± 1.6^{AB}	13.2 ± 1.6^{abB}	20.4 ± 0.9^{A}
	A1	8.0 ± 4.4^{C}	16.4 ± 1.8^{B}	8.9 ± 3.2^{bC}	22.5 ± 0.5^{A}
	A2	7.0 ± 5.8^{C}	16.7 ± 1.2^{B}	19.2 ± 2.1^{aAB}	21.7 ± 1.5^{A}
Lysozyme	C	ND[3]	18.0 ± 1.3^{B}	18.5 ± 2.7^{aB}	24.4 ± 1.0^{A}
	A1	ND	16.7 ± 1.6^{B}	12.3 ± 3.1^{bB}	24.2 ± 0.6^{A}
	A2	ND	18.4 ± 1.0^{B}	23.1 ± 0.9^{aAB}	24.3 ± 0.9^{A}

[a,b]Means without common index within SCC group and target gene differ significantly ($P < 0.05$) between milk fractions.

[A–C]Means without common index within milk fraction and target gene differ significantly ($P < 0.05$) between SCC groups.

[1]Fractions: C = cisternal; A1 = first 400 g of alveolar milk; A2 = remainder of alveolar milk.

[2]SCC groups: 1 = $<12 \times 10^3$/mL; 2 = 12 to 100×10^3/mL; 3 = 100 to 350×10^3/mL; and 4 = $>350 \times 10^3$/mL.

[3]ND = Not done (due to technical reasons).

wai et al., 1999). These factors were investigated during milking and dependent on SCC. Tumor necrosis factor-α and IL-1β are important proinflammatory cytokines, and therefore, play a major role in the defense against mastitis (Blum et al., 2000; Riollet et al., 2000). The impact of TNF-α as one of the cytokines mediating the acute phase response was demonstrated because an increase of SCC occurred concomitantly with the rise of TNF-α mRNA expression (Figure 2); IL-1β showed the same pattern. It is known that these 2 cytokines stimulate IL-8 secretion (Persson et al., 1993), which is an important mediator of PMNL migration. The influx of PMNL into the mammary gland affects the progress of the infection. The course of milking affected mRNA expression marginally.

Compared with TNF-α and IL-1β, an increase of Lf mRNA expression was detected with increasing SCC levels. Lactoferrin is known to increase in bovine milk during clinical mastitis (Kawai et al., 1999), and is mainly produced within the immune cells by stimulated PMNL (Prgomet et al., 2005). A significant increase of Lz mRNA expression was also obvious, with peak values in SCC group 4. These numerically increasing expression levels of Lz with SCC level indicate a possible relevance of Lz in the mammary gland immune defense due to its bacteriostatic effects on udder pathogens (Carlsson et al., 1989). Increased synthesis of prostaglandins and leukotrienes with increasing SCC was shown based on the increase of COX-2 mRNA expression from SCC groups 1 to 4.

The results indicate that most of the factors investigated show significantly higher expression levels with increasing SCC. The higher levels of mRNA expressions occurred without any experimental induction of mastitis; for example, with LPS injection (Schmitz et al., 2004). This means that natural stimuli like pathogens cause the effect of an upregulation of all inflammatory factors. Changes in mRNA expression of the housekeeping genes ubiquitin and glyceraldehyde-3-phosphate dehydrogenase did not occur. Thus, the changes of mRNA represent specific responses of the mammary gland to certain stimuli that correlate with increasing SCC levels.

CONCLUSIONS

The milk fraction during the course of milking and SCC level have a crucial influence on the distribution of leukocyte populations as well as on the composition of milk. The surprisingly low content of macrophages and PMNL and concomitantly low mRNA expression of inflammatory factors in quarters with SCC $<12 \times 10^3$/mL indicates a different and possibly reduced readiness of the immune system to respond to invading pathogens. In contrast, the increased percentage of macrophages and PMNL in quarters with higher SCC is also reflected by high cytokine mRNA expression. The importance of somatic cells for mammary gland defense is well known and does not need to be emphasized. The results of this work suggest that not only is the total number of immune cells important in mounting an immune response-the cell type, subtype, their products, and activities may affect the progress of the infection.

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Importance of the Sampled Milk Fraction for the Prediction of Total Quarter Somatic Cell Count

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ABSTRACT

This study investigated the changes in somatic cell counts (SCC) in different fractions of milk, with special emphasis on the foremilk and cisternal milk fractions. Therefore, in Experiment 1, quarter milk samples were defined as strict foremilk (F), cisternal milk (C), first 400 g of alveolar milk (A1), and the remaining alveolar milk (A2). Experiment 2 included 6 foremilk fractions (F1 to F6), consisting of one hand-stripped milk jet each, and the remaining cisternal milk plus the entire alveolar milk (RM). In Experiment 1, changes during milking indicated the importance of the sampled milk fraction for measuring SCC because the decrease in the first 3 fractions (F, C, and A1) was enormous in milk with high total quarter SCC. The decline in SCC from F to C was 50% and was 80% from C to A1. Total quarter SCC presented a value of approximately 20% of SCC in F or 35% of SCC in C. Changes in milk with low or very low SCC were marginal during milking. Fractions F and C showed significant differences in SCC among different total SCC concentrations. These differences disappeared with the alveolar fractions A1 and A2. In Experiment 2, a more detailed investigation of foremilk fractions supported the findings of Experiment 1. A significant decline in the foremilk fractions even of F1 to F6 was observed in high-SCC milk at concentrations $>350 \times 10^3$ cells/mL. Although one of these foremilk fractions presented only 0.1 to 0.2% of the total milk, the SCC was 2- to 3-fold greater than the total quarter milk SCC. Because the trait of interest (SCC) was measured directly by using the DeLaval cell counter (DCC), the quality of measurement was tested. Statistically interesting factors (repeatability, recovery rate, and potential matrix effects of milk) proved that the DCC is a useful tool for identifying the SCC of milk samples, and thus of grading udder health status. Generally, the DCC provides reliable results, but one must consider that SCC even in strict foremilk can differ dramatically from SCC in the total cisternal fraction, and thus also from SCC in the alveolar fraction.

