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FULL PAPER

Immunology

Innate immune response of mammary gland induced by intramammary infusion of *Bifidobacterium breve* in lactating dairy cows

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ABSTRACT. This study aimed to evaluate innate immune responses of mammary glands induced by intramammary infusion of *Bifidobacterium breve* in dairy cows. Somatic cell counts in quarters of cows showed a marked increase following *B. breve* infusion on days 1 and 2. Opsonized-stimulated chemiluminescence response in quarter milk was significantly ($P<0.05$) increased by *B. breve* infusion on days 1 to 3 compared to that of pre-infusion. Lactoferrin concentrations in *B. breve*-infused quarter milk increased significantly ($P<0.05$) on days 2 to 4 and 6 compared to those of pre-infusion. IgG and IgA concentrations in *B. breve*-infused quarters significantly ($P<0.05$) increased on days 2 to 4 for IgG and days 3, 4, 6 and 8 for IgA compared to those of pre-infusion. Interleukin (IL)-1β and IL-8 mRNA levels in somatic cells from *B. breve*-infused quarters were significantly ($P<0.05$) upregulated on day 1 compared to those on days 0 and 14. Conversely, IL-6 mRNA levels in somatic cells from *B. breve*-infused quarters on days 0, 1 and 14 and NF-κB mRNA levels on day 0 were significantly ($P<0.05$) down-regulated compared to those of control. IL-1β, tumor necrosis factor (TNF)-α and IL-6 concentrations increased on days 1, 3 and 7 after *B. breve* infusion in quarters. Intramammary infusion of *B. breve* (3×10^9 cfu) induces a massive influx of leukocytes and enhances innate immune response in mammary glands. This event may contribute to the enhancing host defense in the mammary gland.

KEY WORDS: *Bifidobacterium breve*, dairy cow, innate immunity, intramammary infusion

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Innate immunity is closely associated with host defense and augmentation of innate immunity could be a rational approach for enhancing host defense against invading pathogens [20, 22, 27]. Intramammary infusion of lactic acid bacteria (LAB) has been proposed for treating bovine mastitis [1, 11, 21, 24]. Cripie *et al.* [5] reported that immune responses in the mammary gland are enhanced by intramammary infusion of *Lactococcus lactis* (*L. lactis*). Probiotic activity of LAB is diverse and may have modulating effects on host defense mechanisms of the mammary gland [3, 21]. It is of great value to explore a possible approach for non-antibiotic therapy in dairy cows with mammary infections.

One of the LABs, *Bifidobacterium breve* (*B. breve*) is primarily located in human breast milk and the gastrointestinal tract and exhibits a symbiotic relationship with host microbiota [16, 23]. In our previous study of *B. breve* infusion into mammary glands in dairy cows with chronic subclinical mastitis, somatic cell counts (SCC) markedly increased and bacteriological cure rate of quarters infected with coagulase-negative staphylococci was about 60% on day 14 after *B. breve* infusion [18]. These findings suggest that intramammary infusion of *B. breve* may enhance innate immune response and increase clearance of minor mastitis-causing pathogens from infected quarters. However, it remains how *B. breve* modulates host defense mechanisms of the mammary gland in lactating dairy cows with mild infection. In addition, the reactivity of *B. breve* to quarters of cows was evaluated in comparison with *Lactobacillus lactis* (*Lb. lactis*) and *L. lactis,* which had been shown as possible stimulants to the bovine mammary gland [5].

This study was to elucidate innate immune response of mammary glands of lactating dairy cows with subclinical mastitis following by intramammary infusion of LAB.

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MATERIALS AND METHODS

Preparation of LAB

A food grade *B. breve*, *Lb. lactis* and *L. lactis* were provided by Dr. Kikuchi, Food Microbiology Laboratory, Rakuno Gakuen University (Ebetsu, Japan), and were used for the analysis of mammary responses to LAB. Each LAB was cultured in medium (GUM culture, Nissui, Tokyo, Japan) at 37°C for 7–10 days. LAB-culture was decanted and washed twice with sterile phosphate buffered saline (PBS, pH 7.2) at 1,750 × *g* for 10 min at 4°C and the number of LAB was finally adjusted to 1×10^9 cfu/ml in sterile PBS. Heat-killed LAB was prepared after heating at 75°C for 30 min and then stored at −20°C prior to use for intramammary infusion.

Animals

A total of 25 Holstein cows, 3.7 ± 1.6 (SD) year-old, at the mid lactation stage, 28–38 kg/day of milk production, was used based on their SCCs in quarter milk and bacteriological culture results of quarter milk from dairy cows at the University farm (Rakuno Gakuen University). Cows were routinely milked twice daily by milking parlor where recommended milking procedures were conducted [17]. Experimental protocol was approved by the Institutional Animal Care and Use Committee of Rakuno Gakuen University.

