



# Somatic cell and innate immune responses in mammary glands of lactating cows to intramammary infusion of *Bifidobacterium breve* at pre-drying off period

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**ABSTRACT.** Intramammary infusion of *Bifidobacterium breve* (*B. breve*)-induced somatic cell (SC) counts, chemiluminescent response (CL), lactoferrin (LF) concentrations and mastitis-causing pathogens from quarters with subclinical mastitis were measured to evaluate innate immune response of mammary glands in dairy cows at 3 to 4 weeks before drying off. SC counts in 7 quarters of 7 control cows and 5 quarters of 6 cows with mastitis increased markedly on day 1 and SC values in control cows were significantly ( $P<0.05$ ) increased and returned to pre-infusion levels on day 5 after *B. breve*-infusion. CL values in both groups increased markedly on day 1 and then decreased after *B. breve*-infusion; however, CL values in cows with mastitis did not return to normal levels on day 5 and at postpartum. The CL values were highly correlated with their SC counts in milk from both groups. LF concentrations increased toward day 3 after *B. breve*-infusion and were higher in cows with mastitis. *B. breve*-infusion eliminated 16.6% (1/6) of pathogens from 6 quarters with chronic subclinical mastitis. *B. breve*-induced SC responses in quarters from 3 cows with mastitis showed characteristic patterns of recovery, persistent and new infections. *B. breve*-induced SC counts in quarters from the cows in the pre-drying off were lower (25.7–70.6%) than those of the cows in mid-lactation. The intrinsic innate immune response in cows on pre-drying off may be decreased and appears to be insufficient to eliminate pathogens from mammary gland in the pre-drying off.

**KEY WORDS:** *Bifidobacterium breve*, innate immune response, lactating cows, mammary gland, pre-drying off

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The physiological and immunological responses in mammary glands of dairy cows vary widely depending on parturition, metabolic changes, and lactation stage [2, 12, 21, 24, 25]. Immunosuppression around the periparturient period in dairy cows results in an increased susceptibility to mammary infections [10–12, 25]; however, host defense response to microorganisms and innate immune response in mammary glands from dairy cows 3–4 weeks prior to drying off in late lactation has not been fully elucidated [25]. SC and immunological responses in mammary glands of dairy cows can change markedly in response to mammary infection, milk yield, and metabolic changes [4, 12, 25]. In previous studies of *Bifidobacterium breve* (*B. breve*) infusion into mammary glands in lactating cows in mid-lactation period, we characterized the enhancing innate immune response and increase clearance of minor mastitis causing pathogens from infected quarters [15, 16].

The pre-drying off period is a critical phase for determining the infection status of mammary glands as indicated by somatic cell (SC) levels and presence of mastitis-causing pathogens in mammary glands before drying off and dry cow therapy administration [7, 26]. The innate immune response is critical to the control of microorganisms and the elimination of pathogens that invade the mammary glands [2, 3, 21, 25]. Determining the innate immune response in mammary glands of dairy cows in the pre-drying off period could further current understanding of the host immune responsiveness of mammary glands and refine the measures implemented in dry cow management [1, 6].

In this study, we evaluated the innate immune responses in mammary glands by measuring *B. breve*-induced SC response, CL

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response, LF concentrations, and the elimination rate of mastitis-causing pathogens in udder quarter milk of lactating cows with and without subclinical mastitis at 3 to 4 weeks before drying off, at dry-off, and within 3 days after parturition. In addition, *B. breve*-induced SC counts and LF concentrations in quarter milk from the cows on pre-drying off were compared with those of the cows in the mid-lactation period.

To evaluate the relationship between host defense response in mammary glands and the type of mammary infection, the recovery from mammary infection, persistent and new infections of mammary glands at pre-drying off, drying off, and in the postpartum period were monitored in lactating cows with naturally occurring mastitis.

