# Postprint: Milk Somatic Cell Typing and Changes in Dairy Cow Lactation Performance at Different Somatic Cell Count Levels

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## Abstract

This experiment aimed to investigate the differential characteristics of milk somatic cells at different somatic cell counts (SCC) and thereby evaluate dairy cow lactation performance. Milk samples were collected from 102 Holstein dairy cows for determination of SCC, milk yield, and milk composition. Based on SCC test results, the 102 milk samples were divided into three groups: low SCC group (SCC < 100,000 cells/mL), medium SCC group (SCC = 100,000–400,000 cells/mL), and high SCC group (SCC > 400,000 cells/mL). Somatic cells in milk samples were sorted by flow cytometry to determine the proportions of lymphocytes, macrophages, and polymorphonuclear neutrophils (PMN). Total bacterial count was enumerated using the standard plate count method, and bacteria in milk samples were identified and classified through bacteriological examination. The results showed that: (1) Within the SCC < 100,000 cells/mL range, when SCC was 13,000–76,000 cells/mL, the lymphocyte proportion was 89.43%, representing the predominant cell population in healthy mammary glands. When SCC increased to 76,000–100,000 cells/mL, the PMN proportion increased extremely significantly ($P < 0.01$) to 79.68%, becoming the predominant cell population within the mammary gland, indicating that early-stage inflammation had already initiated. When SCC > 200,000 cells/mL, PMN proportion was positively correlated with SCC; lymphocyte proportion was negatively correlated with SCC; and macrophage proportion showed no significant correlation with SCC. (2) The major pathogens identified included Staphylococcus aureus, coagulase-negative staphylococci (CNS), and Streptococcus agalactiae; minor pathogens included Pseudomonas aeruginosa, Bacillus, and Lactococcus lactis. The PMN proportion in milk samples from the major pathogen group (48.93%) was extremely significantly higher than that in the pathogen-free group (17.23%) ($P < 0.01$) and significantly higher than that in the minor pathogen group ($P < 0.05$). (3) In bacteriologically positive milk samples, cows infected with contagious pathogens exhibited significantly decreased

milk yield ($P < 0.05$) and significantly reduced casein/protein ratio and lactose content ($P < 0.05$); in bacteriologically negative milk samples, significantly reduced casein and lactose contents were observed in medium SCC (10,000–40,000 cells/mL) and high SCC (SCC > 40,000 cells/mL) milk samples ($P < 0.05$), and significantly decreased milk protein content was observed in high SCC milk samples ($P < 0.05$). These results demonstrate that somatic cell differential analysis can be employed for more detailed assessment of mammary gland health status, particularly for earlier identification of inflammatory initiation in low-SCC milk. The onset of early-stage mammary inflammation occurred at SCC levels markedly below the currently recognized threshold for healthy mammary gland milk (<100,000 cells/mL), and CNS may be potential pathogens that induce early-stage mammary inflammation. In bacteriologically negative samples, inflammation caused significant reductions in casein/protein ratio and lactose content in high-SCC milk.

## Full Text

## Abstract

This study aimed to evaluate lactation performance in dairy cows by examining the characteristics of milk somatic cell classification under different somatic cell counts (SCC). Milk samples were collected from 102 Holstein dairy cows and analyzed for SCC, milk yield, and milk composition. Based on SCC results, the 102 samples were divided into three groups: low SCC group (SCC < 100,000 cells/mL), medium SCC group (SCC between 100,000 and 400,000 cells/mL), and high SCC group (SCC > 400,000 cells/mL). Flow cytometry was used to sort somatic cells in milk samples and determine the proportions of lymphocytes, macrophages, and polymorphonuclear neutrophils (PMN). The standard plate count method was employed to enumerate total bacterial colonies, and bacteriological examination was conducted to identify and classify bacterial species in milk samples. The results showed: 1) Within the SCC range of <100,000 cells/mL, when SCC was between 13,000 and 76,000 cells/mL, the lymphocyte proportion was 89.43%, representing the dominant cell population in healthy mammary glands. When SCC increased to 76,000–100,000 cells/mL, the PMN proportion increased dramatically ($P < 0.01$) to 79.68%, becoming the predominant cell population in the mammary gland and indicating the initiation of early inflammation. When SCC > 200,000 cells/mL, PMN proportion was positively correlated with SCC, while lymphocyte proportion was negatively correlated with SCC; macrophage proportion showed no significant correlation with SCC. 2) The main pathogens identified were *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), and *Streptococcus agalactiae*; secondary pathogens included *Pseudomonas aeruginosa*, *Bacillus* spp., and *Lactococcus lactis*. The PMN proportion in milk samples from the major pathogen group (48.93%) was significantly higher than in the no-pathogen group (17.23%) ($P < 0.01$) and significantly higher than in the secondary pathogen group ($P < 0.05$). 3) In cows infected with contagious pathogens, milk yield decreased

