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Seasonal variations in the concentration of antimicrobial components in milk of dairy cows

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Abstract

The incidence of bovine mastitis and the bulk milk somatic cell count (BMSCC) are influenced by season, which may be associated with innate immune functions, including antimicrobial components in mammary glands. Therefore, the present study was conducted to examine the effect of season on antimicrobial components in milk. Rectal temperature and plasma cortisol, thyroxine, and derivatives of reactive oxygen metabolites (d-ROMs) were measured as stress parameters. Concentrations of lactoferrin (LF), lingual antimicrobial peptide (LAP), psoriasin (S100A7), and Immunoglobulin A (IgA) in milk were measured as indicators of innate immune function. LF and LAP concentrations were significantly lower in summer than in winter and spring, respectively, whereas the concentration of S100A7 was significantly lower in winter than in spring and autumn. The rectal temperature was significantly higher in summer than in other seasons, whereas plasma cortisol, thyroxine, and d-ROMs did not exhibit any seasonal variation. In conclusion, even though stress parameters were not changed, the concentration of antimicrobial components, such as LF and LAP, decreased in summer, which may explain the frequent occurrence of mastitis during this season.

KEYWORDS

antimicrobial components, bovine mastitis, heat stress, innate immunity, season

1 | INTRODUCTION

Bovine mastitis is an inflammation of the mammary glands caused by pathogenic invasion, and is known to have significant economic repercussions in the dairy industry (Hogeveen, Huijps, & Lam, 2011; Seegers, Fourichon, & Beaudeau, 2003). Mastitis can be caused by several bacterial species, such as those belonging to *Staphylococcus*, *Streptococcus*, and Enterobacteriaceae (Thomas et al., 2015). In addition, some causative pathogens of mastitis, such as *Staphylococcus aureus*, threaten public health, because a contamination of dairy products by these bacteria or their enterotoxins can lead to foodborne illnesses (Bianchi et al., 2014; Johler et al., 2018).

The innate immune functions protect bovine mammary glands against pathogens. Antimicrobial components, including lactoferrin (LF), lingual antimicrobial peptide (LAP), and psoriasin (S100A7), which are components of the innate immune system, are synthesized in bovine mammary glands (Isobe, 2017). LF synthesized in the mammary epithelium and leukocytes inhibits bacterial growth, due to its high affinity to iron molecules (Huang, Morimoto, Hosoda, Yoshimura, & Isobe, 2012; Hurley & Rejman, 1993). LAP, a β-defensin, is a cationic antimicrobial peptide (Isobe, Nakamura, Nakano, & Yoshimura, 2009) secreted by the mammary epithelial cells into bovine milk (Isobe, Hosoda, & Yoshimura, 2009; Isobe, Nakamura, et al., 2009). S100A7, a calcium-binding protein that inhibits bacterial growth, such as that of *Escherichia coli* (Regenhard et al., 2009), was first reported in epithelial cells from human psoriatic skin (Madsen et al., 1991), and was later found in bovine and goat mammary teat epithelium (Tetens et al., 2010; Zhang, Lai, Yoshimura, &

Isobe, 2014). Antimicrobial components are active against a wide spectrum of bacteria, including causative bacteria of bovine mastitis (Langer et al., 2017; Regenhard et al., 2009). Immunoglobulin (Ig) G, IgA, and IgM, which are known factors of acquired immunity, are also found in bovine milk (Cakebread, Humphrey, & Hodgkinson, 2015). However, natural antibodies have recently been reported as important components of innate immunity that bind bacterial components, such as lipopolysaccharides (LPS) and lipoteichoic acid (LTA; Ploegaert et al., 2011). In particular, natural IgA is important for mucosal defense against bacterial invasion (Cakebread et al., 2015; Schroten et al., 1998).