## MATERIALS AND METHODS

### Preparation of *B. breve*

A food grade of lactic acid bacteria, *B. breve*, was used [16]. *B. breve* was cultured in medium (GUM culture; Nissui, Tokyo, Japan) at 37°C for 7–10 days. *B. breve*-culture was decanted and washed twice with sterile phosphate buffered saline (PBS, pH 7.2) at  $1,750 \times g$  for 10 min at 4°C and the number of *B. breve* was adjusted to  $1 \times 10^9$  colony forming unit (cfu)/ml in sterile PBS. Heat-killed *B. breve* was prepared after heating at 75°C for 30 min and then stored at –20°C prior to use.

### Animals

Udder quarters from lactating cows on pre-drying off were screened for this study based on monthly SC counts  $>30 \times 10^4$  cells/ml for 1–2 months by a routine dairy herd monitoring, and the quarters with high SC counts were subsequently identified by bacteriological analysis. Holstein lactating cows, 3 to 8-year-old, at 3 to 4 weeks before drying off (pre-drying off), 14 to 18 kg/day of milk production, were used as the cows in the pre-drying off period. The cows were routinely milked twice daily in a milking parlor system and dry cow therapy using antibiotics was not administered to the cows.

Seven lactating cows, 7 quarters, on pre-drying off were used as normal control (without mammary infection) based on their SC counts showing  $<30 \times 10^4$  cells/ml and bacteriological culture results of quarter milk showing no pathogen or  $<300$  cfu/ml. Six lactating cows, 6 quarters, with chronic subclinical mastitis were showing  $>30 \times 10^4$  cells/ml and mastitis-causing pathogen positive-results of quarter milk of  $>300$  cfu/ml. The mastitis-causing pathogens isolated were coagulase negative staphylococci (CNS) in all 6 quarters. These cows were used to evaluate the effects of intramammary infusion of *B. breve* on the elimination of mastitis-causing pathogens from mammary glands.

In comparison of SC response and LF concentrations in mammary secretions from lactating cows in mid-lactation, age-matched 9 Holstein cows, 22 to 37 kg/day of milk production, involving 5 cows without mammary infection and 4 cows with mammary infection caused by CNS on 100 to 200 days after parturition, were used as the cows in mid-lactation.

To reveal the characteristic changes in *B. breve*-infusion induced SC counts, CL response, and LF concentrations in quarters, 3 cows on pre-drying off with naturally occurring mastitis caused by CNS and *Escherichia coli* were evaluated on pre-drying off, drying off, and postpartum period.

The proposed study protocol was compliant with the University's Guidelines on the Care, Use and Management of Dairy Cows (Rakuno Gakuen University, Hokkaido, Japan).

### Infusion of *B. breve* to quarter

Teats of the lactating cows for *B. breve* infusion were wiped with 70% alcohol after regular milking. Three ml of *B. breve* ( $1 \times 10^9$  cfu/ml) were infused aseptically once into quarter via the streak canal using a blunted smoothed tip with elastic tube (polyvinyl chloride,  $2.2 \times 150$  mm; MediTop, Tokyo, Japan) connected with a 5 ml plastic syringe.

### Collection of quarter milk

Five to 8 ml of quarter foremilk samples were collected aseptically into a sterile culture tube (Eiken Co., Tokyo, Japan) for bacteriological tests, SC analysis and measurement of LF concentration. Quarter milk samples were taken from the cows as follows: (1) 2 to 3 days before infusion of *B. breve* for screening of the mammary infection in quarter milk, (2) immediate before infusion on the day of *B. breve* infusion, and (3) on days 1, 2, 3, 5 and 7 after *B. breve* infusion to lactating cows around 4 to 3 weeks before drying off, at drying off, and in the immediate postpartum period, respectively.

### Bacteriological analysis

Milk samples (10  $\mu$ l) collected aseptically from quarter milk were swirl plated onto trypticase soy blood agar plates containing 5% sheep blood (Nissui) and incubated aerobically for 24 to 48 hr at 37°C. The identification of mastitis-causing pathogens grown on the blood agar plate was carried out according to the procedure described by National Mastitis Council [17], based on colony morphology and hemolytic patterns on blood agar, Gram's staining, and additional biochemical tests. Quarter milk was considered as a bacteriologically positive if  $>300$  cfu/ml were detected in a quarter milk. The cultural results were used for classification of mammary secretions as the quarter with and without mammary infection, respectively.

