



# Cellular and soluble components decrease the viable pathogen counts in milk from dairy cows with subclinical mastitis

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**ABSTRACT.** The present study was undertaken to clarify the factors that reduce the viable pathogen count in milk collected from the udders of subclinical mastitic cows during preservation. Milk was centrifuged to divide somatic cells (cellular components, precipitates) and antimicrobial peptides (soluble components, supernatants without fat layer); each fraction was cultured with bacteria, and the number of viable bacteria was assessed prior to and after culture. In 28.8% of milk samples, we noted no viable bacteria immediately after collection; this value increased significantly after a 5-hr incubation of milk with cellular components but not with soluble components (48.1 and 28.8%, respectively). After culture with cellular components, the numbers of bacteria (excluding *Staphylococcus aureus* and *Streptococcus uberis*) and yeast decreased dramatically, although the differences were not statistically significant. After cultivation with soluble components, only yeasts showed a tendency toward decreased mean viability, whereas the mean bacterial counts of *S. uberis* and *T. pyogenes* tended to increase after 5-hr preservation with soluble components. These results suggest that most pathogens in high somatic cell count (SCC) milk decreased during preservation at 15 to 25°C, due to both the cellular components and antimicrobial components in the milk. Particularly, the cellular components more potently reduced bacterial counts during preservation.

**KEY WORDS:** antimicrobial components, dairy cow, milk, pathogen, somatic cell count

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Mastitis is an inflammation of the udder in cows, generally caused by bacterial infection. It is the costliest infectious disease in the dairy industry, because it causes decreases in milk quality and quantity, and the death of cows [17, 20]. Bacterial examination of milk samples is important to determine the clinical treatment method for the mastitis. However, approximately 10 to 40% of milk samples from mastitic udders show no notable pathogens [6, 15, 16]. The time from milking to culture is usually several hours. Hisaeda *et al.* [6] reported that the viable bacterial count in milk from cows with subclinical mastitis decreased during a 5-hr preservation period at room temperature after milking. This reduction was observed in coliforms, *Corynebacterium bovis*, yeasts, coagulase-negative staphylococci (CNS) and streptococci, with the exception of *Streptococcus agalactiae* and *Streptococcus uberis* (ST). The mammary gland is protected by defense systems, such as innate and acquired immunity. The innate immune mechanisms of protection include  $\beta$ -defensins, lactoferrin, lactoperoxidase, neutrophil and macrophages [4, 8, 10–13, 25]. Reductions in milk bacterial counts during preservation are reportedly related to lingual antimicrobial peptide (LAP, a  $\beta$ -defensin) and lactoferrin concentrations, lactoperoxidase activity and high somatic cell counts (SCC containing neutrophil and macrophages) [6]. However, the relationship between bacterial count reduction and innate immune components (both cellular and soluble components) has not been fully clarified. The objective of the present study was to clarify the factors that change the viable pathogenic bacterial counts in milk collected from the udders of subclinical mastitic cows during preservation.

## MATERIALS AND METHODS

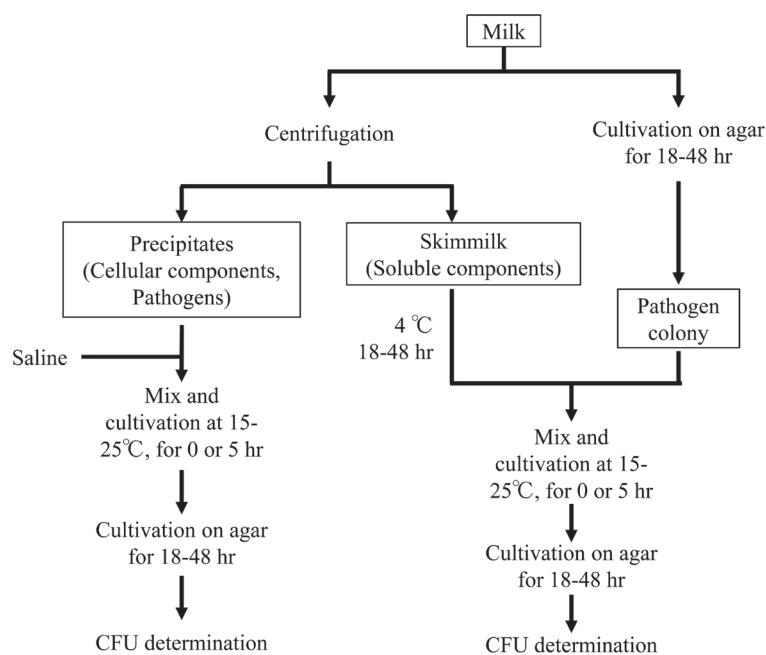
Thirty-eight Holstein Friesian cows (52 quarters) from 9 farms were enrolled in this study. The cows were managed with tie stalls in 7 farms, a free barn in 1 farm and free stalls in 1 farm. This study was performed in accordance with the regulations of the