The effect of season on intramammary infection in dairy cows has previously been reported. According to Moosavi, Mirzaei, Ghavami, and Tamadon (2014) in Iran, clinical mastitis during the late lactation period is more frequent in summer, whereas it occurs more frequently during the early lactation period in winter. In the Netherlands, the milk somatic cell count (SCC), which is an indicator of intramammary infection, has been reported to be higher in August (Khatun, Bruckmaier, Thomson, House, & García, 2019; Olde Riekerink, Barkema, & Stryhn, 2007). The cows that calved in late summer to autumn (August–November) were at lower risk of developing high SCC than those that calved in other seasons (Bobbo, Penasa, Finocchiaro, Visentin, & Cassandro, 2018). Thus, it is evident that the prevalence of mastitis in dairy cows is affected by season, although the results remain controversial.

The immune function in dairy cows can also be affected by season. The production of cytokines, such as interleukin-4 (IL-4), IL-5, IL-6, interferon γ (IFNγ), and tumor necrosis factor-α (TNF-α), are inhibited by an increase in the blood cortisol concentration under heat stress (Bagath et al., 2019). Conversely, another report showed that heat stress can result in an increase in cytokines, including IL-1β, IL-6, IFNγ, and TNF-α (Chen et al., 2018). However, the effect of season or heat stress on the innate immune functions of the mammary glands has not been elucidated. Therefore, the aim of this study was to identify the effect of season on the antimicrobial components in milk of dairy cows.

2 | MATERIALS AND METHODS

2.1 | THI and bulk milk SCC in Hiroshima Prefecture in 2018

Temperature humidity index (THI) was calculated as described previously (Mirzad et al., 2018) for each month in 2018. The temperature and humidity data in Hiroshima city, as a representative of Hiroshima Prefecture, were obtained from the Japan Meteorological Agency website (https://www.jma.go.jp/jma/indexe.html). The average bulk milk somatic cell count (BMSCC) was calculated for each month in 2018, using samples from 124 commercial dairy farms located in the Hiroshima Prefecture (a Hiroshima cooperative society of dairy industry).

2.2 | Seasonal comparison of antimicrobial components in milk, rectal temperature, and circulating levels of endocrine hormones and d-ROMs

2.2.1 | Herd selection and animals

Samples were collected from 96 randomly selected Holstein-Friesian cows from 20 commercial dairy farms in the Hiroshima Prefecture. Of these 20 dairy farms, 18 farms adopted the tie-stall system, whereas of the remaining two, one had the free-stall system and the other had the free-barn system.

2.2.2 | Sampling design

Blood and quarter milk samples were collected from January to December 2018. To exclude the effect of lactation period on the concentration of antimicrobial components in milk (Cakebread et al., 2015; Isobe, Shibata, Kubota, & Yoshimura, 2013) and the concentration of endocrine hormones in blood (Endo, Kitamura, Okubo, & Tanaka, 2019; Hudson, Mullford, Whittlestone, & Payne, 1976), samples were collected 5–8 days after calving. To exclude the effect of mammary inflammation on the concentration of antimicrobial factors in milk, only the milk samples with SCC < 300,000 cells/ml were selected. January–February, April–May, July–August, and October–November were defined as winter, spring, summer, and autumn, respectively. This study was conducted in accordance with the guidelines for animal experiments issued by the Hiroshima University (E19-3).

2.2.3 | Milk and blood sampling

Before collecting milk samples, teats were debrided and the foremilk was disposed. Ten milliliters of milk samples were collected and immediately stored at 4°C. Milk samples were centrifuged at 5,000× *g* for 5 min at 4°C, and the skim milk was stored at −30°C for enzyme immunoassay, and the precipitates were used to determine the SCC using Countess® II FL (Thermo Fisher Scientific), as described previously (Purba, Ueda, Nii, Yoshimura, & Isobe, 2020). Blood samples were collected from the jugular vein and transferred to a tube containing heparin, and then immediately stored at 4°C. Blood samples were centrifuged at 1,700× *g* for 15 min at 4°C, and the plasma obtained was immediately stored at −30°C for enzyme immunoassay and measurement of derivatives of reactive oxygen metabolites (d-ROMs). The samples were collected at daytime between milking. The rectal temperature of each cow was recorded during sampling. A total of 368, 92, and 72 samples were available for milk, plasma, and rectal temperature, respectively (Table 1).