Based on the bacteriological results and SCC data 5 to 7 days before the study, 18 quarters from 18 cows with subclinical mastitis based on the results of the presence of mastitis causing pathogens >300 cfu/ml and SCCs of >30~150 × 10^4 cells/ml in quarter milk were used as cows with subclinical mastitis. The mastitis causing pathogens, coagulase negative staphylococci (CNS) and environmental streptococci were isolated as minor pathogens from quarter milk from the cows with subclinical mastitis. Seven quarters from 7 healthy lactating cows having no pathogen and SCCs <20 × 10^4 cells/ml in quarter milk were used as control quarters.

The cows used for the study were properly handled according to the regulations on food safety and recommendations for good dairy management (Hokkaido, Japan).

Infusion of LAB to quarter

Teats of lactating cows for LAB infusion were wiped with 70% alcohol after immediate regular milking. Three ml of each LAB (3×10^9 cfu) were infused into the teat sinus via the streak canal inserted to a depth of 10 mm using a blunted smoothed tip with elastic tube (polyvinyl chloride, 2.2 × 150 mm, Medi Top, Tokyo, Japan) connected with a 5 ml plastic syringe (Terumo, Tokyo, Japan). *B. breve* was infused into the quarter of the lactating cows immediately after regular milking twice daily for 1 to 3 days, and other quarters were left untreated as control which parameters showed normal range of SCCs and negative culture results.

Collection of milk samples from quarters

Three to 5 ml of quarter foremilk samples were collected aseptically into a 10 ml sterile culture tube (Eiken, Shimotsuga, Japan) for bacteriological analysis. Twenty ml of quarter milk were collected for the analysis of milk and milk SCCs. To compare the somatic cell response in quarters after *B. breve* infusion, quarter milk samples (20 ml) were taken immediately before milking on the day before *B. breve* infusion (day −5 to −7), immediately before infusion (day 0) and on days 1 to 7 after *B. breve* infusion. Quarter milk samples of 200~400 ml were collected for the measurement of gene expression of milk somatic cells on days 0, 1, 3, 14 and 21 after *B. breve* infusion.

Bacteriological analysis

Milk samples (10 µl) 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 pathogens grown on the blood agar plate was carried out according to the procedure described by National Mastitis Council [19], 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 growth of >300 cfu/ml was counted [17]. The results of the bacteriological findings were used for assigning the quarter milk as normal or mammary infection as described above.

Parameters for quarter milk

The number of SCCs in quarter milk was determined by a Fossomatic cell counter (N90, Foss, HillerØd, Denmark) [17]. Concentrations of lactoferrin (LF) and immunoglobulin G and A (IgG & A) in quarter milk were measured by single radial immunodiffusion assay kits (Ecos, Sendai, Japan) [17].

Chemiluminescence (CL) response in quarter milk

Opsonized zymosan (OPZ)-stimulated CL response in whole milk was measured in a luminescencer (AB 2200, Atto, Tokyo, Japan). Fifty µl of quarter milk were mixed with 400 µl of Hank's balanced salt solution and 20 µl 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 µl of opsonized zymosan (final 450 µg/ml) for 5 min as stimulated CL counts.

Cytokine production

Production of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6 and IL-12 from quarter milk from cows by intramammary infusion of *B. breve* were measured according to the procedures as described [7]. Quantification of bovine IL-1β, TNF-α, IL-6 and

Table 1. Sequences of oligonucleotide primers

Gene name	Primer sequence (5′-3′)	Amplicon size (bp)	Accession number
β-Actin	F: AGC AAG CAG GAG TAC GAT GAG R: ATC CAA CCG ACT GCT GTC A	241	NM_173979.3
YWHAZ	F: GCA TCC CAC AGA CTA TTT CC R: GCA AAG ACA ATG ACA GAC CA	120	GU817014.1
IL-1β	F:AGT GCC TAC GCA CAT GTC TTC R: TGC GTC ACA CAG AAA CTC GTC	114	NM_174093.1
IL-6	F: ATC AGA ACA CTG ATC CAG ATC C R: CAA GGT TTC TCA GGA TGA GG	145	NM_173923.2
IL-8	F: GAA GAG AGC TGA GAA GCA AGA TCC R: ACC CAC ACA GAA CAT GAG GC	142	NM_173925.2
TNF-α	F: TCT TCT CAA GCC TCA AGT AAC AAG C R: CCA TGA GGG CAT TGG CAT AC	418	NM_173966.3
LF	F: GTG GAT GGC AAG GAA GAC TTG R: CAA AGA GCT GGA AGC TCC GA	90	NM_180998.2
NF-κB	F: ATA CGT CGG CCG TGT CTA T R: GGA ACT GTG ATC CGT GTA GG	187	XM_024987643.1

bp, base pair; F, forward; R, reverse; YWHAZ, tryptophan 5-monooxygenase activation protein zeta polypeptide; IL, interleukin; TNF, tumor necrosis factor; LF, lactoferrin; NF, nuclear factor.