Key words: foremilk, milk fraction, somatic cell count

INTRODUCTION

The SCC in milk is an indicator of the activity of the cellular immune defense of the udder (Sordillo et al., 1997; O'Brien et al., 1999; Leitner et al., 2000). These somatic cells, mainly leukocytes, are part of the natural defense mechanism, and SCC is often used to distinguish between infected and uninfected quarters. Milk from uninfected quarters generally contains a physiological basal cell count of $<100 \times 10^3$ somatic cells/mL (Hillerton, 1999). A striking elevation of SCC greater than this concentration is abnormal, and high individual cow SCC are known to be positively correlated with mastitis (Kehrli and Schuster, 1994; Kelly et al., 2000). A rapid increase in SCC reflects activation of the mammary immune response in the early acute phases of infection.

Mostly, foremilk or composite quarter milk samples are used for SCC measurement, and results have shown the high impact of SCC on the interpretation of udder health status (Woolford et al., 1998; Schukken et al., 2003). In addition, herds and cows with very low SCC have been examined, and there is evidence that risk of severe mastitis is increased in those with low SCC in comparison with those with greater SCC before infection (Sol et al., 2000; Suriyasathaporn et al., 2000; Sarikaya et al., 2006).

It is generally accepted that cells are important in the defense of the udder and the SCC is used to monitor udder health status. Because SCC differs in foremilk and composite milk, we hypothesized that SCC would also differ between fractions of the foremilk. A new cell-counting technology allows sample sizes of <100 μL. Therefore, it is important to know the influence of a specific milk fraction on the SCC results. In this context, the study aimed to investigate the importance of the sampled milk fraction to predict total quarter milk SCC and udder health status.

MATERIALS AND METHODS

Cows and Husbandry

Two experiments were carried out. In Experiment 1, 36 Brown Swiss cows in their first to fifth lactation were

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used. Ten animals were in early stages of lactation (12 to 98 d), 15 were in midlactation (112 to 206 d), and 11 in a late stage of lactation (224 to 421 d). Experiment 2 included 25 Brown Swiss cows in their first to fourth lactation. Ten cows were in early (10 to 87 d), 8 in mid- (120 to 201 d), and 7 in late lactation (233 to 395 d).

Cows were fed a diet consisting of 22 kg of maize silage, 12 kg of grass silage, 2 to 3 kg of hay, and 6 to 8 kg of concentrates. Water was available ad libitum. Average milk production on the day of investigation was 23 ± 2 kg per cow. Cows were kept in a loose-housing barn and were milked twice daily at 5 a.m. and 4 p.m.

Experimental Design

Experiment 1. This study included fractionized milking during routine milking times with special quarter milking equipment (Sarikaya et al., 2005). This device allowed separation of single quarter milk samples into 4 fractions: strict foremilk (**F**), cisternal milk (**C**), first 400 g of alveolar milk (**A1**), and the remaining alveolar milk (**A2**). To obtain F and C free of alveolar milk, milking was performed without any udder preparation to avoid milk ejection and mixing of milk fractions (Bruckmaier and Blum, 1996). According to previous studies (Bruckmaier and Hilger, 2001), no milk ejection is expected during the first 50 s after initiating tactile teat stimulation. Therefore, the F and C samples were removed during this period. In this case, F represented the first 2 stripped jets of milk, and C was the remaining milk before milk ejection (i.e., within 50 s after initiating sampling). The A1 fraction consisted of the first 400 g of milk after milk ejection. This fraction contained mainly alveolar milk, but it is possible that a portion of cisternal milk also was included. The remaining alveolar milk was defined as A2.

Experiment 2. This study also included fractionized milking, but with a different setup of the fractions. Milking was performed without any udder preparation. The first 6 fractions (**F1** to **F6**) consisted of one hand-stripped milk jet each (i.e., each fraction represented the volume of the teat cistern capacity). By definition, F1 to F6 were all fractions of strictly foremilk. The remaining quarter milk was collected by normal machine milking and defined as remaining milk (**RM**). This fraction included the remaining cisternal and the entire alveolar milk.

In both experiments, each cow was sampled only once. Later, samples were collected from a single quarter of each cow. All samples were immediately stored at 4°C and transferred for further processing on the same day.

Measurement of SCC

As a basis for this study, the validity of measuring SCC by an automated cell counter [DeLaval cell counter (**DCC**); DeLaval, Tumba, Sweden] was tested. The validation considered the statistical traits of repeatability, recovery rate, and potential matrix effects in various milk samples.

Table 1. Grouping and number of investigated quarter milk samples according to their total quarter SCC in Experiments 1 and 2

SCC group	Experiment 1, SCC/mL	No.	Experiment 2, SCC/mL	No.
1	$<20 \times 10^3$	5	$<50 \times 10^3$	8
2	20 to 50×10^3	14	50 to 100×10^3	6
3	50 to 100×10^3	9	100 to 350×10^3	6
4	$>100 \times 10^3$	8	$>350 \times 10^3$	5

Mathematical Calculations

For calculation of repeatability, each sample was measured twice. Hereby, the REML method of estimating variance components was performed. The recovery rate, which emphasizes the reliability of the measurement, was determined by adding different volumes of cell suspensions with a defined number of cells to untreated low-SCC milk. To achieve a suspension with a defined number of cells, milk was centrifuged for 30 min at 1,500 $\times g$ at 4°C. The separated cell pellet was washed with PBS and resuspended in PBS. Afterward, the cell count of the suspension was determined by hemocytometric counting with a Neubauer chamber (Sarikaya et al., 2004). Hereafter, the cell count per milliliter of suspension was calculated. Five different amounts of cell suspensions (i.e., with 5 different cell numbers) were then added to the untreated low-SCC milk. Each step was measured in addition to the original milk sample. The recovery rate showed the ratio of the difference between the measured SCC (before and after adding the cells) and the expected value. Individual sample recovery also was calculated.

Potential matrix effects were investigated by performing 6 serial dilutions of milk samples with PBS buffer. Dilution factors ranged from 1 to 6. In each series of dilutions, Pearson's coefficient of correlation was calculated. The correlation coefficient is a quantity that gives the quality of a least-squares fitting to the original data.

SCC

The SCC of all milk samples in Experiments 1 and 2 was measured with a DCC (DeLaval). The DCC was particularly suitable for the low amounts of milk available, because it requires a minimum sample size of only 60 μL (Sarikaya et al., 2006). Milk samples were categorized into 1 of 4 groups based on the total quarter SCC (Table 1).

