

Postprint: Changes in Milk Somatic Cell Typing and Lactation Performance of Dairy Cows under Different Somatic Cell Counts

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Abstract

This study aimed to evaluate dairy cow lactation performance by investigating the differential characteristics of milk somatic cells under different milk somatic cell counts (SCC). One hundred and two Holstein dairy cows were selected for milk sample collection, and SCC, milk yield, and milk composition were measured. Based on SCC test results, these 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). Milk somatic cells were sorted using flow cytometry to determine the proportions of lymphocytes, macrophages, and polymorphonuclear neutrophils (PMN). The standard plate count method was employed to enumerate total bacterial count, and bacteriological examination was performed to classify and identify bacteria in milk samples. The results indicated: 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 inflammation had already initiated. When SCC > 200,000 cells/mL, PMN proportion was positively correlated with SCC; lymphocyte proportion was negatively correlated with SCC; macrophage proportion showed no significant correlation with SCC. 2) The identified major pathogens included *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), and *Streptococcus agalactiae*; minor pathogens included *Pseudomonas aeruginosa*, *Bacillus*, and *Lactococcus lactis*, among others. 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 milk samples with positive bacterial culture, cows infected with contagious pathogens exhibited significantly

decreased milk yield ($P < 0.05$), and significantly reduced casein/total protein and lactose contents ($P < 0.05$); in milk samples with negative bacterial culture, 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 reduced milk protein content was observed in high SCC milk samples ($P < 0.05$). These findings demonstrate that somatic cell differential analysis can be utilized for more detailed assessment of mammary health status in dairy cows, particularly for earlier identification of inflammatory initiation in low SCC milk. The onset of early inflammatory response in the mammary gland occurs significantly below the currently recognized SCC threshold for healthy mammary gland milk ($<100,000$ cells/mL), and CNS may be a potential pathogen inducing early mammary inflammation. In bacterial culture-negative samples, inflammation caused significant reductions in casein/total protein and lactose contents in high SCC milk.

Full Text

Milk Somatic Cell Classification and Changes in Lactation Performance of Dairy Cows under Different Somatic Cell Counts

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Abstract: This study aimed to evaluate lactation performance in dairy cows by investigating the characteristics of milk somatic cell classification under different somatic cell counts (SCC). Milk samples were collected from 102 Holstein dairy cows to determine SCC, milk yield, and milk composition. Based on SCC 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). Flow cytometry was used to sort somatic cells in milk samples and detect 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 classify and identify bacteria in milk samples. The results showed: 1) Within the SCC range of $<100,000$ cells/mL, when SCC was between 13,000–76,000 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 extremely significantly ($P < 0.01$) to 79.68%, becoming the predominant cell population in the mammary gland and indicating that early inflammation had already begun. When $SCC > 200,000$ cells/mL, the PMN proportion was positively correlated with SCC, while the lymphocyte proportion

was negatively correlated with SCC; no significant correlation was observed between macrophage proportion and 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 extremely 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 bacteriologically positive milk samples, cows infected with contagious pathogens showed significantly decreased milk yield ($P < 0.05$) and significantly reduced casein/milk protein and lactose content ($P < 0.05$). In culture-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 ($P < 0.05$), while milk protein content was significantly reduced in high SCC milk samples ($P < 0.05$). These findings demonstrate that somatic cell classification can be used for more detailed analysis of dairy cow mammary gland health status, particularly for earlier 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 gland milk (<100,000 cells/mL), and CNS may be a potential pathogen triggering early mammary inflammation. In culture-negative samples, inflammation caused significant reductions in casein/milk protein and lactose content in high-SCC milk.