IL-12 in quarter milk was performed by enzyme-linked immunosorbent assay (ELISA) kits (IL-1β and IL-6: Thermo, Franklin, MA, USA; TNF-α and IL-12: Uscn Life Science, Houston, TX, USA).

Total RNA isolation and cDNA synthesis

Total RNA (tRNA) was extracted from milk somatic cells obtained using the Pure Link RNA kit (Ambion, Austin, TX, USA). Following DNAse digestion with TURBO DNA-free DNAse (Ambion), tRNA was quantified via spectrophotometry using a BioSpec-nano (Shimadzu, Kyoto, Japan). ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and oligo dT primers (Toyobo) were used to synthesize cDNA from 1 µg of tRNA. A parallel negative control reaction was performed without reverse transcriptase for each reaction. PCR was used to amplify β-actin, and bands were analyzed on 1.5% agarose gels stained with ethidium bromide and visualized on a UV transilluminator.

Quantitative RT-PCR (qRT-PCR) analysis

The reaction was performed using a Thunderbird SYBR qPCR mix (Toyobo, Tokyo, Japan) and a MyiQ-iCycler (Bio-Rad, Hercules, CA, USA) as described previously [7]. Information on the primers for IL-1β, -6, -8, TNF-α, LF and nuclear factor (NF)-*κ*B is depicted in Table 1. A BLAST search was performed to confirm that the primer sequences amplified only the target gene of interest. Thermal cycling consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. For specificity to ascertain that only one product was amplified, the melting temperature of the PCR product was determined by melt curve analysis, which was performed by heating the PCR product from 55 to 95°C and monitoring the fluorescence change for every 0.5°C increase. We calculated the number of copies of each gene expressed in the bovine peripheral blood mononuclear cells using a standard curve. β-actin and tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) were used as reference genes. The results of cytokine mRNA expressions on milk somatic cells after *B. breve* infusion were expressed as compared with those of 4 cows without subclinical mastitis (control).

Statistical analysis

The values were expressed as the mean ± standard deviation (SD) or standard error (SE). The values of CL response, LF concentrations, immunoglobulins and cytokine measures in quarter milk were analyzed by linear mixed-effects models using package lme4 of R version 3.6.3.(https://www.r-project.org) accounting for the fixed effect of days after infusion of *B. breve* and the random effects of quarter. The *P*-values of <0.05 were regarded as significantly different.

RESULTS

Somatic cell response of mammary gland of cows against B. breve, Lb. lactis and L. lactis

The number of SCCs in quarter milk after *B. breve* infusion was measured in comparison with their counts of *Lb. lactis* and *L. lactis* intramammary infusion. Each LAB preparation, *B. breve*, *Lb. lactis* and *L. lactis*, was individually infused after regular milking into 6 quarters of 6 cows with different SCCs: 3 quarters from 3 cows with subclinical mastitis showed increased SCCs (>30 × 10^4 cells/ml) and 3 quarters from 3 control cows had lowered SCCs (<30 × 10^4 cells/ml), respectively. SCCs in milk from these quarters were measured on days 1 to 7 after LAB infusion (Fig. 1). SCCs in quarter milk showed 7 to 100- fold increase after LAB infusion, compared to pre-infusion and peaked on day 1 after infusion. Thereafter, SCCs gradually decreased and reached pre-infusion values on