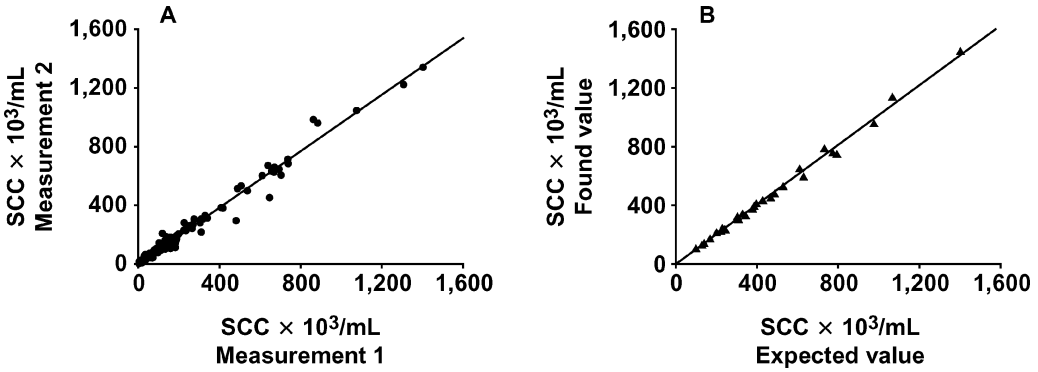

Figure 1. Regression curves of the repeatability (A) and recovery rate (B) resulting from the validation of SCC as measured by the DeLaval cell counter (DeLaval, Tumba, Sweden).

Statistical Analyses

Data are presented as means ± standard errors of the means (SEM). Differences between itemized fractions and SCC groups were tested for significance by ANOVA using the MIXED models procedure of SAS (SAS Inst., Inc., Cary, NC). The MIXED model included the cow and milk fraction as class variables. Cow was the repeated term during the course of milking. The matrix structure of compound symmetry was used. Statistical significance between fractions was tested by the least significance difference test.

RESULTS

Mathematical Calculations (Statistical Parameters of Validation)

A repeatability of 0.99 was achieved based on 180 samples that were measured in duplicate. The recovery, calculated as the ratio between the difference of the measured SCC before and after adding cells and the expected value, was 99.3 ± 0.8% based on 30 measurements (Figure 1). Individual sample recovery ranged from 93 to 106%. Potential matrix effects, investigated by performing serial dilutions of milk with buffer, produced a Pearson's correlation coefficient of >0.99 in each series of dilutions.

Milk Cells

All investigated milk samples were assigned to 1 of 4 categories according to their total quarter milk SCC. Mean SCC in groups 1, 2, 3, and 4 were 15 ± 1, 32 ± 2, 68 ± 4, and 198 ± 24 × 10^3/mL, respectively, in Experiment 1, and 26 ± 4, 79 ± 5, 181 ± 33, and 864 ± 135 × 10^3/mL, respectively, in Experiment 2.

Experiment 1. No significant changes in SCC were detected during the course of milking in groups 1, 2, and 3. The SCC in group 4, however, decreased ($P < 0.05$) from F to C and further to A1, and increased slightly again toward A2. The decrease in SCC of A2 was significant only in the F fraction. The SCC of fractions F and C was lowest in group 1 and increased ($P < 0.05$) with the SCC group number. This significance was not detected in fractions A1 and A2 (Figure 2). The mean volume of each fraction and its respective proportion of total milk harvested are summarized in Table 2.

Experiment 2. No significant changes in SCC were detected during the course of milking in groups 1, 2, and 3. A decrease ($P < 0.05$) in SCC during milking was observed in group 4. Here, the greatest SCC concentrations were achieved in F1. A slight decrease in SCC took place in fractions F2 and F3. Fractions F4 and F5

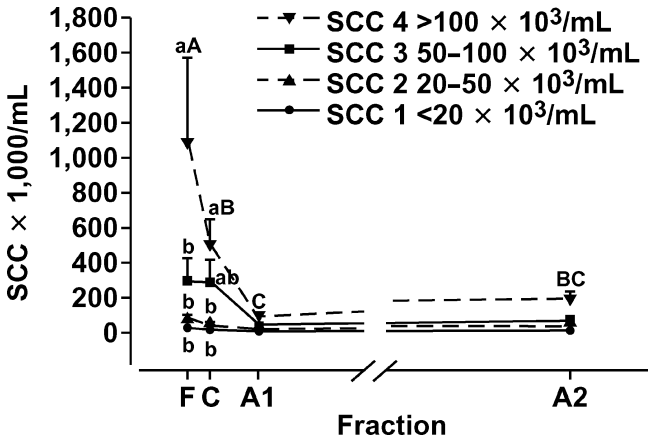

Figure 2. Changes in SCC in the defined milk fractions of strict foremilk (F), cisternal milk (C), first 400 g of alveolar milk (A1), and the remaining alveolar milk (A2) obtained via fractionized milking in Experiment 1. Fractions were additionally categorized into 4 different SCC groups according to their total quarter SCC. [a,b]Means with different superscript letters within a milk fraction differ ($P < 0.05$) between groups. [A–C]Means with different superscript letters within a group differ ($P < 0.05$) between milk fractions.

Table 2. Classification of collected milk fractions (Experiment 1)

Abbreviation	Milk fraction	Volume,[1] mL	Percentage of total milk[2]
F	Strict foremilk[3]	9.4 ± 0.8	0.3 ± 0.0
C	Cisternal milk[4]	140.5 ± 12.5	4.3 ± 0.4
A1	First 400 g of alveolar milk	396 ± 8	13.6 ± 1.1
A2	Remaining alveolar milk	2,820 ± 184	81.8 ± 1.2

[1]Fraction mean volume ± SEM.

[2]Proportion of total milk harvested.

[3]First 2 stripped jets of milk without any udder preparation.

[4]Remaining milk before milk ejection.

contained fewer ($P < 0.05$) SCC than F1. This decrease ($P < 0.05$) continued in F6 and RM. The SCC of all 6 foremilk fractions (F1 to F6) of group 4 were increased ($P < 0.05$) compared with the SCC of their identical fractions in groups 1, 2, and 3. The RM showed a numerical increase from group 1 to 4 (Figure 3). The mean volume of each fraction and its representative proportion of total milk harvested are summarized in Table 3.

DISCUSSION

As the trait of interest, SCC was measured directly by use of the DCC. The quality of measurement was tested. Statistically interesting factors (repeatability, recovery rate, and potential matrix effects) proved that the DCC is a useful tool for identifying the SCC of milk samples and thus for grading the health status of the udder.