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**Fig. 1.** Flowchart of milk handling. Cultivation on agar was carried out under both aerobic and anaerobic condition. CFU: colony forming unit.

Hiroshima University Animal Research Committee.

The quarter milk collected from cows with no clinical symptom of mastitis, was subjected to the California Mastitis Test (CMT) before collection, and only CMT-positive milk was collected. The SCC in milk was measured with fluorescence optical somatic cell-measuring equipment (SomaScope Series; Milestone-General, Kawasaki, Japan). CMT-positive milk with SCC >300,000/ml, classified as coming from a cow with subclinical mastitis, was used in the present study. The collected quarter milk was divided into 2 parts as shown in Fig. 1. One part of the milk (1 ml) was subjected to centrifugation (3,000 ×g, 15 min, 4°C), and the skim milk was kept at 4°C for 18 to 48 hr until use, as described below. The pellet (cellular components), containing leukocytes and pathogens, was resuspended in 1 ml/saline without adjusting pathogen number and kept at 15 to 25°C for 0 or 5 hr. Then, this solution, containing bacteria, was plated onto 5% sheep blood agar (BBL, Tokyo, Japan) and cultured at 37°C for 18 to 48 hr to determine the number of colony forming units (CFUs). The other part of the milk was plated onto 5% sheep blood agar and cultured at 37°C for 18 to 48 hr. The pathogens isolated from the developed colonies were dissolved in skim milk (soluble components, prepared using the first part of the milk), kept at 15 to 25°C for 0 or 5 hr and then plated onto 5% sheep blood agar followed by culture at 37°C for 18 to 48 hr to determine the number of CFUs. *S. aureus* and *S. uberis* were identified by a positive coagulase test using rabbit serum (Usagi plasma EIKEN; EIKEN Chemical, Tokyo, Japan) and an identification kit (MIYARISAN Medicine manufacture, Tokyo, Japan), respectively. *Bacteroides* spp. were identified by gram-staining method following anaerobic culture using AnaeroPack-Anaero (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) at 37°C for 18–24 hr. Other pathogens were identified in accordance with the law, as described elsewhere [7].

The proportions of pathogen-free samples were compared by chi square analysis between groups. The mean ratio of the viable pathogen count after 5 hr of cultivation was compared to that at 0 hr with the nonparametric Wilcoxon signed-rank test. A probability value of  $P < 0.05$  was considered statistically significant.