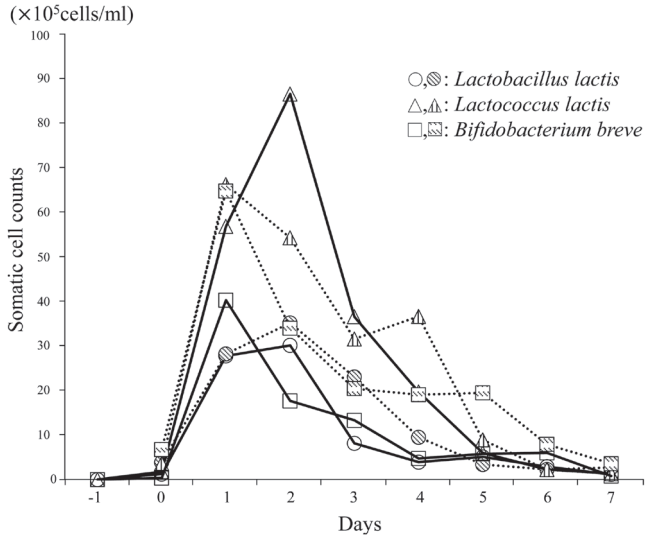

Fig. 1. Somatic cell response in quarter milk from dairy cows following intramammary infusion of *Bifidobacterium breve* (*B. breve*), *Lactobacillus lactis* (*Lb. lactis*) and *Lactococcus lactis* (*L. lactis*). Three LAB preparations (3×10^9 cfu), *B. breve, Lb. lactis* and *L. lactis,* were infused individually into 6 quarters from 6 lactating cows with different SCCs: 3 quarters (normal: circles, triangles and squares) ranging from 3.5 to 17.6×10^4 cells/ml and 3 quarters (subclinical mastitis: circles, triangles and squares with diagonal lines) ranging from 36.3 to 67.6×10^4 cells/ml, respectively. SCCs were measured before and after LAB infusion on days 1 to 6 and 7.

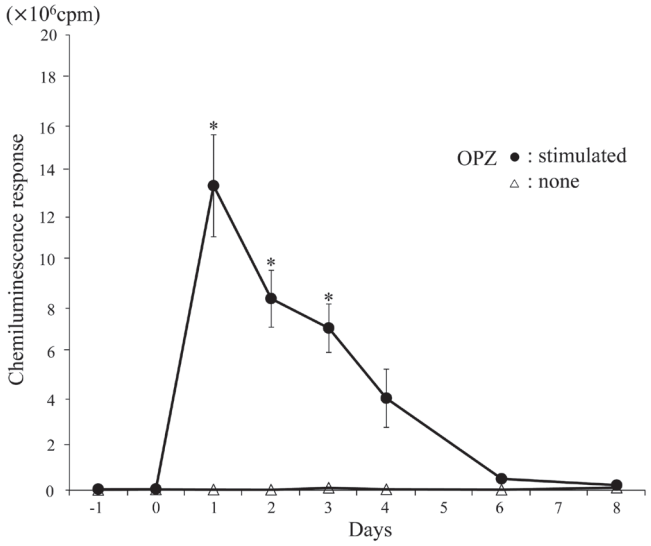

Fig. 2. Opsonized zymosan-stimulated chemiluminescence (CL) response in 5 quarter milk from 5 lactating cows with subclinical mastitis before and after *Bifidobacterium breve* (*B. breve*) intramammary infusion on days 1 to 4, 6 and 8. ●: OPZ-stimulated CL response; △: unstimulated CL response. Mean ± SE (n=5). **P*<0.05.

day 7. The mode of changes and peak SCCs in quarter milk following intramammary LAB infusion were amongst the three bacterial species tested. *B. breve* was used to analyze the innate immune response in quarters in our study.

SCCs in quarters with subclinical mastitis following *B. breve*-infusion were significantly ($P<0.05$) increased compared to those of normal quarters (data not shown). Increase in net SCCs, expressed as the differential of before and after *B. breve*-infused SCCs, in quarters with subclinical mastitis was 1.98-fold higher than that of normal quarters (data not shown).

To evaluate the times of *B. breve* infusion on SCCs in quarter milk, single to triple *B. breve* infusions were carried out (data not shown). Significantly ($P<0.05$) increased SCCs were maintained for 2 days in quarters following *B. breve* infusion once daily for 2 to 3 days compared to that of single infusion.

CL response in quarter milk

B. breve (3×10^9 cfu) was infused once after regular milking and OPZ-stimulated CL response in quarter milk from 5 lactating cows with subclinical mastitis was evaluated (Fig. 2). OPZ-stimulated CL response in *B. breve*-infused quarter milk was significantly ($P<0.05$) increased on days 1 to 3 after infusion compared to pre-infusion. OPZ-stimulated CL response decreased to pre-infusion values on day 6.