The results of the intramammary infusion of *B. breve* were expressed as effective which is based on the results of no growth of pathogen or  $<300$  cfu/ml of original mastitis-causing pathogen and SCC  $<30 \times 10^4$  cells/ml in quarter milk on days 5 and 7 after *B. breve* infusion.

### Quantification of LF

LF concentrations in quarter milk were measured by single radial immunodiffusion assay kits (Ecos, Sendai, Japan). Mean  $\Delta$ LF concentrations which expressed by subtracting *B. breve* pre-infused LF concentrations from peak LF concentrations were used to compare net LF values in quarter milk from the cows on pre-drying off and the cows in mid-lactation.

### Determination of SC counts

The number of SC counts in quarter milk was determined by a SC counter (Fossomatic N90, Foss, Hillerød, Denmark) and count data were expressed as SC counts/ml and logarithmically transformed values. Mean  $\Delta$ SC counts which expressed by subtracting *B. breve* pre-infused SC counts from peak SC counts were used to compare net SC counts in quarter milk from the cows in the pre-drying off and mid-lactation.

### N-acetyl- $\beta$ -D-glucosaminidase (NAGase) activity

NAGase activity was measured using a fluorometric procedure with 4-methylumbelliferyl-N-acetyl- $\beta$ -D-glucosaminide (Sigma Chemicals, St. Louis, MO, USA) as described previously [14]. The enzymatic reaction was started by adding 30  $\mu$ l of a milk sample to a tube containing 200  $\mu$ l of 2 mM 4-methylumbelliferyl-N-acetyl- $\beta$ -D-glucosaminide in 0.25 M citrate buffer (pH 4.4), incubated for 10 min at 37°C. The enzyme activity was expressed as arbitrary units of 4-methylumbelliferone fluorescence liberated.

### CL response

Opsonized zymosan (OPZ)-stimulated CL response in whole milk was measured in a luminescencer (AB 2200, Atto, Tokyo, Japan) [16]. Fifty  $\mu$ l of quarter milk were mixed with 400  $\mu$ l of Hank's balanced salt solution and 20  $\mu$ l of luminol (final  $10^{-4}$  M) were added. After incubated at 37°C for 5 min, CL counts (cpm) for 5 min were read as unstimulated CL and then CL counts were read after addition of 20  $\mu$ l of OPZ (final 450  $\mu$ g/ml) for 5 min as stimulated CL counts.

### Statistical analysis

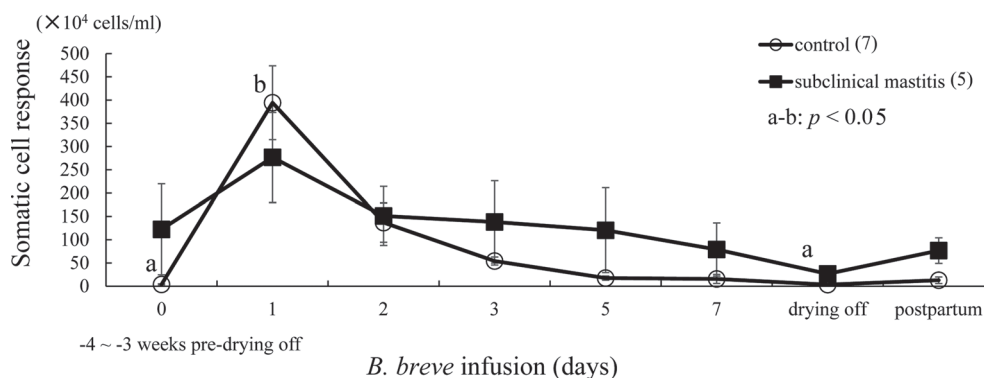
The values were expressed as the mean  $\pm$  standard deviation (SD) or standard error (SE). The values of SC counts, CL response and LF concentrations in quarter milk from lactating cows with and without mammary infection were analyzed by Friedman test and Scheffe's multiple comparison test. The net SC counts ( $\Delta$ SC counts) and LF values ( $\Delta$ LF values) in quarter milk from the cows in the pre-drying off and mid lactation period were compared by Student's *t*-test. The *P*-values of <0.05 were regarded as significantly different.