## RESULTS

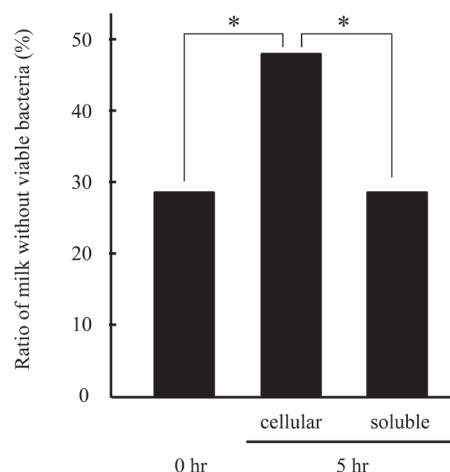
The pathogens derived from the high SCC milk samples from the cows with subclinical mastitis are shown in Table 1. *S. uberis*, *S. aureus* and CNS were detected in more than 10% of the milk samples. Other microbes, including *Nocardia farcinica*, *Pseudomonas aeruginosa* and *Bacteroides* spp., were also detected. In 28.8% of milk samples, no viable pathogen growth was noted immediately after collection. In these samples, no viable pathogen was also detected just after the resuspension of precipitates with saline. However, the proportion of bacteria-free samples increased significantly after 5-hr incubation with milk cellular components but not soluble components ( $P < 0.05$ , 48.1 and 28.8%, respectively, Fig. 2).

The mean ratio of the viable bacterial count after 5 hr of cultivation to the count at 0 hr is shown in Fig. 3a. The viable bacterial counts of ST, CNS, coliforms, *T. pyogenes* and yeasts cultured with cellular components were lower at 5 hr than those at 0 hr, but the differences were not significant. After incubation in the presence of soluble components, only the viable cell counts for yeasts decreased, whereas the counts for *S. uberis* and *T. pyogenes* increased. The viable bacterial counts of *Bacteroides* spp. after incubation with either cellular or soluble components were 0 ( $n=1$ ). The viable bacterial counts of *P. aeruginosa* and *N. farcinica*

**Table 1.** Pathogens derived from high somatic cell count milk from cows with subclinical mastitis

Pathogen	Number (%) of milk samples
Coliforms	3 (5.8)
<i>Streptococcus uberis</i>	10 (19.2)
ST	4 (7.6)
<i>Staphylococcus aureus</i>	7 (13.5)
Coagulase-negative staphylococci	6 (11.5)
Yeasts	2 (3.8)
<i>Trueperella pyogenes</i>	2 (3.8)
Others	3 (5.8)
None detected	15 (28.8)

ST: streptococci except for *Streptococcus agalactiae* and *Streptococcus uberis*.



**Fig. 2.** Ratio of milk without viable pathogen before and after preservation for 5 hr with cellular or soluble components. \*Significant differences between two ratios ( $P < 0.05$ ).

did not decrease during cultivation with cellular components (n=1 each).

Percentages of each category of viable pathogen counts after 5-hr cultivation compared with the count before cultivation are shown in Fig. 3b. In all bacteria cultured with cellular or soluble components, except for *T. pyogenes* cultured with soluble components, most samples had decreased bacterial counts after incubation; in particular, 100% of samples had lower counts of ST, *T. pyogenes* and yeasts. For *S. aureus*, half samples cultured with cellular components, had a viable bacterial count ratio <1 after cultivation.