LF, IgA and IgG in quarter milk

LF concentrations in quarter milk from 5 lactating cows with subclinical mastitis following *B. breve*-infusion increased on days 2 to 4 compared with those of pre-infused quarters (Fig. 3). Mean LF concentrations (μg/ml) in *B. breve*-infused quarters from 5 lactating cows were significantly ($P<0.05$) higher on days 2 to 4 (1192) and day 6 than pre-infusion values (347). Mean IgA and IgG concentrations (mg/ml) in *B. breve*-infused quarters from 5 lactating cows were significantly ($P<0.05$) increased on days 3 (0.53), 4 (0.68), 6 (0.53) and 8 (0.47) for IgA and days 2 (1.52) to 4 (1.29) for IgG than those of pre-infused quarters (0.18 and 0.65 for IgA and IgG), respectively (Fig. 4).

Cytokine profile

IL-1β, TNF-α, IL-6, IL-8, LF and NF-κB mRNA expression in somatic cells from quarters of cows with subclinical mastitis were measured before and after *B. breve* infusion on days 0, 1 and 14 (Fig. 5). IL-1β and IL-8 mRNA levels were significantly ($P<0.05$) increased on day 1 compared to those of control on days 0 and 14. TNF-α mRNA expression was increased on day 1 compared to those of control and on day 14. IL-6 mRNA expression on days 0, 1 and 14 were significantly ($P<0.05$) decreased compared to those of control quarters. NF-κB mRNA expression was significantly ($P<0.05$) decreased on day 0 compared with those of pre-infusion and on day 14 after infusion.

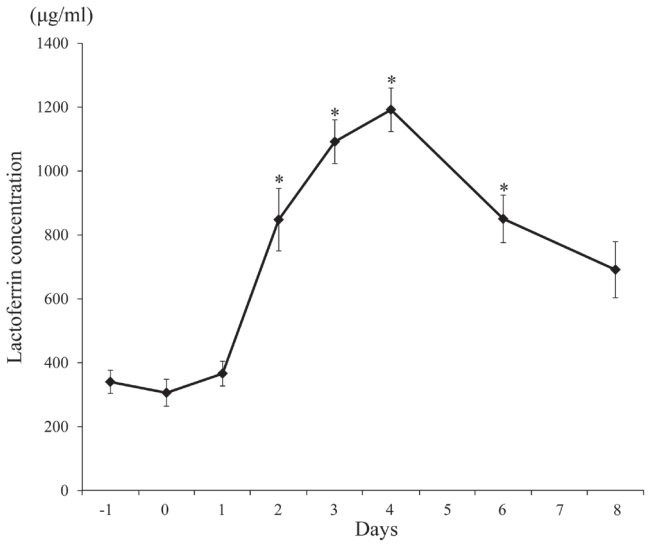

Fig. 3. Lactoferrin (LF) concentration in 5 quarter milk from 5 cows with subclinical mastitis before and after *Bifidobacterium breve* (*B. breve*) intramammary infusion on days 1 to 4, 6 and 8. Mean ± SE (n=5). **P*<0.05.

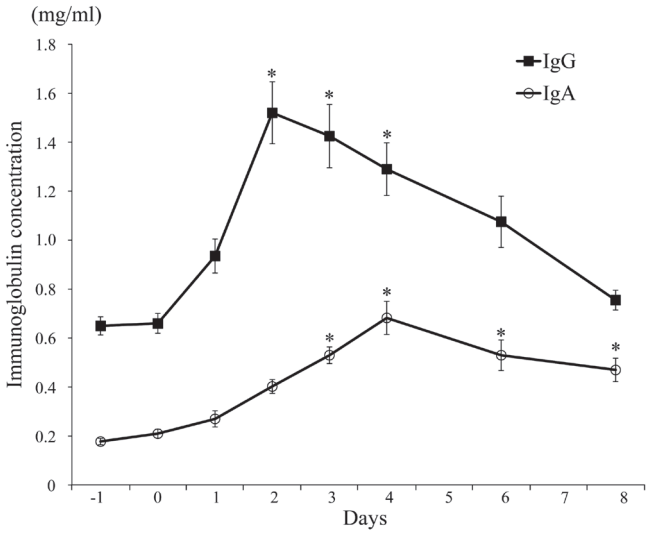

Fig. 4. Immunoglobulin IgA and IgG concentrations in 5 quarter milk from 5 lactating cows with subclinical mastitis before and after *Bifidobacterium breve* (*B. breve*) intramammary infusion on days 1 to 4, 6 and 8. ○: IgA; ■: IgG. Mean ± SE (n=5). **P*<0.05.