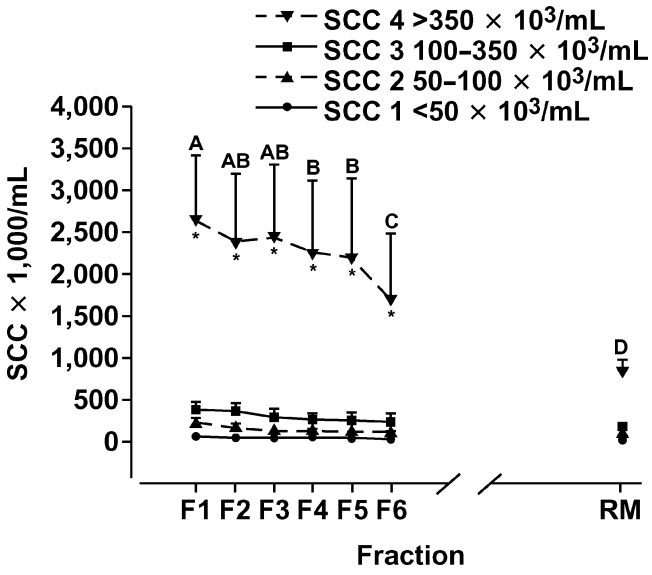

Figure 3. Changes in SCC in the defined milk fractions F1 to F6, consisting of one hand-stripped milk jet each, and the remaining cisternal plus alveolar milk (RM) in Experiment 2. Fractions were additionally assigned to 4 different SCC groups according to their total quarter SCC. *Means within a milk fraction differ ($P < 0.05$) between groups. $^{A–C}$Means within a group without common superscript letters differ ($P < 0.05$) between milk fractions.

Furthermore, our results demonstrated changes in milk SCC based on the milk fraction and total quarter SCC. In Experiment 1, collected milk fractions from a single quarter were defined as strict foremilk (F), cisternal milk (C), first 400 g of alveolar milk (A1), and remaining alveolar milk (A2). All investigated milk fractions of each quarter were assigned to groups (1 to 4) according to their total quarter SCC.

The SCC groups 1 to 3, representing the quarters with a total SCC of $<100 \times 10^3$/mL, showed minor changes during milking. A significant change in SCC during milking was observed in SCC group 4 belonging to quarters with a total SCC above 100×10^3/mL. Milk samples containing somatic cells above this concentration were assumed to emanate from inflamed quarters, subclinically or clinically. The F in SCC group 4 had the greatest concentrations and represented a fivefold increase in somatic cells per milliliter, compared with the total SCC, even though it represented only 0.3% of the total milk volume. A significant decrease in SCC was observed from F to C and further to A1. Fraction C, representing the next 4% of total milk, already showed half the SCC of F. In A1, the SCC was 10% of F. The main milk fraction composite as A2 had an SCC similar to A1. These changes during milking, indicating the importance of the sampled milk fraction for measuring SCC as the change in the first 3 fractions, were remarkable. Fractions F and C also showed significantly different SCC concentrations among the 4 SCC groups. This difference could not be observed in the later A1 and A2 fractions. Thus, the expressiveness of the SCC changes was according to which SCC fraction was used. Fraction F presented an alarming SCC that indicated a highly inflamed udder, even in a clinical way. Fraction C ranged in the subclinical inflammation area, whereas A1 showed a slight increase in SCC.

Taking into account the results of Experiment 1, we conducted Experiment 2. Here, the foremilk fraction was

Table 3 Classification of collected milk fractions (Experiment 2)

Abbreviation	Milk fraction	Volume,[1] mL	Percentage of total milk[2]
F1	First foremilk jet	2.7 ± 0.3	0.1 ± 0.0
F2	Second foremilk jet	2.3 ± 0.3	0.1 ± 0.0
F3	Third foremilk jet	3.1 ± 0.3	0.1 ± 0.0
F4	Fourth foremilk jet	3.3 ± 0.3	0.1 ± 0.0
F5	Fifth foremilk jet	3.4 ± 0.3	0.2 ± 0.0
F6	Sixth foremilk jet	3.7 ± 0.3	0.2 ± 0.0
RM	Remaining cisternal and alveolar milk	2,560 ± 236	99.2 ± 0.1

[1]Fraction mean volume ± SEM.

[2]Proportion of total milk harvested.

investigated in greater detail. Therefore, foremilk was categorized into 6 fractions consisting of one hand-stripped milk jet each. In this case, the SCC groups 1 to 3 represented the quarters with an SCC of $<350 \times 10^3$/mL, and they showed no significant changes during milking. A significant decrease in SCC was observed in group 4. Even in the 6 foremilk fractions F1 to F6, the decrease was significant. This was very interesting because one fraction presented only 0.1 to 0.2% of the total milk harvested. Fraction F6 represented only two-thirds and RM only one-third of the SCC of F1. Fractions F1 to F6 of group 4 also were increased significantly to their identical fractions in groups 1, 2, and 3. Surprisingly, this significance was not detected in RM. In this context, Bruckmaier et al. (2004) investigated the effect of milk ejection on the sensitivity of mastitis indicators such as physicochemical factors and somatic cells. They showed that the significances between high- and low-SCC quarters before milk ejection were striking.

In conclusion, SCC measurement by the DCC provided reliable and precise results; in particular, in those quarters having a high SCC, the sampled milk fraction had a crucial influence on the measured SCC value. One must consider that even SCC in strict foremilk can dramatically differ from that in the total cisternal fraction. The practical consequence of our findings was that any interpretation of the milk SCC must consider the fraction from which the milk sample was removed.

ACKNOWLEDGMENT

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The Effect of Centrifugation on Somatic Cell Content and Distribution in Milk

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ABSTRACT

The effect of centrifugation of milk at different relative centrifugal forces (RCF), centrifugation times and temperatures on the distribution of somatic cells (SC) in the evolving phases fat, skim milk and cell pellet was investigated. Furthermore, the commercially produced whole milk and whipping cream were investigated for somatic cell count (SCC) changes during processing. Results show that the degree of separation of SC is dependent on RCF. The number of pelleted cells increased to a maximum up to a RCF of 1850 x*g* at constant time (30min) and temperature (4°C). Higher RCF led to a decline of pelleted cells likely due to destruction of cells. The additionally tested cell viability in the pellet showed that increasing RCF led to increasing number of dead cells. The distribution in the three phases showed that most of the cells remained in the fat, although higher RCF led to a decrease of this effect. The cells in the skim milk increased slightly with increasing RCF. In a second step the centrifugal force was kept constant at 1850 x*g* and centrifugation time and temperature were varied. A longer duration of centrifugation up to 30 min resulted in a higher amount of separated cells in the pellet and correspondingly decreased number of cells isolated from the fat. This effect of more separated SC from the fat into the cell pellet was enforced by increasing the temperature to 55°C. The product line whole milk showed a continuous decrease of SCC during processing, so that the final product contained only 3% of the initial SCC. However, even here the milk after separator contained still about 50% and the cream about 30% of the initial SCC. The SCC in the production line whipping cream decreased from the initial milk to the separated cream and increased temporarily after the cream heater.

