FULL PAPER *Physiology*

Change in viable bacterial count during preservation of milk derived from dairy cows with subclinical mastitis and its relationship with antimicrobial components in milk

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ABSTRACT. The objectives of the present study were to investigate the change in the number of viable pathogens during preservation of milk obtained from cows with subclinical mastitis and the association between the decreasing ratio of viable bacteria during preservation and the somatic cell count (SCC) and the values of lingual antimicrobial peptide (LAP), lactoferrin (LF) and lactoperoxidase (LPO). After preservation of milk at room temperature for 0, 0.5, 1, 2, 3, 4 and 5 hr, the bacterial colonies in the milk were counted to determine the number of colony forming units (CFUs). Fresh skim milk was used to determine the values of LAP, LPO and LF. Bacteria were not detected in 19.4% of milk samples, and this percentage increased up to 30% after 5 hr of preservation. The number of *Staphylococcus aureus* and *Streptococcus uberis* in milk did not change significantly during the 5-hr incubation, whereas significant decreases were observed in the number of coliforms, coagulase-negative staphylococci, yeasts and *Corynebacterium bovis*. High SCC significantly decreased CFUs of *S. aureus* and yeast after preservation of milk for 4 to 5 hr. High LF concentration in milk was associated with decrease in CFU of *S. aureus* during 4-hr preservation. These results suggest that the viable counts of some pathogens in milk decreased during preservation at room temperature after collection, which may be attributed to the leukocytes and antimicrobial components present in milk.
KEY WORDS: dairy cow, milk, pathogen, preservation, somatic cell count

Mastitis is an inflammation of the udder, typically caused by bacterial infection in bovine and other animals. It reduces milk production, consequently incurring economic losses for the dairy industry. Identification of causal bacteria is necessary to treat mastitis. Therefore, bacteriological examination of milk is important to determine the clinical treatment for mastitis. However, approximately 10–40% of milk samples collected from mastitic cows show "no significant bacterial growth" in routine clinical culture assays, the exact reason for which is currently unknown [18, 19]. It could be attributed to infection caused by bacteria present in low numbers, without a reduction in the somatic cell count (SCC). Other influencing factors include the sampling procedure, treatment of milk samples, media used in the bacteriological examination, presence of pathogens below current detection thresholds or absence of bacteria at culture initiation; alternatively, the mastitis may be caused by non-bacterial microorganisms [7, 15]. Since the selection of antibiotics largely depends on the bacterial species infecting the milk, it is important to clarify why the milk tested negative for pathogens.

The innate immune function of the bovine mammary gland is highly developed in order to protect it from invading pathogens. Different antimicrobial components (one of the innate immune factors), such as lingual antimicrobial peptide (LAP), cathelicidins, lactoferrin (LF), lactoperoxidase (LPO) and S100 protein, are produced in mammary epithelial cells and leukocytes and secreted in milk [6, 8, 9, 11, 20, 24, 26, 27]. Their concentrations in mastitic milk are higher than those in healthy milk [4, 10, 13, 16, 26, 27]. LAP exhibited antimicrobial activity against gram-positive and –negative bacteria and fungi [23]. LF has antimicrobial activity against *E. coli* and *Staphylococcus aureus* (*S. aureus*) [14]. Milk LPO catalyzes, in the presence of hydrogen peroxide, the oxidation of thiocyanate (SCN^-) to yield hypothiocyanite ($OSCN^-$) and hypothiocyanous acid (HOSCN) [22]. These compounds are effective against staphylococci and *E. coli* [12].

The process from milking to culturing spans several hours, and it may be possible that the pathogens present in milk are killed by the antimicrobial components during this intervening period. However, the possibility of this phenomenon remains to be elucidated. Therefore, the objective of the present study was to investigate the change in the number of live pathogenic microbes during preservation of milk collected from udders of mastitic cattle, and the association between the number of viable pathogens and the SCC and the levels of LAP, LPO and LF.

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MATERIALS AND METHODS

Animals: In total, 62 Holstein Friesian cows (77 quarters; 2–38 weeks postpartum) grazed in 12 private dairy farms were included in the study. The cows were managed with tie stalls in 10 farms, a free barn in one farm and free stalls in one farm. This study was performed in accordance with the regulations of Hiroshima University Animal Research Committee.