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

Introduction

Diagnosis of bovine mammary gland inflammation, particularly subclinical mastitis, has traditionally relied on somatic cell count (SCC) and bacteriological examination. SCC measures inflammation based on the total number of cells in milk. While bacteriological examination can identify the exact causative agent of infection, 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 a healthy mammary gland as having 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 can vary continuously depending on lactation status, age, milking time and frequency, particularly mastitis 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 cells in milk for diagnosing bovine mammary inflammation. Loken et al. [7] demonstrated that beyond SCC determination, somatic cell differential counts provide a more detailed description of the actual health status of the bovine mammary gland. In the mammary gland, the number and distribution of milk immune cells play important roles in the inflammatory response. Lymphocytes regulate the 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 greatly with the degree of inflammation. Auld et al. [8-9] reported that in healthy milk, macrophages are the main cell type, while lymphocytes are the predominant cell population in healthy mammary glands. Different cell classification techniques can affect somatic cell typing results. Bannerman et al. [10] investigated cell patterns during infections with different pathogens and at different 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 the mammary gland. 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 gland health is closely related to lactation performance, with different pathogens eliciting different immune responses in the mammary gland. Depending on the etiology, significant differences can be observed in both SCC trends and milk composition. Although literature on the relationship between SCC and milk composition is abundant, few studies have addressed the relationship between specific mastitis pathogens and changes in milk composition. Therefore, this study investigated the characteristics of milk somatic cell classification under different SCC levels to provide a more detailed assessment of dairy cow mammary gland 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, or 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 mammary gland was disinfected with a teat dip, cleaned with individual towels,

and then cleaned again with alcohol. After discarding the first three streams of milk, 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 classification, bacteriological examination, and milk composition detection, and all samples were stored at -80°C .

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); incubators [$(36 \pm 1)^{\circ}\text{C}$, $(30 \pm 1)^{\circ}\text{C}$]; refrigerators (-80°C , $2-5^{\circ}\text{C}$); water bath [$(46 \pm 1)^{\circ}\text{C}$]; 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 classification: 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 Classification Somatic cell classification was performed using flow cytometry. Fourteen milliliters of milk were added to a 15 mL centrifuge tube and centrifuged at $690 \times g$ for 10 min. After centrifugation, the upper milk 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 $690 \times g$ 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 cytometry tube for cell counting. One hundred microliters of cell suspension were placed in another 5 mL flow cytometry tube, and 10 μL propidium iodide (PI) was added for viability detection [12].

1.3.3 Total Bacterial Colony Count in Milk Five grams of tryptone, 2.5 g of yeast extract, 1.0 g of glucose, and 15 g of agar were dissolved in 1000 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 1000 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 vortexing 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. When preparing the 10-fold incremental dilutions, 500 μL of sample homogenate was pipetted into sterile agar plates, with two plates per dilution gradient. Simultaneously, 500 μL 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 $(36 \pm 1)^\circ\text{C}$ for 48 h [13].

Total colony counts were enumerated according to GB 4789.2-2010 “National Food Safety Standard - Food Microbiological Examination - Determination of Total Colony Counts.” The calculation formula for total colony count was:

$$N = \frac{\sum C}{(n_1 + 0.1n_2)d}$$

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

1.3.4 Bacteriological Examination of Milk Ten microliters of each milk sample were spread onto blood agar containing 5% defibrinated sheep blood. Plates were incubated aerobically at $(37 \pm 1)^\circ\text{C}$ 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 divided into staphylococci and streptococci by catalase reaction. Coagulase tubes in rabbit plasma were used to differentiate *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 between somatic cell classification and 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 extremely significant.

Results

2.1 SCC and Somatic Cell Classification in Milk

As shown in Table 1 and Table 2, among the 102 milk samples, the average SCC was 351,720 cells/mL, with 44 samples having SCC $< 100,000$ cells/mL (low SCC group), 28 samples with SCC between 100,000-400,000 cells/mL (medium SCC group), and 30 samples with SCC $> 400,000$ cells/mL (high SCC group). Table 2 shows that in 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 \pm 32.44)%]; lymphocyte proportions varying from 3.37% to 91.07% [(48.50 \pm 23.39)%]; and macrophage proportions varying from 2.37% to 55.71% [(21.74 \pm 12.07)%].