## RESULTS

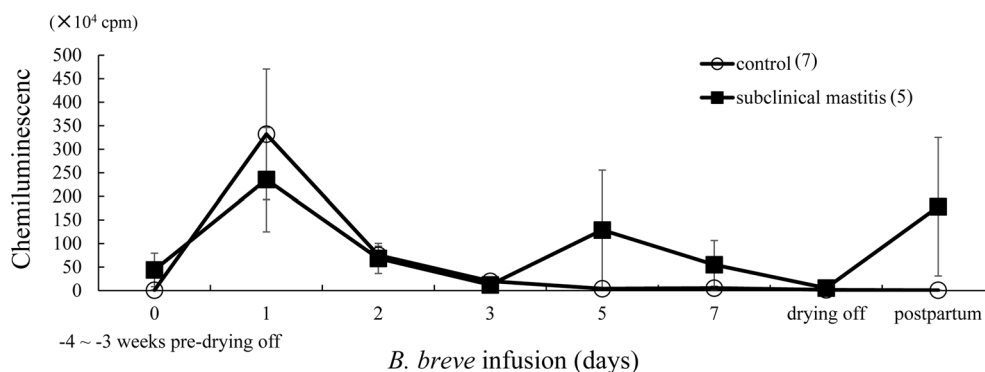
### Effects of intramammary infusion of *B. breve* on SC counts, CL values, and LF concentrations in the pre-drying off period

SC counts in quarter milk from the cows without mammary infection (control) and the cows with subclinical mastitis were measured on days 1 to 7 after *B. breve*-infusion, at drying off, and in the immediate postpartum period (Fig. 1). SC counts of control cows were significantly (*P*<0.05) increased on day 1 after *B. breve*-infusion compared with those of pre-infusion and drying off. SC counts of the cows with subclinical mastitis remained in the range of mammary infection at drying off and in the immediate postpartum period.

The CL values of control cows and cows with subclinical mastitis on pre-drying off were increased remarkably on day 1 after *B. breve*-infusion compared with those at pre-infusion. The CL values thereafter decreased to pre-infusion values on day 5 in control cows (Fig. 2). In contrast, the CL values of the cows with subclinical mastitis showed increased levels on day 5 after *B. breve*-infusion and in the postpartum period.



**Fig. 1.** Somatic cell counts in quarter milk from 7 lactating cows without mammary infection (control) and 5 lactating cows with subclinical mastitis after *Bifidobacterium breve* intramammary infusion at pre-drying off, at drying off, and during postpartum. Mean  $\pm$  standard deviation. .



**Fig. 2.** Chemiluminescence response in quarter milk from 7 lactating cows without mammary infection (control) and 5 lactating cows with subclinical mastitis after *Bifidobacterium breve* intramammary infusion at pre-drying off, at drying off, and during immediate postpartum. Mean  $\pm$  standard error.

To elucidate the relationship between the CL values and the SC counts in quarter milk, the CL values and the SC counts of the 6 quarters with subclinical mastitis and 7 quarters with control cows were measured after *B. breve*-infusion on day 1 (Fig. 3). The CL values in milk from both groups were highly correlated with their SC counts in quarter milk from the cows with subclinical mastitis ( $r=0.814$ ,  $n=6$ ) and the cows with control ( $r=0.733$ ,  $n=7$ ), respectively (Fig. 3).

The LF concentrations of both control cows and cows with subclinical mastitis increased on day 3 after *B. breve*-infusion and subsequently decreased at drying off and in the postpartum period (Fig. 4). The LF values were higher in the cows with subclinical mastitis, but the difference with those of control cows was not statistically significant. After *B. breve* intramammary infusion in cows with subclinical mastitis, 16.6% (1/6) of mastitis-causing pathogens were eliminated from 6 quarters in 6 cows in the pre-drying off period.