Milk collection: The quarter milk was subjected to California Mastitis Test (CMT) before collection, and only CMT-positive milk was collected from cows with no clinical symptoms of mastitis. The SCC in milk was measured by a fluorescence optical somatic cell-measuring equipment (SomaScope Series; Milestone-General, Kawasaki, Japan). The CMT-positive milk with an SCC >300,000/m*l* (indicative of subclinical mastitis) was used in the present study (n=77). Milk fat was removed following centrifugation (3,000 × *g*, 15 min and 4°C), and skim milk was frozen at −20°C until the measurement of innate immune factors. The remaining mastitic milk was kept at 15 to 25°C for 0, 0.5, 1, 2, 3, 4 and 5 hr. Thereafter, 50 *µl* of the milk was plated onto 5% sheep blood agar (BBL, Tokyo, Japan) and cultured at 37°C for 18–48 hr to determine the count colony forming units (CFUs). *S. aureus* was identified by a positive coagulase test using rabbit serum (Usagi plasma EIKEN; EIKEN Chemical Co., Ltd., Tokyo, Japan). *Streptococcus uberis* (*S. uberis*) was identified using an identification kit (MIYARISAN Medicine manufacture Co., Ltd., Tokyo, Japan). Other pathogen identification was conducted in accordance with the law described elsewhere [7].

Measurement of innate immune factors: The concentration of LAP in skim milk was measured as previously described [11], LF concentration was measured by an ELISA quantification set, as per the manufacturer's protocol (Bethyl Laboratories, Inc., Montgomery, TX, U.S.A.), and LPO activity was determined as described [9].

Statistics: Nonparametric data were compared using the Kruskal-Wallis test and Friedman's test, while Dunnett's single diffusion and Bonferroni's single diffusion were used to compare the differences among groups with parametric data. Correlation analysis was performed using Spearman's rank correlation coefficient in case of nonparametric data, and Pearson's moment correlation analysis in case of parametric data. $P<0.05$ was considered statistically significant.

RESULTS

As shown in Table 1, S. *uberis* was detected in 10.4% of milk samples with high SCC, although more than 25% of the milk samples contained streptococci except for *S. agalactiae* and *S. uberis* (ST). Coliforms were observed in 14.2% of milk samples. *S. aureus* and coagulase-negative staphylococci (CNS), Yeast and *Corynebacterium bovis* (*C. bovis*) were also detected in less than 10% of milk samples.

In 19.4% of milk samples, no viable bacterial growth was noted just after collection. However, the percentage of milk samples without viable bacteria increased to 30% after pres-

Table 1. Pathogens derived from milk with high somatic cell count

Pathogen	Number (%) of milk samples
Coliforms	11 (14.2)
Streptococcus uberis	8 (10.4)
ST	20 (25.9)
Staphylococcus aureus	7 (9.0)
Coagulase-negative staphylococci	7 (9.0)
Yeasts	5 (6.5)
Corynebacterium bovis	4 (5.1)
Not detected	15 (19.4)
Total	77

Milk samples were collected from 77 quarters in 62 Holstein Friesian cows. ST: streptococci except for *S. agalactiae* and *S. uberis*.
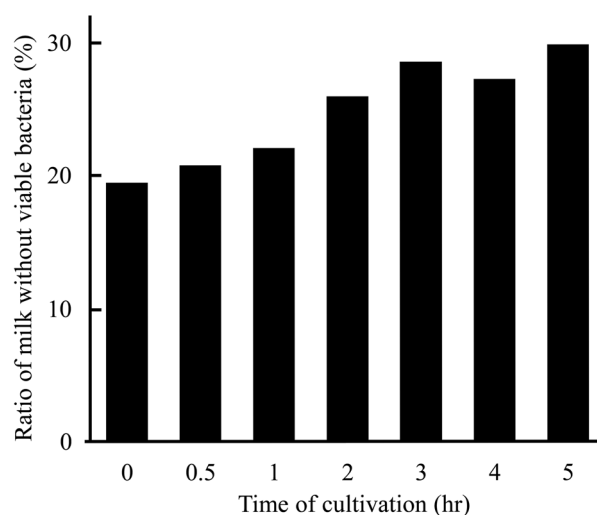

Fig. 1. Changes in the percentage of milk samples without viable bacteria in milk during preservation. Bars denote the percentage of milk samples without viable bacteria (n=77).

ervation for 3–5 hr (Fig. 1).