INTRODUCTION

Milk and dairy products still gain increasing importance in modern nutrition and its image can be summarized as pure. Therefore, it is reasonable that the food industry has steady

requirements which suffice their high quality standards, both from nutritional and technological points of interest. Hereby the hygienic quality of milk and dairy products plays a major role, as the high acceptance of milk by the consumers has to be maintained or even improved.

The somatic cell count (SCC) in milk is used in many countries to determine the hygienic quality of milk. Thus SCC is often considered as an economic milk value and as an indicator for udder health (O`Brien et al. 1999; Leitner et al. 2000). At this the consequential effects of high SCC while processing the milk are neglected. These effects can be lipolysis and proteolysis which lead to off – flavours and reduce the products shelf-life (Ma et al. 2000a; Santos et al. 2003). Furtheron cheese making is negatively influenced by diminished cheese yield and modified cheese composition (Klei et al. 1998; Politis et al. 1988a, b). Therefore it is important to avoid high SCC milk during processing of milk. However it is also possible to reduce the SCC before production. One crucial step in milk processing is centrifugal separation which includes the removal of solid impurities, including somatic cells (SC), from milk prior to pasteurisation. Apart from that it separates the fat from the skim milk.

The aim of this project was the compilation of concise data on the efficiency of centrifugation. Main focus of investigations was set on the methodology of centrifugation and detailed studies of the successional cell distribution in the three phases fat, skim milk and cell pellet. In this context the effects of relative centrifugal force (RCF), time and temperature were investigated. The questions of interest were, how large the proportion of total cell count is that remains in the supernatant after centrifugation as it is usually applied in food technology and how the usual methods of centrifugation can be optimised to achieve maximal, gentle and if possible complete removal of the milk cells. In addition investigations on several steps of processing in the production lines of whole milk and whipping cream were

carried out. This should provide an insight into the efficiency of commercial dairy industry processes.

MATERIALS AND METHODS

Animals and Husbandry

In both laboratory trials the milk of Brown Swiss cows in their first to fourth lactation was used. Trial 1 included twenty one animals, of whom six were in early stage of lactation (34-89 d), ten in mid lactation (110-192 d) and five in a late stage of lactation (231-405 d). Trial 2 included eight animals. Four were in early (45-92 d) and four in mid (145-196 d) lactation.

The average milk production on the day of investigation was 22 kg per cow. Cows were kept in a loose-housing barn and milked twice daily at 5 a.m. and 4 p.m.

Laboratory Procedures

For the investigations composite quarter milk samples directly from the farm were used.

Cell Separation

Within half an hour after collecting the sample 2 x 50 ml was centrifuged in a conical tube to separate the cells. This procedure divides the milk into the three phases fat, skim milk and cell pellet. The fat layer on top can be removed by a spattle and the skim milk decanted. The cell pellet remains on the bottom of the tube.

SCC and Viability in the Cell Pellet

The cell pellet was further cleared by washing with phosphate buffered saline (PBS). After resuspending the pellet in 1-4 ml of PBS, haemocytometric cell counting is performed by use of a Neubauer Counting Chamber. This is a specialised microscopic slide and allows a quantitative calculation of the cell content. In addition to the SCC the viability of the cells

was investigated by the exclusion method with the dye trypane blue (Sarikaya et al. 2004). The cell suspension was kept on ice during the whole procedure.

Measurement of SCC in Fat and Skim Milk

The measurements of SCC were carried out by flow cytometric measurement (Fossomatic®, FOSS, 3400 Hillerød, Denmark). Skim milk causes no problems for this kind of measurement. But it is not possible to measure pure fat in the flow cytometer as it would occlude the cuvette. Therefore, the same sample was centrifuged in duplicate. This allows a measurement of skim milk in the one preparation and the measurement of skim milk + fat in the second preparation. The difference in SCC of these two measurements provides the SCC in the fat.

Experimental Design

The three variable parameters of the centrifugation setup are RCF, time and temperature. These parameters have been investigated systematically.

Effect of RCF – Trial 1

In this trial the effect of RCF on the SC in milk was investigated. RCF was varied in the four steps 200, 850, 1850 and 3300 x*g*. The fixed parameters were time (30 min) and temperature (4°C). The SCC and viability of cells was investigated in the achieved cell pellet. Skim milk and fat were tested for SCC.

Effect of Time and Temperature – Trial 2

In this trial RCF was kept constant (1850 x*g*) based on the results achieved in trial 1. The varied parameters were time (15, 20, 30 min) and temperature (4, 25, 38, 55°C). Investigations were focussed on the SCC distribution on the phases fat, skim milk and cell pellet.

Commercial Milk Processing

In a further step milk samples from dairy processing have been investigated. The chosen production lines of interest were whole milk (n=7) and whipping cream (n=7). Attention has been put on special steps of processing. The list of the investigated steps of processing in the two production lines is shown in Table 1. The parameter of interest was SCC, whereas the initial value for SCC in the first step of processing (i.e. tank) was set as 100%. The change in SCC was calculated according to this value.

Statistical Analyses

Data are presented as means ± standard error of means (SEM). Differences between itemized milk phases, RCF, centrifugation time and centrifugation temperature were tested for significance ($P < 0.05$) by analysis of variance using the MIXED models procedure of the SAS program package. The MIXED model included the animal and the milk phase as class variables. The animal was the repeated term during the course of centrifugation. The matrix structure Compound Symmetry was used. Statistical significance between phases was tested by Least Significance Difference – (LSD) – test.

RESULTS

Effect of RCF – Trial 1

The number of separated cells in the pellet strongly depended on RCF. To analyse the results independent from the total SCC of the quarter milk sample, the number of separated cells at RCF 200 x*g* was set 1. The number of separated cells in the three further steps (RCF of 850, 1850 and 3300 x*g*) shows the x-fold amount relative to RCF of 200 x*g*. The number of pelleted cells increased to a maximum up to a RCF of 1850 x*g* ($P<0.05$; Fig. 1). Higher RCF led to a decline of pelleted cells ($P<0.05$). In addition, the viability of the cells in the pellet

was investigated. Hereby increasing RCF led to an increasing number of dead cells, i.e. the ratio of dead cells to viable cells increased ($P<0.05$; Fig. 1).