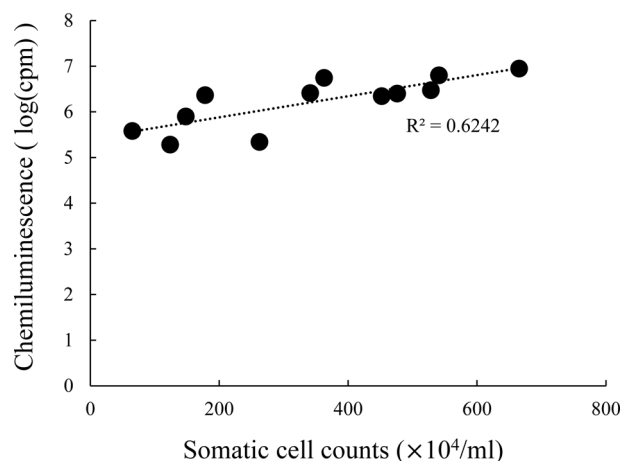
#### Comparison of *B. breve* intramammary infusion-induced SC response and LF concentrations in quarter milk from the cows at pre-drying off and mid-lactation

*B. breve* infusion-induced SC counts (net  $\Delta$ SC values) and LF concentrations (net  $\Delta$ LF values) in quarter milk from the cows at pre-drying off and in mid-lactation were compared to evaluate the differences in responses at the different lactation stages (Table 1).

*B. breve* infusion-induced SC counts and net  $\Delta$ SC counts of 13 cows in the pre-drying off period, which included 7 control cows and 6 cows with subclinical mastitis, decreased (25.7–70.6%) compared with 9 mid-lactation cows (5 control and 4 cows with subclinical mastitis) (Table 1). The net  $\Delta$ SC counts (log) of 6 cows with subclinical mastitis at pre-drying off was significantly ( $P<0.05$ ) lower compared with those of 4 cows in mid-lactation with subclinical mastitis. The net  $\Delta$ LF values (mean  $\pm$  SD), which is derived by subtracting pre-infusion LF values from peak LF values, on day 3 after *B. breve* infusion in 6 quarters from 6 control in the cows at pre-dry off ( $185.7 \pm 123.7$   $\mu$ g/ml) were lower (62.3%) compared with those from the 5 control cows in mid-lactation ( $298 \pm 183.3$   $\mu$ g/ml).

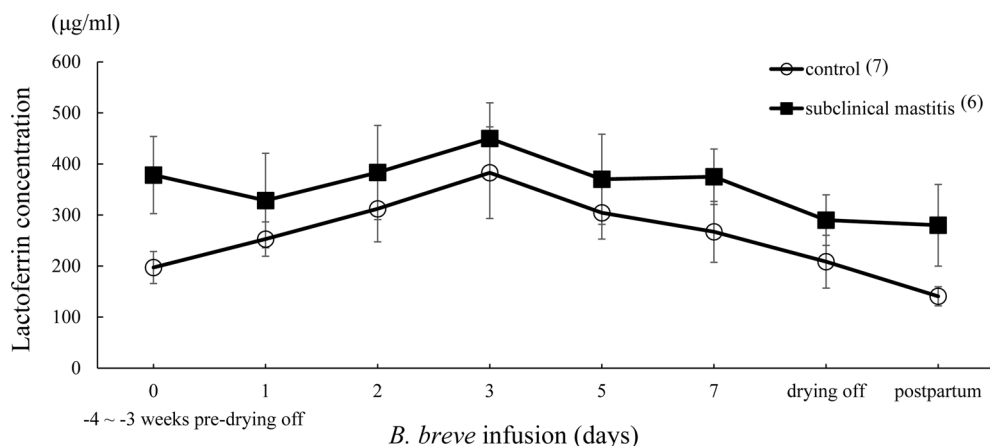
#### *B. breve* intramammary infusion-induced SC counts, CL response and LF concentrations in cows with mammary infection from pre-drying off to postpartum