significantly ($P < 0.05$), and casein/milk protein ratio and lactose content were significantly reduced ($P < 0.05$). In culture-negative milk samples, significant reductions in casein and lactose contents were observed in medium SCC (10,000–40,000 cells/mL) and high SCC (SCC > 40,000 cells/mL) samples ($P < 0.05$), while milk protein content was significantly reduced in high SCC samples ($P < 0.05$). These findings demonstrate that somatic cell classification can provide more detailed analysis of mammary gland health status, particularly for early identification of inflammatory onset in low-SCC milk. The initiation of early mammary inflammation occurs well below the currently accepted SCC threshold for healthy mammary glands (<100,000 cells/mL), and CNS may be a potential pathogen triggering early mammary inflammation. In culture-negative samples, inflammation leads to significant reductions in casein/milk protein ratio and lactose content in high-SCC milk.

**Keywords:** dairy cows; somatic cell count; somatic cell classification; mammary health

## Introduction

Diagnosis of bovine mammary inflammation, particularly subclinical mastitis, has traditionally relied on somatic cell count (SCC) and bacteriological examination. SCC provides a measure of inflammation based on total cell numbers in milk. While bacteriological examination can identify the exact causative agent, it is time-consuming and requires experienced personnel. Polymerase chain reaction has been proposed as an alternative to bacteriological examination, offering rapid detection but at high cost [1]. Numerous studies have evaluated and discussed various SCC thresholds for distinguishing infected from uninfected mammary glands [2]. The European Union defines healthy mammary glands as those with SCC < 200,000 cells/mL, while the German Veterinary Association (DVG) recommends a threshold of 100,000 cells/mL [3-4]. China' s raw milk collection standard specifies SCC < 400,000 cells/mL [5]. Different geographical environments and climates significantly affect SCC, and SCC varies continuously depending on lactation status, age, milking time and frequency, particularly mammary infection status [6]. Therefore, more sensitive inflammatory indicators are needed to enhance mastitis management in dairy farms.

Recent studies have revealed the advantages of identifying immune cell types in milk for diagnosing bovine mammary inflammation. Loken et al. [7] demonstrated that beyond SCC determination, somatic cell differential counts provide more detailed characterization of actual mammary health status. In the mammary gland, the quantity and distribution of milk immune cells play important roles in the inflammatory response. Lymphocytes regulate induction and suppression of immune responses by recognizing antigens through specific biomembrane receptors of invading pathogens. Macrophages can ingest bacteria, cellular debris, and residual milk components. Polymorphonuclear neutrophils (PMN) primarily defend against invading bacteria at the onset of acute inflammatory processes. However, the proportion of each cell type varies considerably

with inflammation severity.

Auldist et al. [8-9] reported that in healthy milk, macrophages are the predominant cell type, while lymphocytes represent the main cell population in healthy mammary glands. Different cell typing techniques can affect somatic cell classification results. Bannerman et al. [10] investigated cell patterns during infections with different pathogens and infection stages, finding that in acute inflammatory mammary glands caused primarily by *Staphylococcus aureus*, PMN were the dominant cell type, accounting for 90% of total leukocytes. In contrast, in chronic mastitis caused by coagulase-negative staphylococci (CNS), PMN numbers were even lower than in uninfected quarters, while macrophage numbers were higher.

Mammary health status is closely related to lactation performance, with different pathogens eliciting different immune responses in the mammary gland. Depending on etiology, distinct differences can be observed in SCC trends and milk composition. Although literature on the association between SCC and milk composition is abundant, few studies have addressed the relationship between specific mastitis pathogens and milk composition changes. Therefore, this study investigated characteristics of milk somatic cell classification under different SCC levels to provide more detailed assessment of dairy cow mammary health status and lactation performance.