The distribution of the cells after centrifugation in the three phases fat, skim milk and cell pellet was investigated, too. Total quarter SCC, determined by Fossomatic®, was defined as 100%. Results showed that most of the cells remained in the fat, although higher RCF led to a decrease of the number of cells in this phase ($P<0.05$; Fig. 2). The cell concentration in the skim milk increased slightly with increasing RCF. This increase became significant at a RCF above 1850 x*g* ($P<0.05$). The number of live and dead cells in the cell pellet showed the same trend as mentioned before (Fig. 2). After summing the cells in the three phases the total quarter SCC could be recovered, except at a RCF of 3300 x*g*, when ~4% of the total SCC could not be recovered.

Effect of Time and Temperature – Trial 2

Results showed that a longer duration of centrifugation increased ($P<0.05$) the number of separated cells in the cell pellet (Fig. 3). This increase could be observed at an extension of centrifugation time from 15 min to 30 min at all four temperature steps (4, 25, 38, 55°C). Increasing temperature caused increasing amounts of cells in the pellet, significant between 4°C and 55°C.

The centrifugation time had no effects on SCC in the skim milk (Fig. 3). Effects could be observed with changing temperature. Higher temperature resulted in higher SCC in this fraction. 15 and 30 min of centrifugation showed a increase ($P<0.05$) in skim milks SCC between 4°C to 25°C and 38°C to 55°C. 20 min centrifugation resulted in a significant increase in SCC between each temperature step.

An extension of centrifugation time from 15 min to 30 min decreased ($P<0.05$) SCC in fat at 25, 38 and 55°C (Fig. 3). The increase in temperature showed also a decrease ($P<0.05$) in SCC at all three centrifugation times.

Commercial Milk Processing

SCC in the production line whole milk decreased ($P<0.05$) during processing (Fig. 4). All investigated steps of processing had the effect of reducing the SCC from the initial milk step by step. The SCC in the production line whipping cream decreased ($P<0.05$) from the initial milk to the separated cream and increased ($P<0.05$) temporarily after the cream heater (Fig. 4). Subsequently, the steps filling tank and the product whipping cream showed decreased ($P<0.05$) levels of SCC comparable to the fraction of the separated cream.

DISCUSSION

Unknown distribution of the SC in the milk can be caused by cell preparation and treatment protocols, which are used in almost all experimental investigations. We investigated the effects of RCF, centrifugation time and temperature on the distribution of the SC in the evolving phases fat, skim milk and cell pellet.

Our results indicate the importance of optimised separation parameters for improving the efficiency of SC separation out of milk. It could be shown that the separation of SC out of milk is dependent on the RCF. The number of separated cells showed an increase up to a RCF of 1850 x*g*, higher RCF led to a decline of separated cells in the pellet. A possible explanation could be the destruction of the SC above a RCF of 1850 x*g*. On the one hand the total number of intact SC decreases. On the other hand, the density of the generated cell fragments is very low, thus they cannot be separated at this level of RCF. Both effects result in a definite loss of cells in the pellet. Furthermore it could be shown that centrifugation has an effect on SC viability. Higher RCF also led to more dead cells in the pellet. These two results should be taken into account if working with milk cells is intended. Many cell culture investigations use separated SC from milk (Boutet et al. 2004; Prgomet et al. 2005). It has to be differentiated between the need of as much cells as possible or as much live cells as possible. In addition,

the distribution of the SC in the phases fat, skim milk and pellet was investigated depending on RCF. Results showed that most of the SC remain in the fat. The percentage declines with increasing RCF, but even at a RCF of 3300 x*g* ~50% of the SC can be detected in the fat (Russell et al. 1977; Lee et al. 1980). The SC distribution in the phases at a RCF value of 3300 x*g* showed an amount of ~4% of "missing cells", i.e. the summary of all detected SC in the phases does not recover the total quarter SCC in the raw milk sample. As described before, the destruction of SC at higher RCF might lead to this discrepancy in cell sum.

In a second step the RCF was kept constant at 1850 x*g*, which has shown an optimum of cell count in the pellet in previous results, and the parameters time and temperature were varied. A longer duration of centrifugation resulted in a higher amount of separated cells in the pellet. Corresponding with this finding, the number of cells isolated from the fat was decreasing. This effect of more separated SC from the fat into the cell pellet was enforced by increasing the temperature to 55°C. This is also the common temperature which the dairy industry is using for milk separation processing.

The systematic investigation of the centrifugation parameters showed a possible link between SC and the milk fat, as the main actors at separation of SC are the fat phase and the achieved cell pellet. This could be partially explained by the fact that both SC and fat globules present a cell membrane. A certain affinity of the SC membrane towards fat globule membrane is obvious. As the fat globules show a dominate amount, they might tear up the cells towards the top fraction during the centrifugal separation (Ma et al. 2000b). This obvious affinity between the SC and the fat could partially be overcome by increased RCF, centrifugation time and temperature.

Having these findings in mind the dairy production lines whole milk and whipping cream were investigated. The product line milk showed a continuous decrease of SCC during processing, so that the final product contained only a few percent of the initial SCC in the raw milk. However, even here the milk after separator contained still ~50% and the cream ~30%

of the initial SCC. The flow chart of the dairy production line reveals that the skimming step is performed before the bactofuge separation. However, the more efficient separation of SC is obtained during bactofugation. It reduced the SCC value of the skim milk to ~5%. It should therefore be considered to connect the bactofugation step before the skimming step. This would allow a centrifugation of the total milk, i.e. including the fat, and maybe thereby more SC could be separated out of the fat.

Due to the observations that a not negligible amount of SC remained in the fat phase more precise investigations have been carried out on the production line whipping cream. The steps of processing showed a correlation between the amount of SC and the fat content. A decrease of SC can only be detected at the step of skimming/creaming from the raw milk to the cream. As no further separation takes place the SC shows no decreasing tendencies in the further steps. The cream heater even causes even a temporary increase in the amount of SC, as the water content decreases marginally. The fat content shows also a temporary increase at this processing step.