*B. breve* infusion-induced SC counts, CL response, and LF concentrations in quarter milk from 3 cows with naturally occurring mastitis on pre-drying off, at drying off, and in the postpartum period were monitored longitudinally (Fig. 5). In association with the increased SC response, CL response, and LF concentration after *B. breve* infusion, the quarter (1027D) in cow case 1 with subclinical mastitis caused by CNS was recovered, while in cow case 2 (1081B), SC counts, CL response and LF concentrations in quarter milk showed a low responded manner, and the quarter with subclinical mastitis persisted. In cow case 3 (1218C), values of SC counts, CL response and LF concentration in quarter milk indicated a lower response in the cow on pre-drying off after *B. breve* infusion. Thereafter, acute clinical mastitis caused by *Escherichia coli* was occurred after immediate parturition, as indicated by marked increases of SC counts, CL response, LF values and NAGase activity.



**Fig. 3.** Relationship between the peak somatic cell counts and the chemiluminescence values in 7 quarter milk from the cows with control and 6 quarter milk from the cows with subclinical mastitis on day 1 after *Bifidobacterium breve* intramammary infusion. The correlation coefficients between the chemiluminescence values and the SC counts in quarters from the cows with subclinical mastitis (filled circle:  $r=0.814$ ,  $n=6$ ) and the cows with control (open circle:  $r=0.733$ ,  $n=7$ ), respectively.





**Fig. 4.** Lactoferrin concentrations in quarter milk from 7 lactating cows without mammary infection (control) and 6 lactating cows with subclinical mastitis after *Bifidobacterium breve* intramammary infusion at pre-drying off, at drying off and during postpartum. Mean  $\pm$  SE.

**Table 1.** Comparison of somatic cell counts and net  $\Delta$  somatic cell (SC) counts in quarter milk from the cows without mammary infection (control) and the cows with subclinical mastitis on pre-drying off and mid-lactation period

Lactation stage	Control cows (without mammary infection)					Cows with subclinical mastitis				
	No. cow <sup>1</sup>	SC counts		$\Delta$ SC	Log <sup>3</sup>	No. cow <sup>2</sup>	SC counts		$\Delta$ SC	Log <sup>4</sup>
		(Pre-infused)	(Infused)				(Pre-infused)	(Infused)		
Mid lactation	5	96.8 $\pm$ 28.3	5,586.2 $\pm$ 3,297.4	5,489.4 $\pm$ 3,286.9	6.42	4	1,351.8 $\pm$ 702.8	10,313 $\pm$ 2,896.0	8,960.8 $\pm$ 3,269.0	6.801
Pre-drying off	7	39.9 $\pm$ 10.5 (41.2%)	3,942.1 $\pm$ 600.3 (70.6%)	3,902.3 $\pm$ 601.6	6.55	6	1,033.8 $\pm$ 749.2 (76.5%)	2,653.5 $\pm$ 897.0 (25.7%)	1,619.7 $\pm$ 563.8	6.091*