## Materials and Methods

### 1.1 Experimental Animals and Sample Collection

Milk samples were collected from Holstein dairy cows at Beijing Chengyuan Shenglong Breeding Co., Ltd. Cows with obvious clinical symptoms (such as metritis, clinical mastitis, displaced abomasum, uterine prolapse, milk fever, clinical ketosis) were excluded. A total of 102 clinically healthy Holstein cows [parity (2–3 lactations), days in milk (152 ± 27) d, milk yield (27 ± 3) kg/d] were selected for milk sample collection. Collection was performed on June 5, 2017, with two milkings at 08:00 and 19:00. Before sample collection, the external udder was disinfected with teat dip, cleaned with individual towels, and then cleaned again with alcohol. After discarding the first three milk streams, approximately 50 mL of milk from all four quarters of each cow was pooled into sterile tubes. The milk samples were then divided into three subsamples for somatic cell typing, bacteriological examination, and milk composition analysis, and all samples were stored at -80°C.

### 1.2 Instruments and Reagents

**1.2.1 Main Instruments** Flow cytometer (NovoCyte 3130, ACEA, USA); micropipettes and tips; flow cytometry tubes; 15 mL centrifuge tubes; centrifuge; milk composition analyzers (Fossomatic 5000, Milkoscan FT6000, FOSS, Denmark); constant temperature incubators [(36 ± 1)°C, (30 ± 1)°C]; refrigerators

(-80°C, 2-5°C); constant temperature water bath [(46 ± 1)°C]; electronic balance (precision 0.01 g); micropipettes; sterile conical flasks (250 and 500 mL); sterile petri dishes (diameter 90 mm); pH meter (precision 0.01); colony counter.

**1.2.2 Main Reagents** Reagents for somatic cell typing: Anti-CD11b antibody (Cat. No.: ab75476); Anti-CD14 antibody [Tuk4] (Cat. No.: ab27545); Anti-mouse IgG (H+L), F(ab' )2 Fragment (Alexa Fluor® 488 Conjugate); Anti-rabbit IgG (H+L), F(ab' )2 Fragment (Alexa Fluor® 647 Conjugate). Reagents for bacterial isolation and identification: tryptone; yeast extract; glucose; agar; distilled water; sodium chloride.

### 1.3 Measurement Indicators and Methods

**1.3.1 Milk SCC and Composition** SCC was measured using a milk composition analyzer (Fossomatic 5000, FOSS, Denmark). Milk fat, protein, lactose, casein, and urea nitrogen contents were measured within 24 h using a milk composition analyzer (Milkoscan FT6000, FOSS, Denmark). Detailed milk protein component contents were determined by reversed-phase high-performance liquid chromatography (RP-HPLC) using skim milk [11].

**1.3.2 Milk Somatic Cell Typing** Somatic cell typing was performed using flow cytometry. Fourteen milliliters of milk were added to a 15 mL centrifuge tube and centrifuged at 690 × g for 10 min. After centrifugation, the upper fat layer was removed and the supernatant was discarded. One milliliter of phosphate-buffered saline (PBS) was added to the tube bottom, transferred to a new 15 mL centrifuge tube, mixed with 10 mL PBS, centrifuged at 690 × g for 10 min, and the supernatant was discarded; this process was repeated three times. Cells were resuspended in 1 mL PBS. Four hundred microliters of cell suspension were placed in a 5 mL flow tube for cell counting. One hundred microliters of cell suspension were placed in a 5 mL flow tube, and 10 L propidium iodide (PI) was added for viability testing [12].