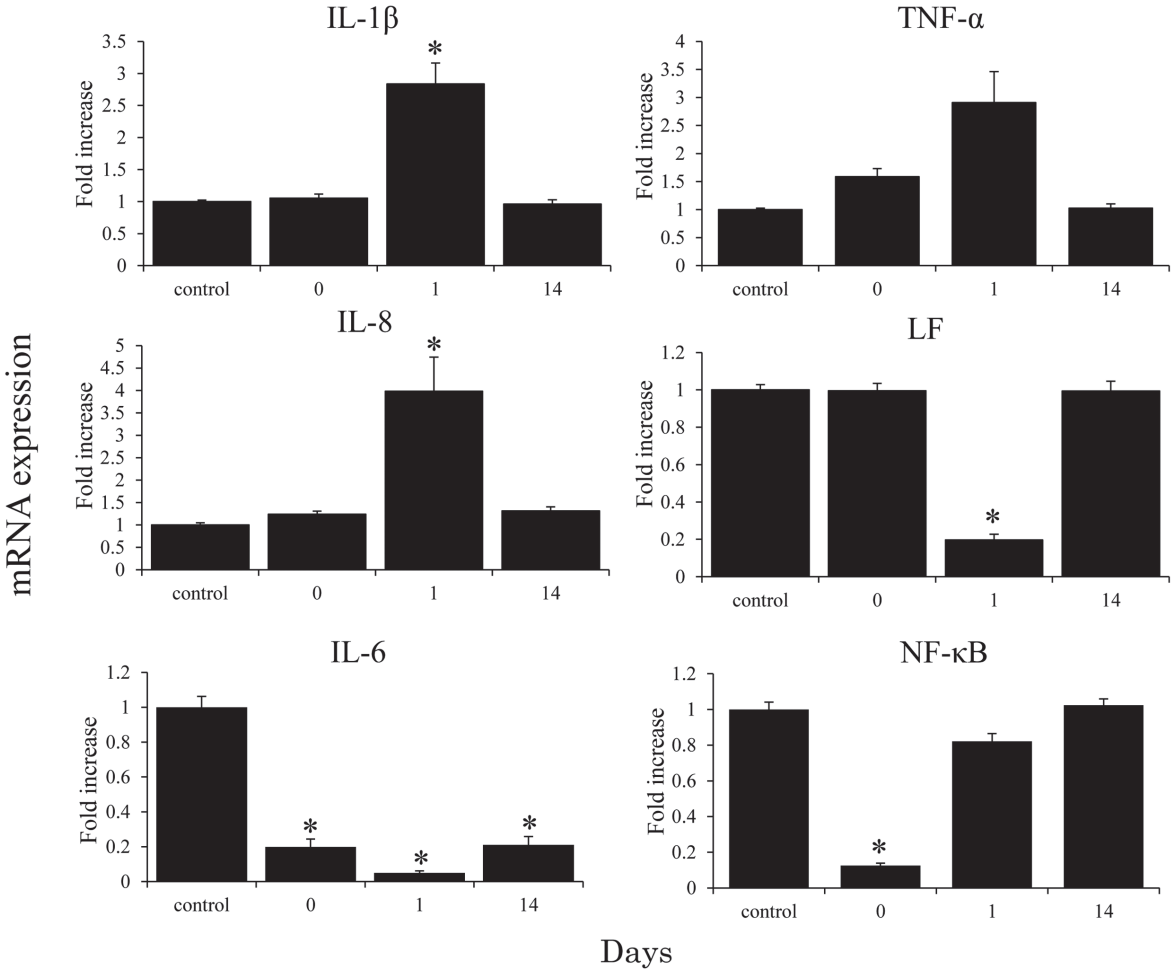

Fig. 5. Cytokine mRNA levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-8, lactoferrin (LF), IL-6 and nuclear factor (NF)-κB in milk somatic cells from 4 lactating cows with subclinical mastitis before and after *Bifidobacterium breve* (*B. breve*) intramammary infusion on days 1 and 14. Their mRNA expressions were compared with those of 4 normal cows (Control). Mean ± SD (n=4). *: *P*<0.05.