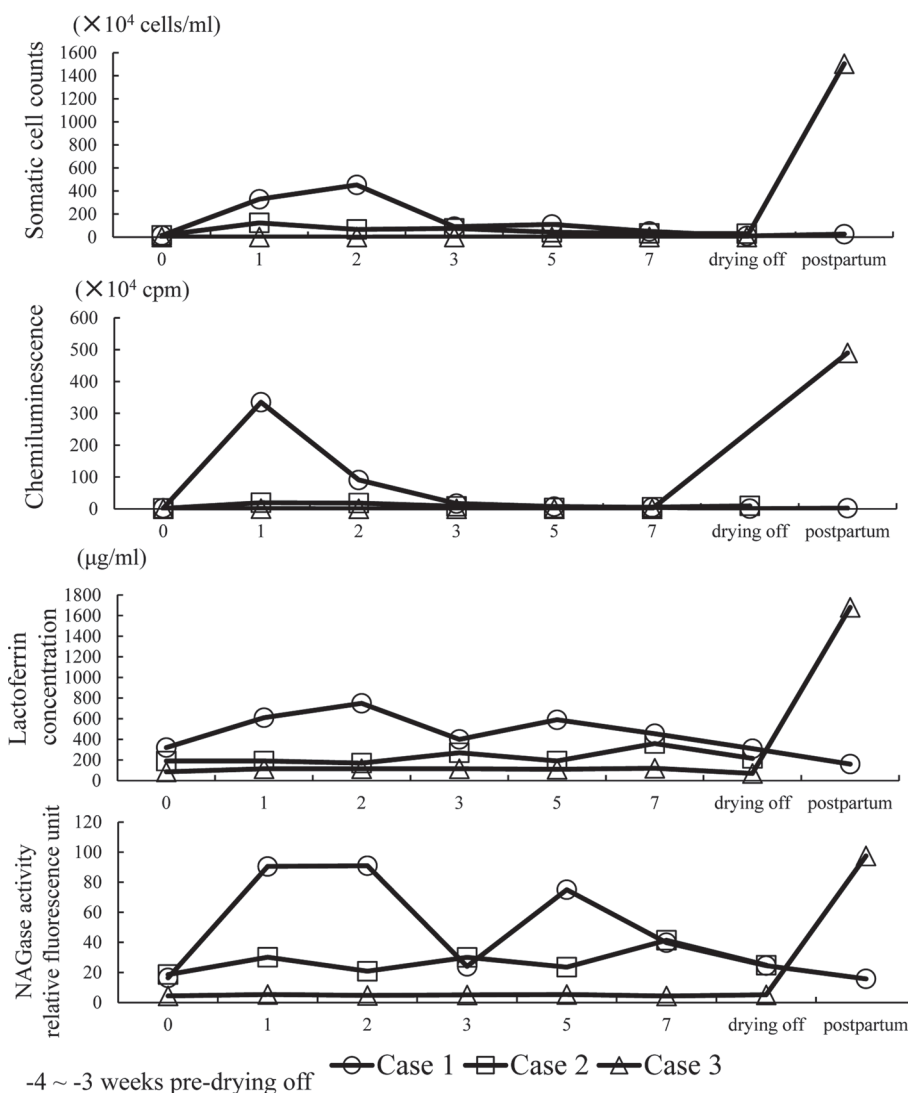
SC counts (Infused): Peak SC counts ( $\times 10^3$  cells/ml) were read on day 1 after *Bifidobacterium breve* intramammary infusion. Delta ( $\Delta$ ) SC values ( $\times 10^3$  cells/ml) were calculated from subtracting SC counts of *B. breve*-pre infusion from their peak SC counts. Mid lactation: Lactating cows around 100–200 days after parturition. <sup>1,2</sup> Number of quarters from the cows. <sup>3,4</sup> Log ( $\Delta$ SC values). Mean  $\pm$  standard error. \* $P < 0.05$ .

## DISCUSSION

The mammary infusion of *B. breve* resulted in a massive influx of phagocytic cells into the mammary glands and enhanced innate immunity and contributed to host defense activity [16, 19]. The SC counts in control cows on day 1 after *B. breve* infusion were significantly ( $P < 0.05$ ) increased from the pre-infusion values. In contrast, SC counts in cows with subclinical mastitis remained in infection range on days 3 to 7 after *B. breve* infusion, at drying off, and during postpartum, suggesting that inflammatory responses persisted during this period, which means mastitis-causing pathogens were not eliminated from the quarters. *B. breve* infusion-induced peak SC counts and CL values in the cows with subclinical mastitis were lower than those of the control cows. It is likely that decreased SC and CL responses in mammary glands in response to *B. breve* are associated with a decrease in host defense activity in the mammary glands, as suggested by Mehrzad *et al.* [13]. As for the relationship between the CL values and their SC counts in quarter milk from both groups, higher correlations found between the peak CL values and the SC counts in quarter milk from the cows with subclinical mastitis and the cows with control, indicating that the increase of CL values in *B. breve*-infused quarters is attributable to the increase in SC count associated with the migration of phagocytic leukocytes into mammary glands [19]. As shown in Fig. 2, the CL values were lowered in the cows with subclinical mastitis as compared with that of the cows with control after *B. breve* infusion on day 1; however, as the similar values of correlation coefficients were found in both cow groups (Fig. 3), we assumed that CL emits per phagocytic cells in quarter milk from the cows with subclinical mastitis are to be less reflected by mammary infection.