**1.3.3 Milk Total Bacterial Count** Five grams of tryptone, 2.5 g of yeast extract, 1.0 g of glucose, and 15 g of agar were dissolved in 1,000 mL distilled water, pH adjusted to 7.0 ± 0.2, boiled to dissolve, dispensed into conical flasks, and sterilized by autoclaving at 121°C for 15 min to prepare agar medium. Eight point five grams of sodium chloride were dissolved in 1,000 mL distilled water and sterilized by autoclaving at 121°C for 15 min to prepare sterile physiological saline. One milliliter of milk sample was aspirated with a sterile micropipette into a test tube containing 9 mL sterile physiological saline, mixed thoroughly to prepare a 1:10 sample homogenate. One milliliter of the 1:10 sample homogenate was aspirated and slowly injected into a sterile test tube containing 9 mL sterile physiological saline, mixed by oscillation to prepare a 1:100 sample homogenate. Following this method, 10-fold serial dilutions were prepared, with four dilution gradients: 1:10, 1:100, 1:1,000, and 1:10,000. For each dilution, 500 L of sample

homogenate was pipetted into sterile agar plates, with two plates per dilution. Simultaneously, 500 L of blank diluent was added to two sterile agar plates as blank controls. After the agar plates cooled and solidified, they were inverted, sealed, and incubated in a constant temperature incubator at (36 ± 1)°C for 48 h [13].

Total bacterial count was enumerated according to GB 4789.2-2010 "National Food Safety Standard - Microbiological Examination of Food - Aerobic Plate Count." The calculation formula was:

$N = (\Sigma C) / (n \times d)$

Where: N is the total bacterial count (CFU); C is the colony count at each dilution (CFU); n is the number of parallel plates at each dilution; d is the dilution factor (first dilution).

**1.3.4 Milk Bacteriological Examination** Ten microliters of each milk sample were streaked onto blood agar containing 5% defibrinated sheep blood. Plates were incubated aerobically at (37 ± 1)°C and examined after 24 and 48 h. Bacteria were identified according to National Mastitis Council (NMC, 1999) guidelines, including morphology, Gram staining, catalase and coagulase reactions, oxidase reaction, biochemical characteristics, and hemolysis patterns. Gram-positive bacteria were differentiated into staphylococci and streptococci by catalase reaction. Coagulase tube testing in rabbit plasma was used to distinguish *Staphylococcus aureus* from CNS. Gram-negative bacteria were identified by oxidase testing and growth characteristics on MacConkey agar and eosin methylene blue agar [14].

### 1.4 Statistical Analysis

Initial data were organized and visualized using Excel 2007. GraphPad Prism 6.01 was used for statistical analysis of graphical data and calculation of Pearson correlation coefficients (r). The MIXED procedure in SAS 9.2 was used to analyze associations among somatic cell typing, SCC, subclinical mastitis-specific pathogens, and milk composition. One-way ANOVA was used for single-factor analysis of variance, and Duncan' s multiple range test was used for mean comparisons. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered highly significant.

## Results

### 2.1 SCC and Somatic Cell Typing in Milk

As shown in and , among the 102 milk samples, the mean SCC was 351,720 cells/mL, with 44 samples having SCC < 100,000 cells/mL (low SCC group), 28 samples with SCC between 100,000 and 400,000 cells/mL (medium SCC group), and 30 samples with SCC > 400,000 cells/mL (high SCC group). shows that across the 102 milk samples, SCC ranged from 13,000 to 1,024,000 cells/mL,

with PMN proportions varying from 4.48% to 87.29% [(39.76 ± 32.44)%]; lymphocyte proportions varying from 3.37% to 91.07% [(48.50 ± 23.39)%]; and macrophage proportions varying from 2.37% to 55.71% [(21.74 ± 12.07)%].

Extensive cellular variation existed within somatic cell populations, particularly for lymphocytes and PMN. Therefore, correlations between somatic cell typing and SCC were analyzed. As shown in [Figure 1: see original paper]-a, when SCC < 200,000 cells/mL, PMN proportion was relatively low at only 27.4%, increasing significantly with SCC; when SCC > 400,000 cells/mL, PMN proportion reached 87.29%, becoming the predominant cell population in inflammatory milk samples. [Figure 1: see original paper]-b shows that when SCC < 200,000 cells/mL, lymphocytes were the main cell population with proportions up to 91.07%, decreasing significantly with increasing SCC. [Figure 1: see original paper]-c shows that macrophage proportions ranged from 2.37% to 55.71% and did not show strong correlation with SCC ($r = -0.1672$, $P > 0.05$).