The changes in the CFUs of different pathogens in milk during preservation are shown in Figs. 2 and 3. The CFUs of coliforms tended to decrease during preservation and showed a significant decrease to 49 ± 14% at 4 hr (Fig. 2). In *C. bovis*, yeast and CNS, CFUs decreased significantly to less than 60% even at 30 min (47 ± 9%, 45 ± 16% and 61 ± 17%, respectively) and continued to decrease thereafter, resulting in <20% CFUs at 5 hr (12 ± 7%, 9 ± 9% and 20 ± 11%, respectively, Figs. 2 and 3). The CFUs of ST decreased significantly when milk was preserved for longer than 1 hr (Fig. 3). The CFUs of *S. uberis* tended to decrease during preservation; however, no significant difference was observed. The CFUs of SA showed no significant reduction during preservation.

When the correlation among SCC, LAP and LF concentrations, and LPO activity was analyzed in all milk samples,
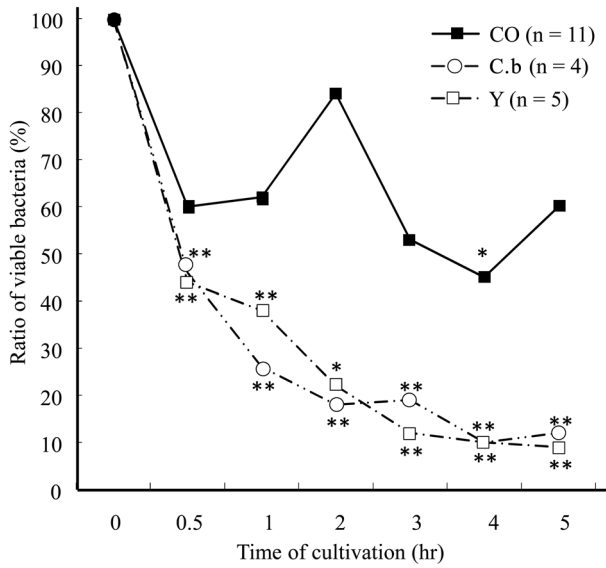
Fig. 2. Changes in the number of viable pathogens that decreased significantly during preservation. The vertical axis represents the ratio of viable pathogen number at each preservation time to the number at the onset of cultivation. CO: Coliforms, C.b: *Corynebacterium bovis*, Y: Yeasts *,**: Significant differences *vs.* viable organism counts at 0 hr (**P<0.01, *P<0.05).
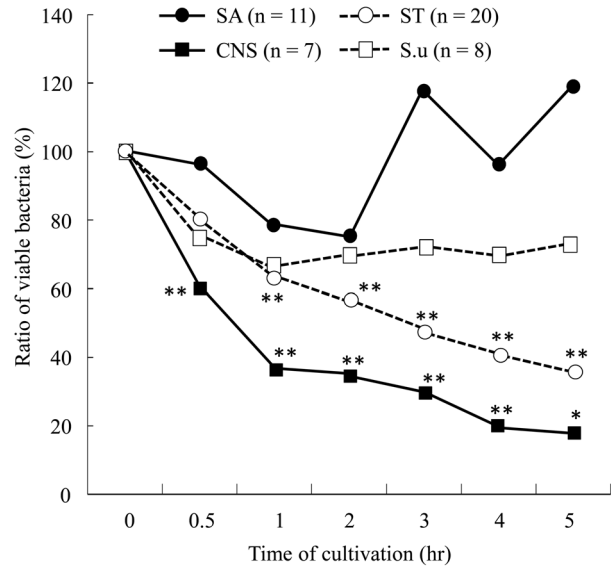