Table 1 SCC in milk samples ($n = 102$)

SCC range (cells/mL)	Sample No.
$< 100,000$	
100,000-400,000	
$> 400,000$	

Table 2 Distribution of somatic cell types in milk samples ($n = 102$)

Items	Mean	SD	Minimum	Maximum
SCC (cells/mL)				
Somatic cell types/%				
PMN				
Lymphocyte				
Macrophage				

Extensive cell variation existed in the somatic cell population, particularly for lymphocytes and PMN. Therefore, the correlation between somatic cell classification and SCC was analyzed. As shown in Figure 1 [Figure 1: see original paper]-a, when SCC $< 200,000$ cells/mL, the PMN proportion was low at only 27.4%, increasing significantly with SCC; when SCC $> 400,000$ cells/mL, the

PMN proportion reached 87.29%, becoming the predominant cell population in inflammatory milk samples. Figure 1-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-c shows that macrophage proportions ranged from 2.37% to 55.71%, without strong correlation with SCC ($r = -0.1672$, $P > 0.05$).

Figure 1 The ratios of PMN (a), lymphocyte (b) and macrophage (c) in milk samples ($n = 102$)

To test 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 [Figure 2: see original paper], in healthy mammary glands, the lymphocyte proportion decreased while the PMN proportion increased with elevated milk SCC. The lymphocyte proportions in Groups I–III (59.35%–81.26%) were extremely 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%) showed no significant differences ($P > 0.05$) but were all extremely significantly lower than in Group IV (82.68%) ($P < 0.01$).

Figure 2 Comparison of differential cell counts within the SCC range of healthy mammary glands (SCC < 100,000 cells/mL)

*** indicated extremely significant difference ($P < 0.01$); * indicated significant difference ($P < 0.05$); NS indicated no significant difference ($P > 0.05$). The same as below.

2.2 Total Bacterial Colony Count and Bacterial Species in Milk

As shown in Table 3, significant differences in total bacterial colony counts existed among different SCC groups. Milk samples with SCC < 100,000 cells/mL had total bacterial counts of only 8.08×10^8 CFU/mL; samples with SCC of 100,000–400,000 cells/mL had total bacterial counts of 46.04×10^8 CFU/mL; when SCC > 400,000 cells/mL, total bacterial counts increased sharply to 94.95×10^8 CFU/mL. However, all bacterial counts in milk samples from each SCC group remained within normal ranges.

Among the 102 milk samples tested, 13.72% were bacteriologically negative, 83.34% were culture-positive, and three samples were contaminated. Based on the transmission characteristics of mastitis pathogens in dairy cows, isolated and identified bacteria were classified as contagious, environmental, and opportunistic pathogens. As shown in Table 4, among culture-positive milk samples,

opportunistic pathogens isolated were CNS (accounting for 34.32% of total samples and 39.77% of culture-positive samples). The average SCC in milk samples infected with CNS was 71,850 cells/mL. Contagious pathogens isolated (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 milk 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.

Table 3 Total number of colonies in milk samples (n = 102)

SCC range (cells/mL)	Total bacteria ($\times 10^4$ CFU/mL)
<100,000	
100,000-400,000	
>400,000	

Table 4 Identification and classification of bacteria in milk samples

Items	Numbers	Ratio (%)	Mean SCC (cells/mL)	SD
Contagious				
<i>Staphylococcus aureus</i>		27.45 (31.81)	824,280	
<i>Streptococcus agalactiae</i>		11.76 (13.64)		
<i>S. aureus</i> + <i>Streptococcus dysgalactiae</i>		8.82 (10.22)		
<i>S. aureus</i> + <i>S. agalactiae</i>		2.94 (3.41)		
<i>S. aureus</i> + <i>Enterococcus</i>		1.96 (2.27)		
<i>Streptococcus uberis</i>		0.98 (1.14)		
Environmental				
<i>Proteus</i> spp.		21.57 (25.00)	338,670	
<i>Pseudomonas aeruginosa</i>		0.98 (1.14)		
<i>Klebsiella</i> spp.		1.96 (2.27)		
		6.86 (7.95)		