To determine whether statistically significant differences existed in the immunological status of healthy mammary glands, all milk samples with SCC < 100,000 cells/mL were divided into four groups based on SCC: Group I (SCC 13,000–34,000 cells/mL, n = 9), Group II (SCC 34,000–55,000 cells/mL, n = 11), Group III (SCC 55,000–76,000 cells/mL, n = 10), and Group IV (SCC 76,000–100,000 cells/mL, n = 14).

As shown in [Figure 2: see original paper], within healthy mammary glands, lymphocyte proportions decreased while PMN proportions increased with elevated milk SCC. Lymphocyte proportions in Groups I–III (59.35%–81.26%) were significantly higher than in Group IV (22.17%) ($P < 0.01$). No significant differences in macrophage proportions were observed among the four groups (19.15%–32.13%) ($P > 0.05$). PMN proportions in Groups I–III (19.17%–31.22%) were not significantly different ($P > 0.05$) but were all significantly lower than in Group IV (82.68%) ($P < 0.01$).

### 2.2 Total Bacterial Count and Bacterial Species

As shown in , total bacterial counts varied considerably among different SCC groups. Milk samples with SCC < 100,000 cells/mL had total bacterial counts of only 8.08 × 10  CFU/mL; samples with SCC between 100,000 and 400,000 cells/mL had counts of 46.04 × 10  CFU/mL; when SCC > 400,000 cells/mL, total bacterial counts increased dramatically to 94.95 × 10  CFU/mL. However, all bacterial counts remained within normal ranges.

Among the 102 milk samples tested, 13.72% were culture-negative, 83.34% were culture-positive, and 3 samples were contaminated. Based on transmission characteristics of mastitis pathogens, isolated bacteria were classified as contagious, environmental, or opportunistic pathogens. As shown in , among culture-positive samples, opportunistic pathogens isolated were CNS (accounting for 34.32% of total samples and 39.77% of culture-positive samples), with an average SCC of 71,850 cells/mL in CNS-infected samples. Contagious pathogens

(accounting for 27.45% of total samples and 31.81% of culture-positive samples) included *Staphylococcus aureus* and *Streptococcus agalactiae* as the main pathogens, with an average SCC of 824,280 cells/mL in samples infected with contagious pathogens. Environmental pathogens (accounting for 21.57% of total samples and 25.00% of culture-positive samples) included *Proteus* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., *Bacillus* spp., *Escherichia coli*, and *Lactococcus lactis*, with an average SCC of 338,670 cells/mL.

### 2.3 Somatic Cell Typing Under Different Pathogen Conditions

Somatic cell typing was analyzed based on different mastitis pathogens detected in milk samples. The 102 milk samples were divided into three groups: no-pathogen group, secondary pathogen group, and major pathogen group. No pathogens were detected in 14 samples (13.72%) (culture-negative). Major pathogens (such as CNS, *Staphylococcus aureus*, *E. coli*, *Streptococcus agalactiae*) were isolated from 47 samples (46.07%), while secondary pathogens (such as *Bacillus* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp.) were detected in 24 samples (23.53%).

As shown in [Figure 3: see original paper], compared with the major pathogen group (31.45%), the lymphocyte proportion in the no-pathogen group (59.48%) was significantly higher ($P < 0.01$), representing the main cell population in healthy mammary glands. Macrophage proportion (21.03%) showed no significant correlation with pathogen status ($P > 0.05$). The PMN proportion in the major pathogen group (48.93%) was significantly higher than in the no-pathogen group (17.23%) ($P < 0.01$) and significantly higher than in the secondary pathogen group ($P < 0.05$).

Although only 13.72% of samples were culture-negative, their average SCC was >200,000 cells/mL (). Therefore, further somatic cell typing comparison was conducted on the 14 culture-negative milk samples, which were divided into three groups based on SCC: culture-negative-H group (no pathogen growth with high SCC, SCC > 400,000 cells/mL, $n = 4$), culture-negative-M group (no pathogen growth with medium SCC, SCC 100,000–400,000 cells/mL, $n = 5$), and culture-negative-L group (no pathogen growth with low SCC, SCC < 100,000 cells/mL, $n = 5$).