Fig. 3. Changes in the number of viable pathogens during preservation. The vertical axis represents the ratio of viable pathogen number at each preservation time to the number at the onset of cultivation. SA: *Staphylococcus aureus*, CNS: Coagulase-negative staphylococci, S.u.: *Streptococcus uberis*, ST: streptococci except for *S. agalactiae* and *S. uberis*. *,**: Significant differences *vs.* viable arganism counts at 0 hr (**P<0.01, *P<0.05).

significant positive correlation was found between LAP and SCC (r=0.232), LAP and LPO (r=0.255), and LPO and LF (r=0.54) (Table 2). The concentrations of LAP and LF and LPO activity in milk containing different pathogen species are shown in Fig. 4. LAP and LF concentrations did not significantly differ among different pathogenic species, although significantly higher LPO activity was found in milk containing yeast rather than coliforms.

To investigate whether the secretion of the innate immune factors is promoted by pathogens, the correlation between CFUs and the levels of innate immune factors in milk before preservation was analyzed (Table 3). We observed that only the CFUs of ST showed significant positive correlation to the SCC. In yeast and *C. bovis*, significant positive correlations were observed between CFUs and LAP, LF concentrations and LPO activity, but not between *C. bovis* and LPO activity. The CFUs of CNS showed a significant positive correlation to LPO activity.

To investigate whether innate immune factors are associated with the decrease in CFUs during milk preservation, the CFUs of pathogens after 4 hr and 5 hr of preservation were divided by those at 0 hr (decreasing ratio), and their correlations to the levels of innate immune components were calculated (Table 4). In SA, high SCC and LF concentration decreased pathogen numbers at 4 hr, although LAP concentration increased it at 5 hr. The correlation between the SCC and decreasing ratio of yeast at 4 hr was significantly negative, whereas a positive correlation between the SCC and decreasing ratio of CNS was observed at 4 hr and 5 hr of preservation. The decreasing ratio of *C. bovis* at 4 hr was

Table 2. Correlation coefficients among somatic cell count (SCC), lingual antimicrobial peptide (LAP), lactoperoxidase (LPO) or lactoferrin (LF) values in milk

items	SCC	LAP	LPO	LF
SCC	-			
LAP	0.23[a)]	-		
LPO	0.16	0.26[a)]	-	
LF	0.13	−0.01	0.54[b)]	-

Values are correlation coefficients. a) *P*<0.05; b) *P*<0.01 (statistically significant correlation between items).

found to be positively correlated to LAP concentration.

DISCUSSION

In the present study, ST were the most frequently isolated pathogens; this finding supports a previous report [17]. Although the milk was cultured immediately after collection, no CFUs were observed in about 19.4% of milk samples. This percentage of milk samples without CFUs increased up to 30% when the samples were preserved for more than 3 hr. However, the DNA of pathogens was detected in the milk by PCR, despite no growth being observed in the clinical culture system [15, 17]. It was reported that milk with a high SCC contained a high concentration of antibacterial peptides [9–11, 26, 27]. Therefore, it is possible that the pathogens were killed by the leukocytes and/or innate immune factors present in the milk, resulting in decreased CFUs during its preservation after collection. Therefore, in the present study,
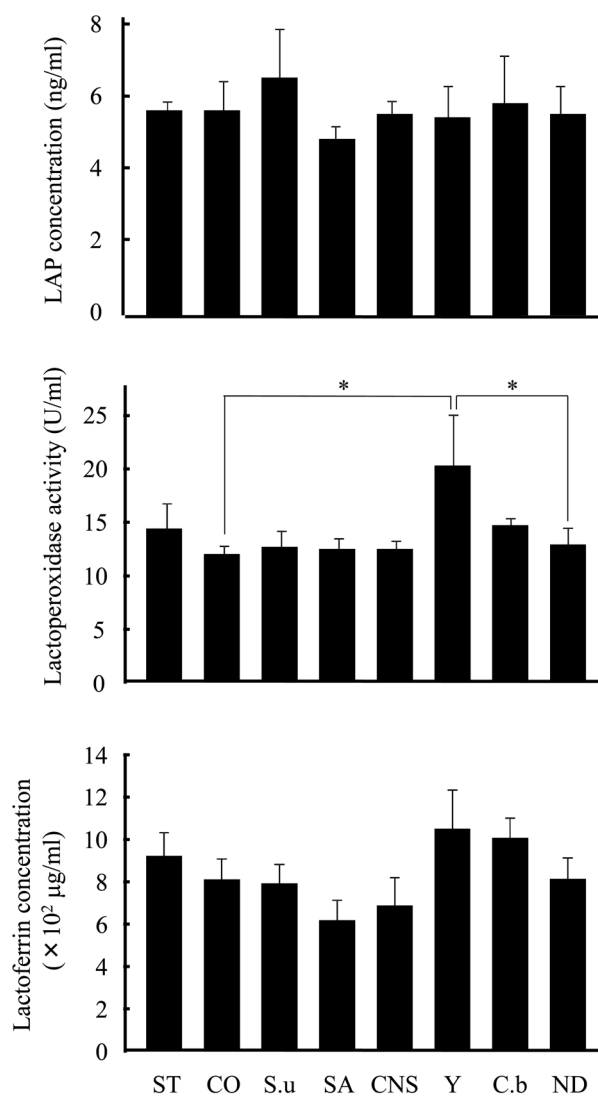
Fig. 4. Concentrations of innate immune factors (LAP, LPO and LF) in milk containing different bacteria. Bars show mean ± SD, and n=20, 11, 8, 7, 7, 5, 4 and 15 in milk contaminated by streptococci except for *S. agalactiae* and *S. uberis* (ST), coliforms (CO), *Streptococcus uberis* (S.u.), *Staphylococcus aureus* (SA), coagulase-negative staphylococci (CNS), yeast (Y) and *Coryne-bacterium bovis* (C. b), and in samples in which pathogens were not detected (ND), respectively. * Significant difference (*P*<0.05).