Items	Numbers (%)	Ratio (%)	Mean SCC (cells/mL)	SD
<i>Bacillus</i> spp.		0.98 (1.14)		
<i>Escherichia coli</i>		7.84 (9.10)		
<i>Lactococcus lactis</i>		1.96 (2.27)		
<i>Aerococcus viridans</i> + CNS		0.98 (1.14)		
Opportunistic		34.32 (39.77)	71,850	
CNS		34.32 (39.77)		
Culture-negative		13.72 (15.90)	324,600	
Contaminated		2.94 (3.41)		

Bacterial proportion data outside parentheses represent the proportion of total samples (n = 102), while data inside parentheses represent the proportion of culture-positive samples (n = 88).

2.3 Different Pathogens and Somatic Cell Classification in Milk

Somatic cell classification 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, *S. aureus*, *E. coli*, *S. agalactiae*) were isolated from 47 samples (46.07%), while secondary pathogens (such as *Bacillus* spp., *P. aeruginosa*, *Klebsiella* spp.) were detected in 24 samples (23.53%).

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

Figure 3 Comparison of somatic cell types in milk samples under different bacterial states

Although only 13.72% of samples were culture-negative, their average SCC was $>200,000$ cells/mL (Table 4). Therefore, further somatic cell classification 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 [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 extremely 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 extremely significantly lower than the other two groups ($P < 0.05$ or $P < 0.01$).

Figure 4 Comparison of somatic cell types in culture-negative milk samples

2.4 Milk Yield and Composition Analysis

As shown in Table 5, milk yield was 26.92 kg/d with considerable variability (coefficient of variation [CV] = 36.87%). Among milk composition analyses, casein/milk protein showed the least variability (CV = 1.60%). Detailed milk protein components determined by RP-HPLC showed CVs ranging from 15.80% to 26.20%, except for lactoferrin (CV = 53.50%).

Table 5 Analysis of milk production, milk composition and milk protein composition

Items	Mean	CV (%)	Minimum	Maximum
Milk yield (kg/d)				
Milk composition				
Milk fat (%)				
Milk protein (%)				
Fat:protein ratio				
Casein (%)				
Casein/milk protein (%)				
Urea (mg/kg)				
Lactose (%)				
Milk protein composition (g/L)				
Total protein				

Items	Mean	CV (%)	Minimum	Maximum
Whey protein				
Casein				
S1-casein				
S2-casein				
-casein				
-casein				
Lactalbumin				
Lactoglobulin				
Lactoferrin				

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

As shown in Table 6, both bacteriologically positive (contagious, environmental, and opportunistic pathogen groups) and culture-negative (culture-negative-L, culture-negative-M, and culture-negative-H groups) statuses affected milk yield, composition, and protein components to varying degrees compared with the healthy group. Among bacteriologically positive milk samples, contagious pathogens caused the greatest harm; cows infected with contagious pathogens showed significantly decreased milk yield ($P < 0.05$) and significantly reduced casein/milk protein and lactose content ($P < 0.05$). Although milk protein, casein, S1-casein, and -casein contents decreased, the differences were not significant ($P > 0.05$). Environmental pathogens significantly affected -casein content, causing a significant reduction ($P < 0.05$).

In culture-negative milk samples (culture-negative-L, culture-negative-H, and culture-negative-M groups), milk yield decreased slightly compared with the healthy group, but the changes were not significant ($P > 0.05$). Lactose content decreased significantly in the culture-negative-H and culture-negative-M groups ($P < 0.05$), casein/milk protein decreased significantly in the culture-negative-H group ($P < 0.05$), and pH increased significantly in the culture-negative-H group ($P < 0.05$). Regarding milk protein components, casein, S1-casein, and -casein contents decreased significantly in the culture-negative-H and culture-negative-M groups ($P < 0.05$), while milk protein and S2-casein contents decreased significantly in the culture-negative-H group ($P < 0.05$).