The results of this work have to be considered from two different points of view. A lot of laboratory work with milk and the SC of milk include somehow a separation or extraction method of the component of interest. If this separation step contains centrifugation, the parameters of centrifugation have to be combined according to the investigators goal. For instance, if working with as many as possible live SC is intended, RCF has to be moderate as high values lead to death and destruction of SC. If a maximum of SC separation is intended the centrifugation time and temperature have to be elevated besides the RCF. Interpreting the results from an industrial point of view would show that one of the critical points in the processing is the skimming step. It is known that high SCC can affect the products shelf-life and lead to off – flavours (Ma et al. 2000a; Santos et al. 2003). Therefore it is very important to remove the SC out of the milk. The separation with the bactofuge seems to be more

effective than the milk separator. Placing the bactofuge in front of the milk separator could enhance the SC separation.

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Figure legends

Figure 1: Change in separation factor of somatic cells and ratio of dead cells depending on relative centrifugal force (RCF);

▲ separation factor; ● ratio dead cells;

a, b, c : means without common letters differ significantly (P<0.05) between RCF.

Figure 2: Distribution of somatic cell counts (SCC) in the three phases cell pellet, skim milk and fat depending on relative centrifugal force (RCF);

dead cells in pellet, live cells in pellet, skim milk, fat;

a, b, c : means without common letters within phase differ significantly (P<0.05) between RCF.

Figure 3: Distribution of somatic cell counts (SCC) in the three phases cell pellet, skim milk and fat depending on centrifugation time and temperature;

pellet, skim milk, fat;

a, b : means without common letters within phase and temperature differ significantly (P<0.05) between time.

A, B, C, D : means without common letters within phase and time differ significantly (P<0.05) between temperature.

Figure 4: Change in SCC during steps of processing in the two production lines milk and whipping cream; SCC in first step of processing was set 100%;

a, b, c,... : means without common letters within production line differ significantly (P<0.05) between steps of processing.

Table 1. Investigated steps of processing in the production lines whole milk and whipping cream

	Milk	Whipping Cream
1.	Tank	Tank
2.	Supply tank	Cream
3.	After Separator	Cream Heater
4.	Cream	Filling Tank
5.	After Bactofuge	Final Product: Whipping Cream
6.	Final Product: Milk	

Fig. 1.

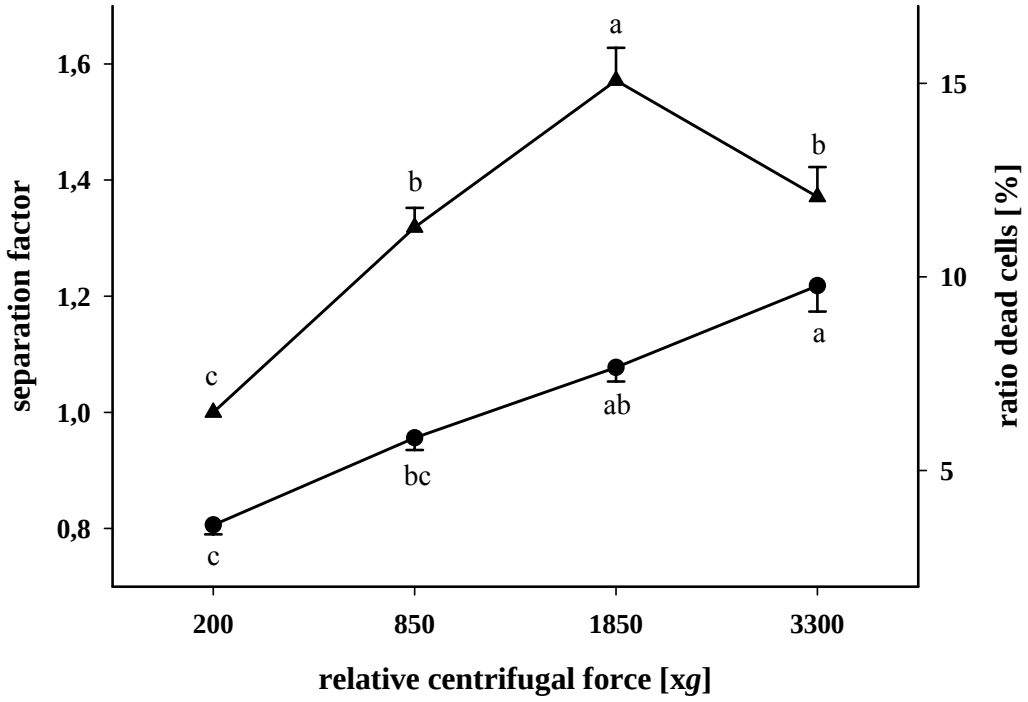

separation factor
1,6
1,4
1,2
1,0
0,8
ratio dead cells [%]
15
10
5
a
b
b
c
ab
a
bc
c
200
850
1850
3300
relative centrifugal force [x*g*]

Fig.2.

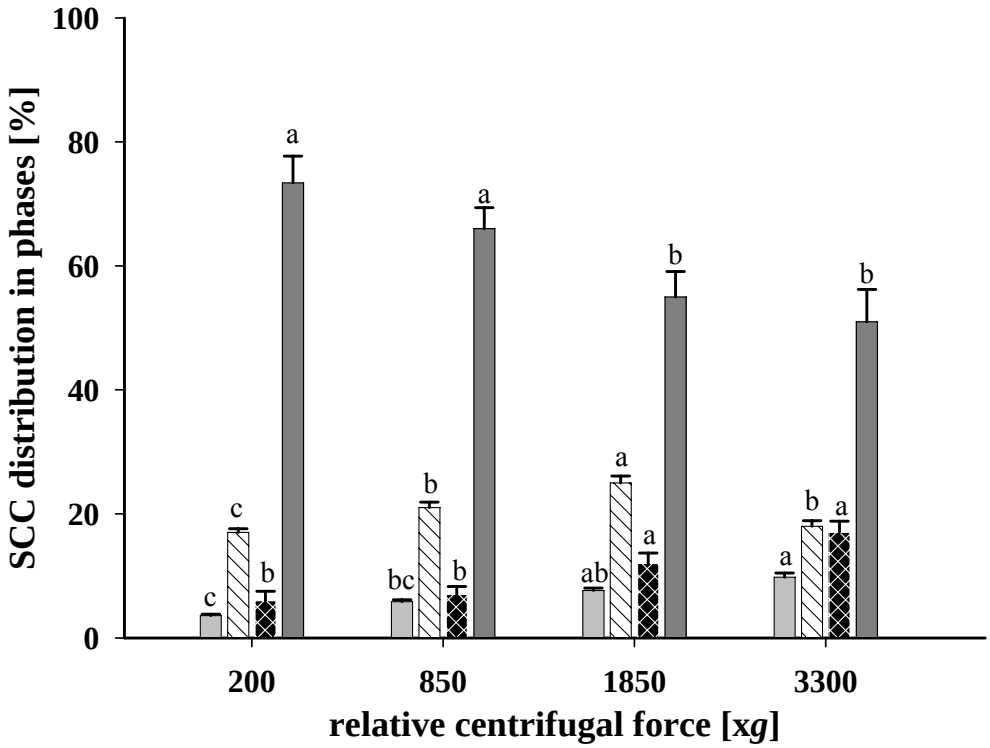

100
80
60
40
20
0
SCC distribution in phases [%]
a
a
b
b
c
c
b
bc
b
ab
a
a
b
a
b
200
850
1850
3300
relative centrifugal force [x*g*]

Fig. 3.