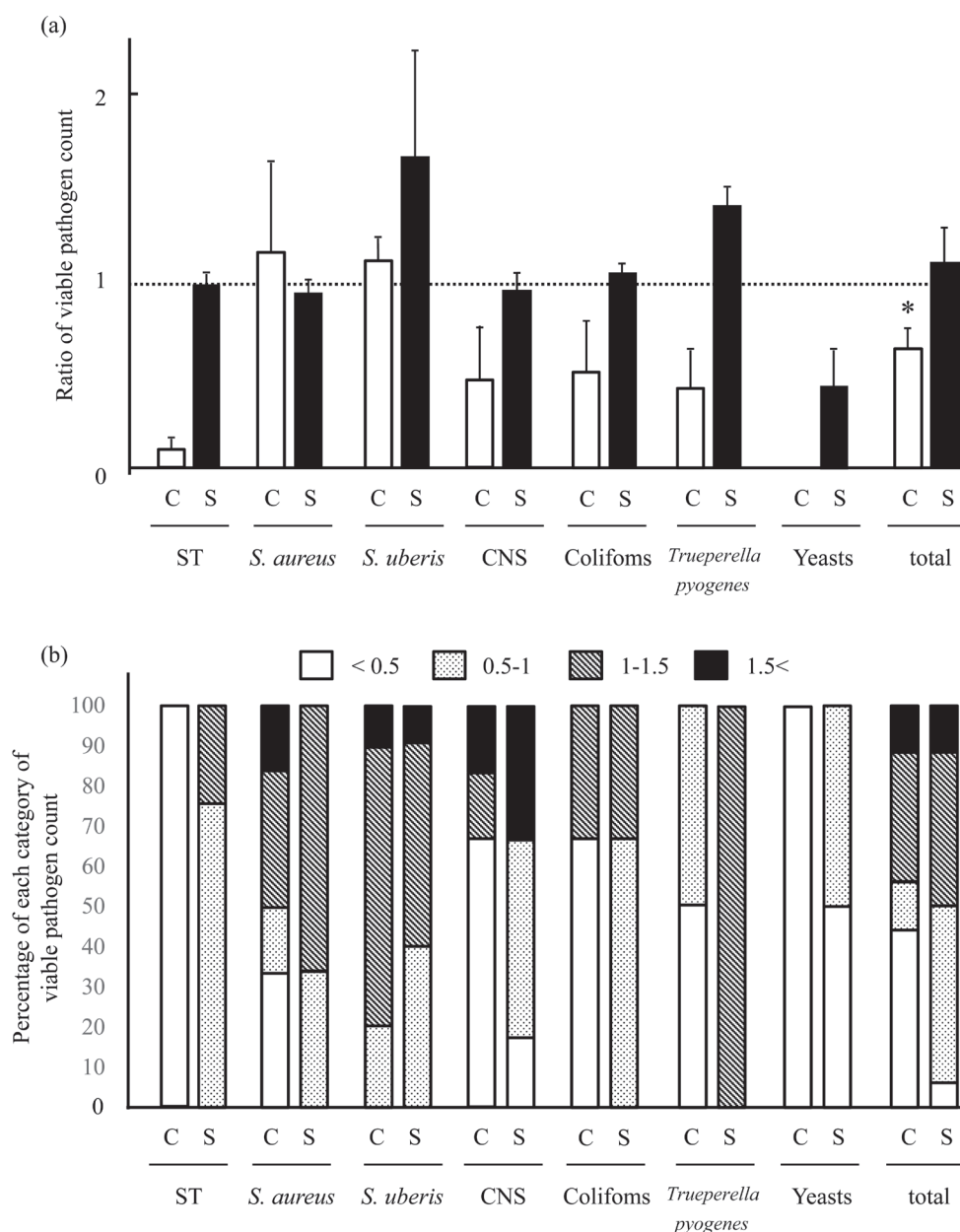
## DISCUSSION

In 28.8% of milk samples, no viable bacterial growth was noted immediately after collection. This result agrees with previous reports [6, 18, 19]. Hisaeda *et al.* [6] reported that this percentage increases after the preservation of milk for 0.5 to 5 hr at room temperature, and suggested that this increase may be associated with the SCC and/or antimicrobial components of the milk. There are many kinds of antimicrobial components in bovine and goat's milk, and their concentrations are elevated by mastitis [9, 10, 12–14, 23–25]. The concentrations of some antimicrobial components in milk may be related to the reduction in viable bacteria during preservation [6]. However, there is no direct evidence that the SCC and antimicrobial components affect viable bacterial counts. Therefore, in the present study, we centrifuged milk to divide the cellular components (somatic cells) and soluble components (antimicrobial peptides), which we then cultured with microbes. The ratio of milk without viable bacteria increased significantly after incubation with milk cellular components but not soluble components (Fig. 2, 48.1 vs. 28.8%). When the viable cell counts were compared before and after cultivation, significantly lower counts were detected in only the milk cultured with cellular components, not with soluble components (Fig. 3a, total). This result suggests that the cellular components contributes more than the antimicrobial components to the reduction in pathogens during preservation.