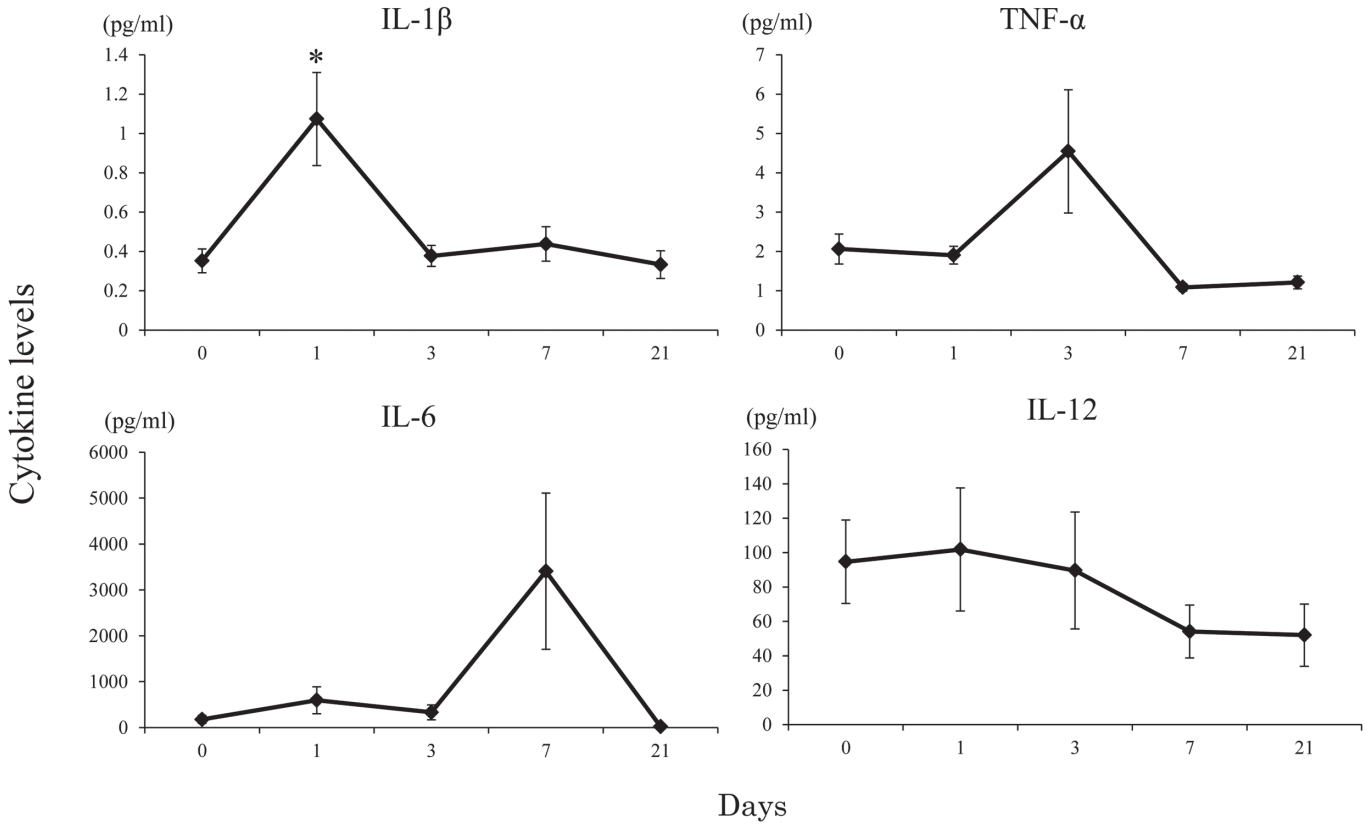

Fig. 6. Cytokine interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6 and IL-12 levels in 3 quarter milk from 3 lactating cows with subclinical mastitis before and after *Bifidobacterium breve* (*B. breve*) intramammary infusion on days 1, 3, 7 and 21. Unit: pg/ml. Mean ± SD (n=3). *P<0.05.

IL-1β, TNF-α, IL-6 and IL-12 protein levels in quarter milk from cows with subclinical mastitis before and after *B. breve* infusion were measured by ELISA kits (Fig. 6). Significantly increased IL-1β in quarter milk was found on day 1, and TNF-α and IL-6 concentrations in quarter milk were increased on days 3 and 7 after *B. breve* infusion compared to pre-infusion. By contrast, cytokine IL-12 concentrations in *B. breve*-infused quarter milk gradually decreased from day 3 to day 21 after *B. breve* infusion.

DISCUSSION

Host defense response to mammary infection corresponds to the magnitude of immune stimulation induced by mammary infections [20]. The present study aimed to determine how *B. breve* intramammary infusion induce the influx of phagocytic cells into quarters and modulates innate immunity in quarters from dairy cows. SCC in quarters from normal cows and quarters from cows with subclinical mastitis markedly increased on days 1, 2 and 3 after infusion of *L. lactis, Lb. lactis* and *B. breve*. SCC changes found in quarters infused with LAB were similar amongst the three bacterial species.