As shown in [Figure 4: see original paper], in the culture-negative-L group, lymphocytes were the dominant cell population, with proportions significantly higher than in the culture-negative-M group ($P < 0.05$) and highly significantly higher than in the culture-negative-H group ($P < 0.01$). Macrophage proportions were 53.1% and 48.72% in the culture-negative-L and culture-negative-H groups, respectively, while the culture-negative-M group was significantly lower than the other two groups ($P < 0.05$). PMN were the predominant cell populations in the culture-negative-H and culture-negative-M groups (72.48% and 61.32%, respectively), with the culture-negative-L group being significantly or highly significantly lower than the other two groups ($P < 0.05$ or $P < 0.01$).

### 2.4 Milk Yield and Composition Analysis

As shown in , milk yield was 26.92 kg/d with considerable variability (coefficient of variation [CV] = 36.87%). Among milk composition analyses, casein/milk protein ratio showed the least variability (CV = 1.60%). Detailed milk protein components determined by RP-HPLC analysis showed CVs ranging from 15.80% to 26.20%, except for lactoferrin (CV = 53.50%).

### 2.5 Relationship Between Mammary Health Status and Milk Yield, Composition, and Protein Components

As shown in , both culture-positive (contagious, environmental, and opportunistic pathogen groups) and culture-negative (culture-negative-L, -M, and -H groups) conditions affected milk yield, composition, and protein components to varying degrees compared with the healthy group. Among culture-positive samples, contagious pathogens caused the most severe damage; cows infected with contagious pathogens showed significantly decreased milk yield ($P < 0.05$) and significantly reduced casein/milk protein ratio and lactose content ($P < 0.05$). Milk protein components were also adversely affected, with decreases in total protein, casein, S1-casein, and -casein contents, though differences were not significant ($P > 0.05$). Environmental pathogens significantly affected -casein content, causing a significant reduction ($P < 0.05$).

Compared with the healthy group, milk yield in culture-negative samples (culture-negative-L, -H, and -M groups) decreased slightly but not significantly ($P > 0.05$). Lactose content was significantly reduced in culture-negative-H and culture-negative-M groups ($P < 0.05$); casein/milk protein ratio was significantly reduced in culture-negative-H group ($P < 0.05$); pH was significantly elevated in culture-negative-H group ($P < 0.05$). Regarding milk protein components, casein, S1-casein, and -casein contents were significantly reduced in culture-negative-H and culture-negative-M groups ($P < 0.05$), while total protein and S2-casein contents were significantly reduced in culture-negative-H group ($P < 0.05$).

## Discussion

### 3.1 SCC and Somatic Cell Typing in Milk

Diagnosis of intramammary infection (IMI) has traditionally been based primarily on SCC and bacteriological examination. Milk samples with SCC < 100,000 cells/mL are currently considered healthy or within normal physiological ranges [14]. However, recent studies suggest that inflammatory responses may already be occurring in these apparently healthy mammary glands [15]. In reality, SCC is low during the initial stage of inflammatory response until invading pathogens are recognized by immune cells that release chemoattractants, stimulating PMN migration [16]. Somatic cell typing may represent a more detailed method for monitoring mammary inflammatory processes because it can identify changes

in relative cell populations before SCC increases during inflammation.