2.2.4 | Measurement of d-ROMs

Of the 92 plasma samples available, 48 randomly selected samples (12 from each season) were used to measure the plasma d-ROM

TABLE 1 Number (*n*) of cows, blood (plasma) and quarter milk samples, and rectal temperature measurements included in the study

	Cow	Blood (plasma)	Milk	Measured rectal temperature
Winter (Jan–Feb)	24	23	91	22
Spring (Apr–May)	25	25	96	21
Summer (Jul–Aug)	30	28	116	21
Autumn (Oct–Nov)	17	16	65	8
Total	96	92	368	72

concentration, by photometric quantification following a commercial protocol (Wismerll Co., Ltd.).

2.2.5 | Enzyme immunoassay

One quarter milk sample with SCC < 300,000 cells/ml was selected per cow for the enzyme immunoassays ($n = 18$ in winter, 20 in spring, 21 in summer, and 16 in autumn). Milk LF and IgA concentrations were measured by ELISA quantification following a commercial protocol (Bethyl Laboratories, Inc.). LAP and S100A7 concentrations were measured as described previously (Isobe, Nakamura, et al., 2009; Zhang et al., 2014).

Cortisol was extracted from the plasma using dichloromethane and measured following the method described in Isobe, Yamada, and Yoshimura (2007). Thyroxine concentration was measured by competitive enzyme immunoassay. Microplates were coated with mouse anti-thyroxine antibody (GeneTex, Inc.) overnight. The solution was then disposed and the plates were washed twice with phosphate-buffered saline (PBS), following which the thyroxine standard (L-thyroxine, Sigma-Aldrich Co. LLC.) and samples diluted with Tris-buffered saline (TBS) were added to the wells and allowed to stand for over 3 hr. Horseradish peroxidase labeled thyroxine (thyroxine-HRP) was then added to these wells and allowed to stand for 30 min. The solution was disposed and the wells were washed four times with PBS. Then, 3,3',5,5'-tetramethylbenzidine (TMB) was added and the optical density was measured at 655 nm wavelength. The HRP was conjugated to L-thyroxine using a commercial kit (Dojindo Laboratories).

2.3 | Statistical analyses

All statistical analyses were performed in EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R foundation for Statistical Computing; Kanda, 2013). The Kolmogorov-Smirnov test was used to check the normality of the data. Plasma thyroxine and d-ROMs and milk S100A7 were normally distributed, whereas plasma cortisol and milk SCC, LF, LAP, and IgA presented a lognormal distribution. Monthly differences in the BMSCC in 2018 were tested using repeated measures ANOVA followed by Bonferroni correction for multiple comparison. The proportion of quarters with SCC < 300,000 cells/ml across the four seasons (winter, spring, summer, and autumn) were compared using Fisher's exact test. Rectal temperature, plasma cortisol, thyroxine, and d-ROMs and milk SCC, LF, LAP, S100A7, and IgA concentrations were compared across the four seasons using one way ANOVA followed by Tukey's test for multiple comparison. Pearson's product-moment correlation was used to test the correlations between milk SCC, LF, LAP, and S100A7. Differences were considered significant for $p < .05$.

3 | RESULTS

Monthly changes in THI and BMSCC are shown in Figure 1. THI was high in July and August, whereas it was low in January and February in 2018. The BMSCC varied significantly by month ($p < .01$). The BMSCC in August was higher than in January, April, May, and June, and that in September was significantly higher than in April and May.