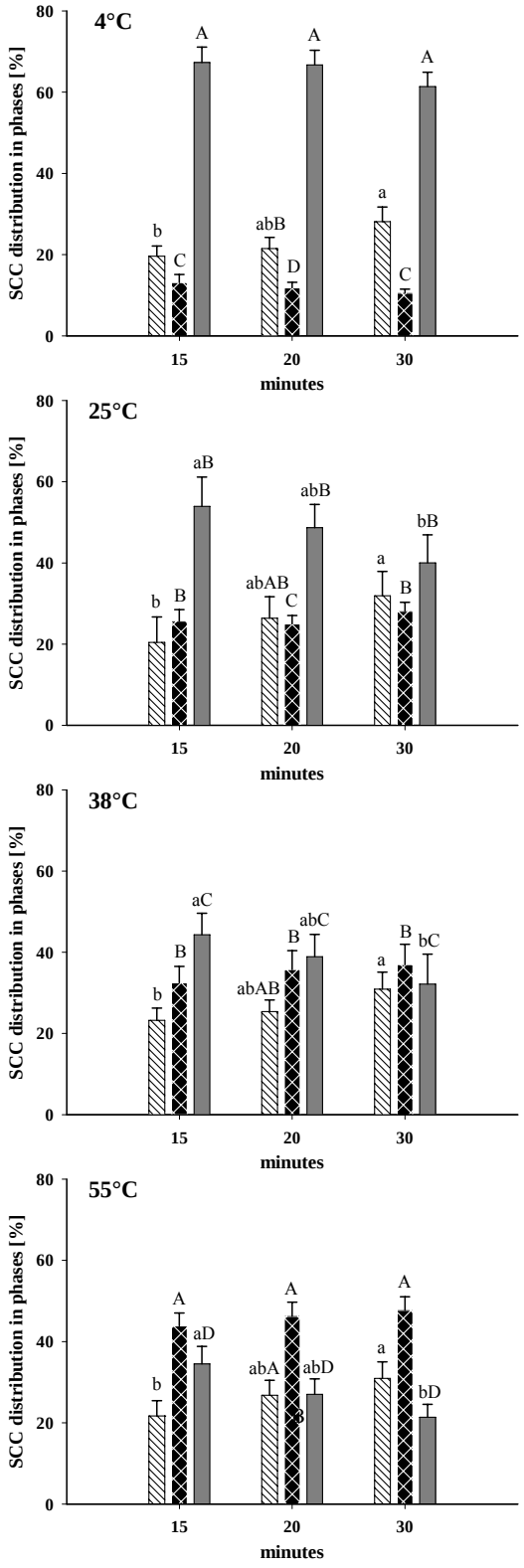
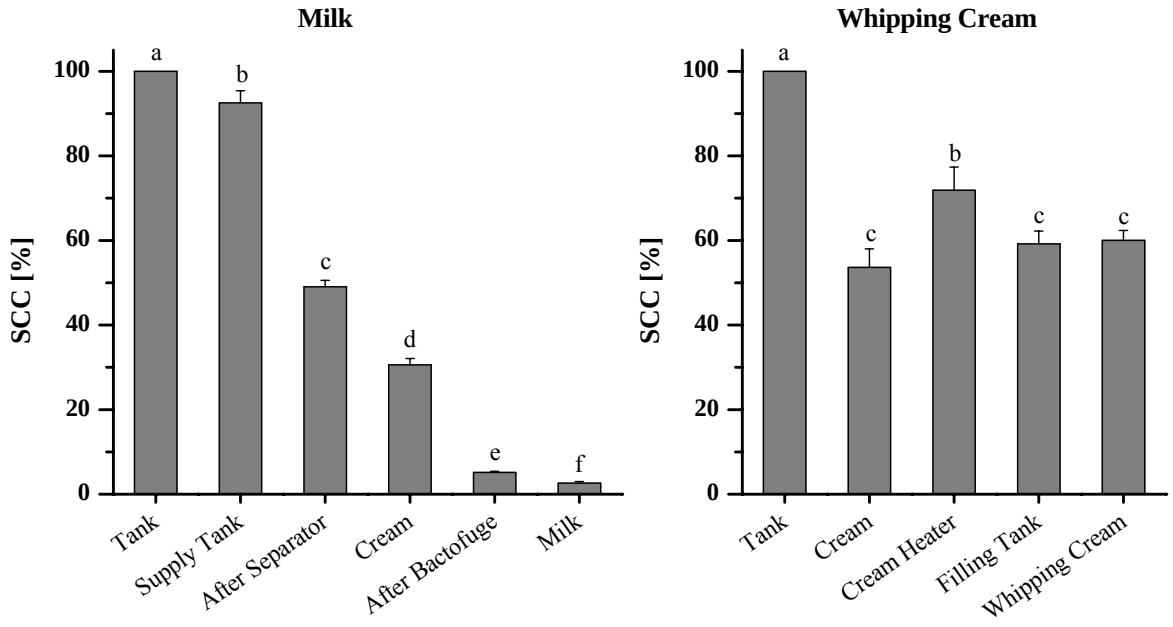

4°C
25°C
38°C
55°C
SCC distribution in phases [%]
minutes
0
20
40
60
80
15
20
30
b
C
A
abB
D
A
a
C
A
b
B
aB
abAB
C
abB
a
B
bB
b
B
aC
abAB
B
abC
a
B
bC
b
A
aD
abA
A
abD
a
A
bD

Fig. 4.


Milk
SCC [%]
0
20
40
60
80
100
a
b
c
d
e
f
Tank
Supply Tank
After Separator
Cream
After Bactofuge
Milk
Whipping Cream
SCC [%]
0
20
40
60
80
100
a
c
b
c
c
Tank
Cream
Cream Heater
Filling Tank
Whipping Cream