Our results demonstrate that when SCC > 400,000 cells/mL, PMN proportion increased significantly with SCC, becoming the predominant cell population in the mammary gland and marking obvious IMI. Lymphocytes, as the dominant cell population in healthy mammary glands, decreased significantly with increasing SCC. Macrophages showed no significant correlation with SCC. These findings are consistent with Kester et al. [17]. However, one cell typing study indicated that lymphocyte proportions remained relatively stable throughout the inflammatory process, while macrophage proportions decreased significantly with increasing SCC [18]. Such discrepancies may result from differences among cell typing techniques. Geary et al. [19] investigated technical factors affecting somatic cell typing in milk, showing that sampling vial material significantly affected cell counts, with plastic centrifuge tubes significantly affecting macrophage/lymphocyte ratios in milk by reducing macrophage numbers due to their adhesive properties. Lymphocytes were minimally affected by vial material. Another technical factor was the number of centrifugation steps. The flow cytometry method used in this study required four centrifugation steps. Research has shown that centrifugation frequency significantly affects cell typing results; fewer centrifugation steps result in less lymphocyte loss because these cells have low density, while macrophages can be found in the fat layer and supernatant of centrifuged milk, making their proportions relatively stable and less affected by centrifugation [20]. Somatic cell typing can be obtained by flow cytometry or optical microscopy. Miller et al. [21] reported high correlation between PMN and lymphocyte proportions identified by these two methods, but lower correlation for macrophage and epithelial cell proportions. Our study found no significant changes in macrophage proportions between healthy and diseased mammary glands, while Miller et al. [21] identified macrophages as the predominant cell population in healthy mammary glands. This difference may be explained by different definitions of healthy mammary glands: our study defined health based on SCC, whereas Miller et al. [21] defined healthy glands as those with negative bacteriological results without specifying SCC. Some studies have indicated that epithelial cells are the main cell type found in uninfected mammary glands [22]. Le Roux et al. [23] noted that distinguishing macrophages from epithelial cells presents difficulties, possibly because epithelial cells transform from macrophages that have ingested indigestible bacteria or experienced long-term stimulation by other antigenic substances. Therefore, the distinction between these cell types is not clear-cut, and some macrophages may be counted as epithelial cells during enumeration. Additionally, because the immunological characteristics of epithelial cells remain unclear, this study did not consider the effect of epithelial cells.

Previous research on bovine mammary glands has focused almost exclusively on infected glands, with few studies addressing the immunological status of healthy mammary glands. Our results indicate that within the SCC range of <100,000 cells/mL, only in milk samples with extremely low SCC (13,000–76,000 cells/mL) did lymphocyte proportions reach 59.35%–81.26%, representing the dominant

cell population in healthy mammary glands. When SCC continued to increase to 76,000–100,000 cells/mL, PMN proportion increased dramatically to 82.68%, indicating that inflammation had already begun at levels below the threshold for healthy mammary glands. Leitner et al. [24] reported that when SCC was 6,250–25,000 cells/mL, PMN proportion was only 17.0%, but when SCC increased to 90,000 cells/mL, PMN proportion had already risen to 54.6%. High PMN proportions in milk are considered important indicators of inflammatory response, yet PMN can also successfully defend against pathogens and prevent mastitis. Therefore, our results suggest that early inflammatory processes may already be present when SCC $<$ 100,000 cells/mL. Another factor that may cause increased PMN proportion is stress; however, this study did not consider stress factors because cows were maintained under optimal conditions throughout the sampling process and followed standard milking procedures, showing no obvious stress symptoms. Dos Reis et al. [25] reported that somatic cell typing is an effective tool for identifying inflammatory processes in milk samples with extremely low SCC. Given that lactating mammary glands are continuously subjected to stress and immune system influences, short-term repeatability information for somatic cell typing is crucial for evaluating its applicability in mastitis and inflammation control programs.

### 3.2 Pathogen Status and Somatic Cell Typing in Bovine Mammary Glands

Our bacteriological examination results showed that opportunistic pathogens isolated from the 102 milk samples, namely CNS, accounted for 34.32% of total samples, with corresponding sample SCC of 71,850 cells/mL. This suggests that the significant increase in PMN proportion when SCC $<$ 100,000 cells/mL may be due to the presence of large numbers of CNS, and that CNS may be a potential inflammatory pathogen triggering early mastitis. The high proportion of contagious pathogens, particularly *Staphylococcus aureus*, is consistent with previous research. The average SCC of these samples was 824,280 cells/mL, indicating that contagious pathogens have stronger pathogenicity and persistent presence compared with the other two pathogen types. Approximately 13.72% of cultured samples were culture-negative; however, the average SCC of these samples was 324,600 cells/mL, far exceeding 100,000 cells/mL. A possible explanation is that some cows were in the healing process at sampling, with infections spontaneously eliminated. In such cases, pathogens may be cleared even though inflammatory responses remain active. Mazal et al. [26] reported that because CNS are commonly found on teat skin, some culture-positive samples may result from contamination of mammary skin during milk collection rather than true gland infection. Additionally, false-negative results may occur in bacteriological examination of these milk samples [27]. Some negative bacteriological results may be due to intermittent shedding of pathogens or shedding below detection limits; the presence of antimicrobial agents or other inhibitors in milk may also contribute. In culture-negative mammary glands, most bacteria are phagocytosed or killed, or survive only within host cells, and significant reduction