LF, an iron-binding glycoprotein in milk that belongs to the transferrin family, is up to about 2 mg/ml and decreases to baseline levels of 0.02 to 0.5 mg/ml during lactation [5, 8, 9, 18]. Anti-bacterial proteins and peptides are secreted in milk and they function effectively and cooperatively with LF [22]. LF concentrations in the cows in the pre-drying off period after *B. breve* infusion were lower than those in the cows in mid-lactation. The reason for decreased LF concentrations in quarter milk from cows with pre-drying off was not clearly explained in our study, it may be related to the quarter conditions of lowered SCC levels of  $< 15 \times 10^4$  cells/ml, no record of subclinical mastitis in lactation period, and primiparous cows.

CNS is the most common bovine mastitis isolates and capable of persisting throughout the lactation and dry periods [18, 20]. In our study, 16.6% (1/6) of CNS-infected quarters with chronic subclinical mastitis were recovered in cows in the pre-drying off period by *B. breve* infusion, whereas 58.8% (10/17) of CNS-infected quarters were recovered in cows in mid-lactation [15]. The reason for the difference in the CNS elimination rate may be reflected by the immune responsiveness of the host defense [2, 3, 13,



**Fig. 5.** Changes in somatic cell counts, chemiluminescence values, lactoferrin concentrations, and NAGase activity in quarter milk from 3 lactating cows with mammary infection after *Bifidobacterium breve* intramammary infusion at pre-drying off, at drying off, and during postpartum. Case 1: Recovery from CNS-caused mammary infection after *B. breve* infusion (1027D). Case 2: CNS-caused subclinical mastitis persisted in quarter after *B. breve* infusion (1081B). Case 3: Acute mammary infection caused by *Escherichia coli* after parturition (1218C). Normal range of NAGase activity in milk: 1.91–14.33 nM of 4-methylumbelliferone produced [14].

19] and other factors [23]. As shown in Fig. 1, SC counts more than  $30 \times 10^4$  cells/ml in the cows with chronic subclinical mastitis on day 7, intramammary infections could persist in the mammary glands at dry-off and in the subsequent postpartum period. The findings suggest that infection tends to persist in the quarters with chronic subclinical mastitis in the pre-drying off period, at drying off, and in the subsequent postpartum period, resulting in the occurrence of mastitis in the early phase of the next lactation.

As shown in Fig. 5, changes in SC response, CL values, and LF concentrations in mammary glands from lactating cows appeared to indicate the infection status of mammary glands. The recovery from mammary infection in case 1 was observed to be associated with the increased SC response induced by *B. breve* infusion. In contrast, the SC response in case 2 with chronic infection caused by CNS and acute mastitis caused by *Escherichia coli* (*E. coli*) at immediate postpartum in case 3 indicated a lower SC response in the pre-drying off period after *B. breve* infusion. This result appears to be consistent with the findings of Mehrzad *et al.* [13] who studied the contribution of neutrophils in quarter milk to the severity of *E. coli* mastitis and found that the low CL response of milk neutrophils prior to infection may be a risk factor for bovine coliform mastitis. Although the changes in SC response, CL values and LF levels of quarters in 3 cases from lactating cows after *B. breve* infusion could not clearly explained, a recovery from mammary infection found in case 1 appears to be reflected by the increased SC response, CL values and LF concentrations. Further studies are needed to evaluate the characteristic changes in innate immune response of quarters at different stages of mammary glands.

*B. breve*-induced increase in SC count (net  $\Delta$ SC counts) in cows with subclinical mastitis in the pre-drying off period was significantly ( $P < 0.05$ ) lower compared with those in the cows in mid-lactation, suggesting that the host defense response in

mammary glands may be decreased in cows in the pre-drying off period. Furthermore, the finding also suggests that mastitis-causing pathogens associated with chronic mammary infection could not easily be eliminated from mammary glands by intrinsic innate immune response in cows in the pre-drying off period. The decreased innate immune response may partly explain the difficulty in eliminating chronic infections in late lactation.

COMPETING INTEREST. The authors declare that they have no competing interest.

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