Table 6 Relationship between mammary gland health status and milk yield, milk composition and protein composition of dairy cows

Items	Healthy	Culture-negative-M	Culture-negative-H	Contagious	Environmental	Opportunistic
Milk yield (kg/d)						

Items	Culture- negative- Healthy	Culture- negative- M	Culture- negative- H	Contagious Enzootic	Iron Contagious	Opportunistic	Stic
Milk composition							
Milk fat (%)							
Milk protein (%)							
Fat:protein ratio							
Casein (%)							
Casein/milk protein (%)							
Urea (mg/kg)							
Lactose (%)							
Milk protein composition (g/L)							
Total protein							
Whey protein							
Casein							
S1-casein							
S2-casein							
-casein							
-casein							
Lactalbumin							
Lactoglobulin							
Lactoferrin							

Values in the same row with the same or no small letter superscripts mean no significant difference ($P > 0.05$), while different small letter superscripts mean significant difference ($P < 0.05$).

Discussion

Diagnosis of intramammary infection (IMI) in dairy cows 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 have shown that inflammatory responses may already be occurring in these apparently healthy mammary

glands [15]. In fact, SCC is low during the initial stage of inflammatory response until invading pathogens are recognized by immune cells that release chemoattractants, thereby stimulating PMN migration [16]. Somatic cell classification may be a more detailed method for monitoring the mammary inflammatory process because it can identify changes in relative cell populations before SCC increases during inflammation.

3.1 SCC and Somatic Cell Classification in Milk

The results of this study showed that when $SCC > 400,000$ cells/mL, the 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 results are consistent with Kester et al. [17]. However, one cell classification study indicated that lymphocyte proportions remained relatively stable throughout the inflammatory process, while macrophage proportions decreased significantly with increasing SCC [18]. These different results may be due to differences between cell classification techniques. Geary et al. [19] investigated the influence of technical factors on somatic cell classification in milk, showing that sampling bottle material significantly affected cell counts. Plastic centrifuge tubes significantly affected the macrophage/lymphocyte ratio in milk by reducing macrophage numbers because macrophages are adhesive and can adhere to plastic surfaces. Lymphocytes were barely affected by sampling bottle material. Another technical factor was the number of centrifugation steps for milk samples. The combination of milk samples and monoclonal antibodies used in this flow cytometry protocol required four centrifugation steps. Studies have shown that the number of centrifugation steps significantly affects cell classification results. Fewer centrifugation steps resulted in less lymphocyte loss because these cells have low density; macrophages could be found in the milk fat layer and supernatant of centrifuged milk, so macrophage proportions were relatively stable and rarely affected by centrifugation [20]. Somatic cell classification can be obtained by flow cytometry or optical microscopy. Miller et al. [21] reported that the correlation for PMN and lymphocyte proportions between these two methods was high, while the correlation for macrophage and epithelial cell proportions was low. This study found no significant changes in macrophage proportions between healthy and diseased mammary glands. Miller et al. [21] showed that macrophages were the main cell population in healthy mammary glands. The difference between these two results can be explained by different definitions of healthy mammary glands. This study defined healthy mammary glands from the SCC perspective, while Miller et al. [21] defined healthy mammary glands based on negative bacteriological examination results without specifying any SCC threshold. Other 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 and epithelial cells is difficult because epithelial cells may transform from macrophages that have

phagocytosed indigestible bacteria or received long-term stimulation from other antigenic substances. Therefore, the distinction between the two is not clear, and some macrophages may be counted as epithelial cells during counting. Additionally, because the immunological characteristics of epithelial cells are not well understood, this study did not consider the influence of epithelial cells.