milk was collected and preserved at room temperature for different timespans, following which bacteriological culture examination was performed. The CFUs of ST, coliforms, CNS, yeasts and *C. bovis* decreased significantly with increasing preservation time after milk collection. These results suggest that the CFUs of some pathogens decreased during preservation of milk at room temperature.

High SCC decreased the number of yeast at 4 hr, suggesting that the decrease in these pathogens during milk preservation depends on their SCC in milk. Similarly, in SA, high SCC or LF concentration was correlated to decreased

bacterial number. However, the mean CFUs of SA were not reduced during preservation. This may be attributed to the fact that some samples with high SCC and LF values showed no decrease in CFUs during preservation, resulting in no significant change in the mean CFUs. Therefore, the sensitivity of SA to antimicrobial components may depend on the microbial strains present in milk.

High SCC and LAP concentrations were correlated to the increase in CFU of CNS and *C. bovis*, respectively. This could be because these pathogens were killed by antibacterial factors other than those evaluated in the present study, such as tracheal antimicrobial peptide (TAP), enteric β-defensin, bovine neutrophil β-defensin (BNBD), S100 protein and cathelicidin, which have also been reported to be present in milk [1, 3, 5, 20, 21, 25].

In mammary glands, invading bacteria are recognized by toll-like receptors (TLRs) of leukocytes and epithelial cells, followed by the secretion of antimicrobial components into milk [5, 10, 26, 27]. Thus, a high number of pathogens in the mammary glands must cause the secretion of a high amount of antibacterial components into milk. Therefore, the correlation between CFUs and the values of antibacterial components in the milk just after collection was analyzed. Significant positive correlations were observed between LF and *S. uberis*, SA and *C. bovis*, LPO and CNS, and between LAP and *C. bovis*. Since these bacteria are gram-positive, these antibacterial components were secreted by stimulation through TLR2 of leukocytes and/or epithelial cells. However, in the present study, each antibacterial component was correlated to a different kind of bacterium. Therefore, the secreted antibacterial components may depend on the bacterial species. It is reported that the concentrations of LAP, LPO and LF in milk increased by stimulation of LPS derived from gram-negative bacteria [10]. However, no correlation was observed between antimicrobial components and coliforms in the present study. Significant positive correlation was found between the CFUs of yeast and the values of LAP, LPO and LF at 0 hr. Dectin was reported to be the receptor for yeast and was localized on the cell membrane of the leukocytes and mammary epithelial cells [2, 28]. Therefore, yeast may be recognized by these dectins, resulting in the secretion of antibacterial components into milk. LPO activity in milk infected with yeast was significantly higher than in milk infected with coliforms and NS (Fig. 4). This result may suggest that compared to bacteria, yeast stimulates more secretion of LPO through dectin receptors.