or cessation of pathogen growth may be another reason for negative bacteriological results [28]. Furthermore, because the milk samples analyzed in this study were composite samples from four quarters, a certain proportion of false-negative results may be due to dilution effects from healthy quarters, preventing detection of 少数菌落 from infected quarters through bacterial culture analysis. Therefore, inflammatory processes can exist even without detectable bacteria, as evidenced by increased SCC and high PMN proportions. This study divided culture-negative samples into three groups based on SCC, suggesting that in high-SCC culture-negative samples, inflammatory status may be at its highest level, but pathogens are engulfed by macrophages and thus cannot be isolated and identified. The interdependence among infection, inflammatory processes, and immune responses in individual mammary glands has been discussed in other literature [29].

### 3.3 Relationship Between Mammary Gland Status and Milk Yield, Composition, and Protein Components

Our results showed that infection with contagious pathogens significantly reduced milk yield in infected cows, while subclinical mastitis infections caused by environmental and opportunistic pathogens did not result in substantial milk production losses. This is consistent with Politis et al. [30]. The effect may be attributed to the pathogenesis of *Staphylococcus aureus*, persistent pathogen presence, and low cure rates. In *S. aureus*-infected quarters, increased protease activity, reduced biosynthesis, and damage to the blood-milk barrier during inflammatory responses contribute to these effects [31]. Additionally, elevated milk protein content was found in milk from mammary glands with *S. aureus*-induced inflammation, possibly due to inflammation-induced influx of soluble proteins from blood into mammary tissue. Pyörälä et al. [32] reported that milk fat, protein, and casein contents were not affected by pathogens causing subclinical IMI. Literature has documented associations between low milk yield and subclinical IMI caused by contagious pathogens and *Streptococcus* spp. In these studies, *E. coli* caused substantial milk yield reductions. However, *S. aureus* and *Klebsiella* spp. also negatively affected milk yield in primiparous and multiparous cows, while CNS infection had no adverse effect on milk production [33].

Piccinini et al. [33] reported no significant changes in milk fat:protein ratio after streptococcal-induced IMI compared with uninfected cows. In contrast, Riollet et al. [34] found that comparisons between infected and uninfected quarters showed varying degrees of changes in total solids, non-fat solids, milk fat, and milk protein contents in IMI caused by different bacteria (*S. aureus*, CNS, *Streptococcus* spp., and *Corynebacterium* spp.). Additionally, higher milk protein contents were found in clinical mastitis caused by staphylococci, streptococci, and *E. coli* due to influx of soluble proteins from blood. Our detailed analysis of milk protein composition by RP-HPLC of skim milk found that whey protein content was not affected by mammary health status, but S1-casein, S2-casein,

and -casein contents were affected. High-SCC milk is characterized by higher proteolytic activity. During innate immune response to infection, activation of the plasminogen system leads to -casein degradation into -casein and proteose-peptone. Therefore, in this study, the apparent inflammatory status in the culture-negative-H group may be associated with greater enzymatic breakdown of casein in high-SCC milk, explaining the approximately 1% reduction in casein/milk protein ratio in culture-negative-H group compared with normal milk. Significantly reduced -casein content was also observed in milk samples infected with environmental pathogens.

Identifying proportions of milk immune cells at different infection stages of bovine mammary glands facilitates more detailed assessment of mammary health. However, because detection technique differences may cause result bias, short-term repeatability of somatic cell typing is an important evaluation criterion. Repeated sampling should also be considered to determine the exact infection stage. Future studies should focus not only on changes in immune cell proportions but also on the concept of immune balance, which is extremely important for ensuring dairy cow health.

## Conclusion

1. Somatic cell classification can provide more detailed analysis of mammary gland health status, particularly for early identification of inflammatory onset in low-SCC milk.
2. The initiation of early mammary inflammation occurs well below the currently accepted SCC threshold for healthy mammary glands (<100,000 cells/mL), and CNS may be a potential pathogen triggering early mammary inflammation.
3. In culture-negative samples, inflammation leads to significant reductions in casein/milk protein ratio and lactose content in high-SCC milk.

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*Note: Figure translations are in progress. See original paper for figures.*