Previous studies on bovine mammary glands have almost exclusively focused on infected glands, while the immune status of healthy mammary glands has rarely been addressed. The results of this study showed 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, the PMN proportion increased extremely significantly to 82.68%, indicating that inflammation had already begun at levels below the threshold for healthy mammary glands. Leitner et al. [24] showed that when SCC was 6,250–25,000 cells/mL, the PMN proportion was only 17.0%, but when SCC increased to 90,000 cells/mL, the PMN proportion had already risen to 54.6%. High PMN proportions in milk are considered important indicators of inflammatory response, while PMN can also successfully defend against pathogens and prevent mastitis. Therefore, the results of this study indicate that early inflammatory processes may already be present when SCC < 100,000 cells/mL. Another factor that may cause increased PMN proportions is stress; however, this study did not consider stress factors because cows were maintained under optimal conditions throughout the sampling process and followed strict milking procedures, so cows showed no obvious stress symptoms. Dos Reis et al. [25] reported that somatic cell classification 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 on somatic cell classification is crucial for evaluating its applicability in mastitis and inflammation control programs.

3.2 Pathogen Status and Somatic Cell Classification in Bovine Mammary Glands

The bacteriological examination results of this study showed that among the 102 milk samples, opportunistic pathogens isolated were CNS, accounting 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 amounts of CNS, and CNS may be a potential inflammatory pathogen triggering early mastitis. The high proportion of contagious pathogens, particularly *Staphylococcus aureus*, is consistent with previous research findings. 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; how-

ever, 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 the time of sampling, with infections spontaneously eliminated. In such cases, pathogens may be cleared even though the inflammatory response remains active. Mazal et al. [26] showed that because CNS are typically found on teat skin, some culture-positive samples may result from contamination of mammary skin during milk sample collection rather than true gland infection. Additionally, false-negative results may occur in the bacteriological examination of these milk samples [27]. Some negative bacteriological results may be due to intermittent shedding of pathogens or shedding below the detection limit of the method; the presence of antimicrobial agents or other inhibitors in milk may also contribute. In bacteriologically negative mammary glands, most bacteria are phagocytosed or killed, or can only survive within host cells; substantial reduction or cessation of pathogen growth may be another reason for negative bacteriological results [28]. Furthermore, because composite milk samples from four quarters were analyzed in this study, a certain proportion of false-negative results may be due to dilution effects from healthy quarters, preventing detection of a few colonies from infected quarters by bacteriological 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 levels. In high-SCC culture-negative samples, the 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

This study found 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 the findings of Politis et al. [30]. This 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 production losses [31]. Additionally, elevated milk protein content was found in milk from *S. aureus*-induced mastitis, possibly due to inflammation causing soluble proteins to flow from blood into the mammary gland. 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 *Streptococcus*-induced IMI compared with uninfected cows. In contrast, Rioulet et al. [34] found that comparisons between infected and uninfected quarters showed that IMI caused by different bacteria (*S. aureus*, CNS, *Streptococcus* spp., and *Corynebacterium* spp.) resulted in varying degrees of changes in total solids, solids-not-fat, milk fat, and milk protein content. Additionally, due to the influx of soluble proteins from blood, milk protein content was higher in clinical mastitis caused by staphylococci, streptococci, and *E. coli*.

Detailed analysis of milk protein composition by RP-HPLC of skim milk in this study revealed that whey protein content was not affected by mammary gland health status, but S1-casein, S2-casein, and κ -casein contents were affected. High-SCC milk is characterized by higher proteolytic activity. During innate immune response infection in the mammary gland, activation of the plasminogen system leads to κ -casein degradation into α -casein and peptones. Therefore, in this study, the obvious inflammatory status in the culture-negative-H group may be related to greater enzymatic decomposition of casein in high-SCC milk, explaining the approximately 1% reduction in casein/milk protein in the culture-negative-H group compared with normal milk. Significantly reduced κ -casein content was also observed in milk samples infected with environmental pathogens.

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

Conclusion

1. Somatic cell classification can be used for more detailed analysis of dairy cow mammary gland health status, particularly for earlier 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 gland milk (<100,000 cells/mL), and CNS may be a potential pathogen triggering early mammary inflammation.
3. In culture-negative samples, inflammation caused significant reductions in casein/milk protein and lactose content in high-SCC milk.

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