We hypothesized that mammary infusion of marked number of *B. breve* (3×10^9 cfu) could act as an attractant of leukocytes in the mammary glands and enhance phagocytic elimination of mastitis-causing pathogens. Cripie *et al.* [5] reported that neutrophil counts in quarters from cows without mammary infection increased from 10^6 cells/ ml on day 1 to 1.8×10^6 cells/ml on day 2 by intramammary infusion of *L. lactis* (2×10^9 cfu). In our study, SCCs in quarters with subclinical mastitis increased from 3 to 5×10^5 cells/ml pre-infusion to 6 to 8×10^6 cells/ml on day 1, i.e., a 12 to 27- fold increase in SCCs occurred following intramammary infusion of *B. breve* (3×10^9 cfu). This finding confirmed that *B. breve* intramammary infusion is functional for immunological contrivance of potentiated neutrophil recruitment in the mammary gland. After *B. breve* intramammary infusion, more increased SCCs in milk were found in quarters from dairy cows with subclinical mastitis than normal quarters. In addition, increased SCCs in quarters were maintained for longer periods by 2 to 3 times infusions. SCCs in quarters returned to pre-infusion values on day 7 after *B. breve* infusion indicating that stimulatory effects of *B. breve* may be alleviated once *B. breve* is eliminated from infused quarters and host defense mechanisms returned to pre-infusion level. This event is considered to be associated with enhanced clearance of the mammary gland and may promote the recovery of immunological responses in mammary gland.

Significantly increased OPZ-stimulated CL response in milk was found in quarters of dairy cows on days 1 to 4 after *B. breve* infusion. The increased CL response appeared to be dependent on neutrophil migration in *B. breve*-infused quarters, as the mean percentage of polymorphonuclear neutrophils increased from 73% on day 1 to 27% on day 4 after infusion (data not shown).

LF, an 80-kDa iron-binding glycoprotein, belongs to the transferrin family in milk. LF is found at physiological concentrations

of up to 2 mg/ml and decreases to baseline levels of 0.02 to 0.5 mg/ml during lactation period [8, 9]. In our study, significantly increased LF concentrations ranging from 347 to 1,192 μg/ml were found in *B. breve*-infused quarters, suggesting that LF concentrations increased in *B. breve*-infused quarters in response to stimuli. There have been several reports on the inhibitory effects of LF on inflammatory responses during mammary infections [14, 15, 25]. LF binds to lipopolysaccharide (LPS) and neutralizes its effects [12, 13]. Moreover, LF activates complement and increases the number of phagocytic cells in milk, further enhancing phagocytosis [10]. LF and lactoferricin stimulate IL-8 release from human neutrophils [26]. These results appear to be associated with activities of immune clearance of mammary gland by increased innate immunity as well as massive neutrophil influx into the mammary gland.

Mammary glands synthesize IgG, IgA and IgM, although synthesis in the mammary gland is less pronounced than in other tissues [2]. IgA-producing cells are predominated located in mammary gland, lung and intestinal tissues [28]. The mammary gland produces Ig locally and transports large amounts of IgG_1 [28]. Higher concentrations of IgG and IgA found in *B. breve*-infused quarters are likely to be induced by immune stimulation and modulation of the host defense in the mammary gland, as previously proposed [6]. Increased IgG and IgA levels in quarter milk following *B. breve* intramammary infusion may be associated with enhancement of the immunologically active motif in mammary glands.

Cytokines involved in inflammatory responses include TNF-α, IL-1β, IL-2 and IL-6 [4]. In the present study, IL-1β, TNF-α and IL-8 mRNA levels were upregulated in somatic cells induced by *B. breve* intramammary infusion compared to cells collected before *B. breve* infusion. Increased IL-1β, TNF-α and IL-6 concentrations were also found following *B. breve* infusion. These findings confirm previous observations showing cytokine gene expression is upregulated in quarter milk in the early phase by *L. lactis* intramammary infusion [1]. The precise mechanism of the enhanced immune responses induced by LAB is not fully understood; however, bacterial cell components may account for immunological responses. It is likely that *B. breve* stimulates leukocyte migration and upregulates mRNA expression of cytokines in association with the recognition of Toll-like receptors expressed on phagocytes. Furthermore, the cytokine network is activated by the micro-environment modified by *B. breve*, LF, immunoglobulins and other physiological substances produced in the mammary gland. Bacterial cell components, such as peptidoglycan, cell wall, LPS and DNA, may activate macrophages and polymorphonuclear leukocytes and release cytokines, as well as stimulate the production of reactive oxygen species by intramammary infusion of *B. breve* in mammary glands.

In conclusion, intramammary infusion of *B. breve* induces massive influx of polymorphonuclear leukocytes into the mammary gland and enhances innate immune response in mammary glands. This event is considered to be associated with enhanced clearance of the mammary gland against invading minor pathogens and may contribute to the enhancing host defense capability in mammary gland.

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

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