In the present study, the CFUs of ST, coliforms, CNS, yeast and *C. bovis* were decreased during milk preservation, which strongly suggests that these pathogens were phagocytosed by neutrophils and/or killed by antimicrobial components in milk. Therefore, it is suggested that cultivation of milk for pathogen identification should be started immediately after milk collection. Further studies are required to investigate the components that affect the decrease in the CFUs of pathogens during preservation.

At 0 hr, LAP concentration was positively correlated to the CFUs of yeast, whereas high LAP concentration was not correlated to a decrease in its CFU at 4 hr and 5 hr of preser-

Table 3. Correlation between pathogen number in milk without culture (0 hr) and somatic cell count (SCC), lingual antimicrobial peptide (LAP), lactoperoxidase (LPO) or lactoferrin (LF) values in milk

Pathogen	SCC	LAP	LPO	LF
ST	0.58[b]	0.25	0.27	0.22
Coliforms	−0.32	−0.07	−0.14	0.22
Streptococcus uberis	−0.03	−0.36	0.35	0.81[b]
Staphylococcus aureus	0.42	0.35	0.57	0.80[b]
Coagulase-negative staphylococci	−0.59	0.72	0.79[a]	0.29
Yeasts	−0.34	0.94[b]	0.98[b]	0.94[b]
Corynebacterium bovis	0.07	0.85[a]	−0.78[a]	0.94[b]

Values are correlation coefficients. ST: streptococci except for *S. agalactiae* and *S. uberis*. a) $P<0.05$; b) $P<0.01$.

Table 4. Correlation between the decreasing pathogen number in milk at 4 hr and 5 hr of preservation and the somatic cell count (SCC), lingual antimicrobial peptide (LAP), lactoperoxidase (LPO) or lactoferrin (LF) values in milk

Pathogen	h	SCC	LAP	LPO	LF
ST	4	−0.13	−0.21	−0.02	0.00
	5	−0.02	−0.02	0.01	0.05
Coliforms	4	−0.27	−0.25	−0.07	0.26
	5	−0.32	−0.28	−0.24	0.29
Streptococcus uberis	4	−0.22	0.50	−0.03	−0.47
	5	−0.25	0.45	−0.14	−0.48
Staphylococcus aureus	4	−0.65[a]	0.34	0.17	−0.61[a]
	5	−0.46	0.66[a]	0.05	−0.55
Coagulase-negative staphylococci	4	0.63[a]	−0.11	−0.29	−0.33
	5	0.77[a]	−0.45	−0.54	−0.15
Yeasts	4	−0.67[a]	−0.17	−0.08	−0.36
	5	−0.59	−0.36	−0.28	−0.55
Corynebacterium bovis	4	0.08	0.95[b]	−0.09	0.41
	5	−0.52	0.24	0.47	−0.29

Values are correlation coefficients. ST: streptococci except for *S. agalactiae* and *S. uberis*. a) $P<0.05$; b) $P<0.01$.

vation. This may indicate that LAP secretion was stimulated by yeast; however, LAP does not kill yeasts.

In the present study, only milk samples from cows with subclinical mastitis were used. *E. coli*, CNS and ST were detected, and these bacteria cause clinical mastitis. Therefore, bacterial number in the milk from clinical mastitis cows may be decreased during preservation. This remains to be elucidated.

Milk samples were preserved at 15 to 25°C in the present study. However, preservation at low temperature attenuates activity of antimicrobial components, which may prevent decrease of bacterial number during preservation. A further study is required to investigate the effect of various temperature for milk preservation on the changes in the bacterial number.

In conclusion, these results suggest that some pathogens in high-SCC milk decreased during preservation at room temperature, which may be associated with the presence of antimicrobial components in milk. Therefore, the reduction in microbial number in milk from the time of its collection to the time of its examination should be taken into consideration while evaluating its contamination.

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