When bacteria were cultured with milk cellular components, the numbers of ST and yeast tended to decrease. All samples infected with ST and yeast had decreased microbes counts after cultivation. These pathogens appear to have higher susceptibilities to the milk-derived cells than other bacteria. Hisaeda *et al.* [6] reported the same phenomenon for yeasts, although they found that ST had no such susceptibility. This difference may be due to the small number of samples with ST in the present study. Dectin on the cell membranes of leukocytes is reported to be the receptor for yeasts [1, 22]. Therefore, yeast may be recognized by these dectins, resulting in phagocytosis by leukocytes.

For CNS and coliforms cultured with cellular components, although we did not observe significant differences, we found that their ratios of viable bacterial counts at 5 hr compared with 0 hr had means <1. Most the samples of these bacteria cultured with cellular components had ratios of <1. Therefore, these bacteria must be susceptible to the cellular components in milk.

When *S. aureus* and *S. uberis* were cultured with either milk cellular or soluble components, the mean ratios of bacteria were near or >1, suggesting that these bacteria had no or low susceptibility to milk components, such as somatic cells and antimicrobial components. Hisaeda *et al.* [6] also found that the bacterial counts of *S. aureus* and *S. uberis* were unchanged during preservation for 5 hr. *S. uberis* has the ability to form biofilms, and *S. uberis* adhesion molecule (SUAM), which mediates its adhesion and invasion into mammary epithelial cells (MECs) and lymphocytes, helps it evade phagocytosis and killing by polymorphonuclear cells and lymphocytes [5, 21] or lactoferrin, an antimicrobial component [2]. *S. uberis* decreases immune gene expression in MECs, which, in turn, reduces the host immune response [15]. *S. aureus* produces phenol-soluble modulins (PSMs) as virulence



**Fig. 3.** The mean ratio of the viable pathogen count after 5-hr cultivation compared with the count before cultivation (a). Dotted line expresses the ratio=1. Percentages of each category of viable pathogen counts after 5-hr cultivation compared with the count before cultivation. Viable pathogen counts are classified into one of four categories as <0.5, 0.5 to 1, 1 to 1.5 and 1.5<(b). Pathogens were cultured with either milk cellular (C) or soluble components (S). \*Significant difference between bacterial numbers before and after cultivation ( $P < 0.05$ ).

factors, which have potent cytolytic activity against many cell types, including neutrophils and monocytes. Furthermore, PSMs decrease the expression of several defensin genes in epithelial cells and inhibit the production of IL-6, IL-8 and IL-32 in bovine MECs, resulting in attenuation of antibacterial function [3]. These components of *S. uberis* and *S. aureus* may prevent the bacteria from being attacked by immune cells and antimicrobial components.

Only yeasts had a mean ratio <1 after cultivation with soluble components, whereas ratios near or >1 were observed for bacteria (Fig. 3a). However, many samples showed less than 1 ratio compared between before and after preservation in bacteria, except for yeasts (Fig. 3b). Therefore, the soluble components in milk had antimicrobial activity, although their activity appeared to be lower than the cellular components. Alternatively, the bactericidal activity of the antimicrobial components may depend on the strains of bacteria in the milk.

The ratio of the bacterial counts before and after cultivation largely depends on the species of the pathogens, whereas pathogen count, such as ST and yeasts, was reduced dramatically by cellular and antimicrobial components. Therefore, it has been suggested

that the cultivation of milk for pathogen identification should be started immediately after milk collection. In the present study, the milk samples were cultured at 15 to 25°C; preservation at low temperature attenuates the activity of antimicrobial components, which may prevent decreases in bacterial counts during preservation. A further study is required to investigate the effects of various temperatures for milk preservation on the changes in bacterial counts.

In 28.8% of milk samples, no viable bacterial growth was noted immediately after collection. This result suggests that bovine inflammatory mediators or innate immune factors, but not bacteria, may be the cause of subclinical mastitis.

In conclusion, these results suggest that the numbers of most pathogens in high-SCC milk decreased during preservation at room temperature, which is due to both the cells and antimicrobial components in milk. However, the cellular components are more potent in reducing bacterial counts during preservation. A further study is required to find methods to keep pathogen numbers in milk during preservation.

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