The mean SCC tended to decrease from winter to autumn (Figure 2a). On categorizing SCC as <300,000, 300,000–1,000,000, and >1,000,000 cells/ml, the proportion of quarters with SCC < 300,000 cells/ml was found to be significantly higher in autumn than in summer ($p = .02$, Figure 2b).

Seasonal differences in mean LF, LAP, S100A7, and IgA concentrations in milk are illustrated in Figure 3. LF, LAP, and S100A7 varied significantly by season ($p = .021$, <.01, and <.01, respectively) whereas the IgA concentrations did not exhibit any significant seasonal variation ($p = .31$). LF concentrations were significantly lower in summer and autumn than in winter ($p = .034$ and .042, respectively), and the LAP concentration was significantly lower in summer and winter than in spring ($p < .01$). The concentration of S100A7 was the lowest in winter, and was significantly different from that in spring and autumn ($p < .01$). The correlations among milk SCC, LF, LAP, S100A7, and IgA are shown in Table 2. A significant negative

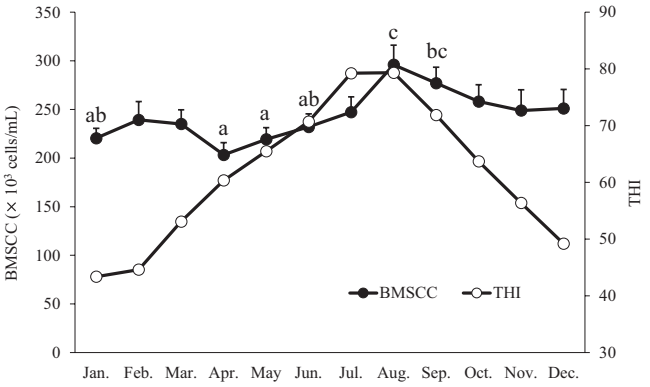

FIGURE 1 Monthly changes in the mean bulk milk somatic cell counts (BMSCC; $n = 124$) and Temperature humidity index (THI) in Hiroshima Prefecture in 2018. Results are presented as mean ± *SEM*. [a,b,c]Values with different letters are significantly different ($p < .05$)

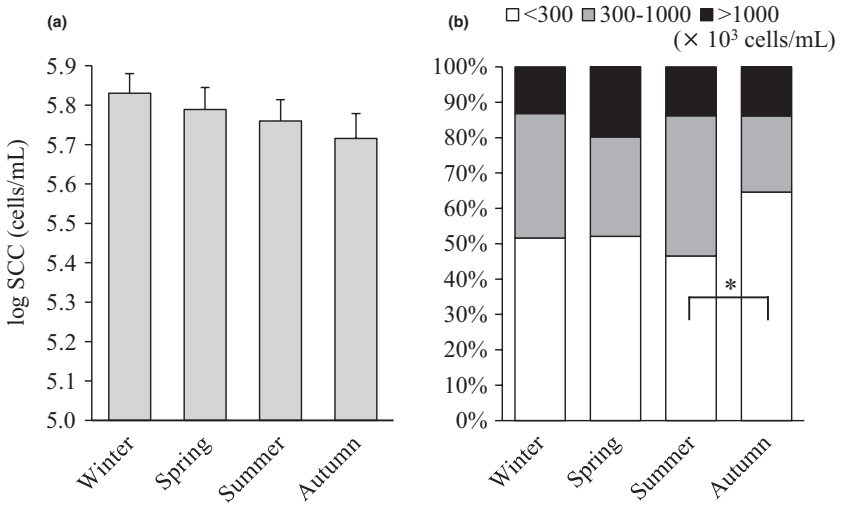

FIGURE 2 Seasonal differences in the mean somatic cell counts (log SCC) of quarter milk ($n = 368$; a) and proportion of quarter milk samples with SCC <300,000, 300,000–1,000,000, and >1,000,000 cells/ml (b). Results in (a) are presented as mean ± *SEM*. *indicates significant difference between groups ($p < .05$)

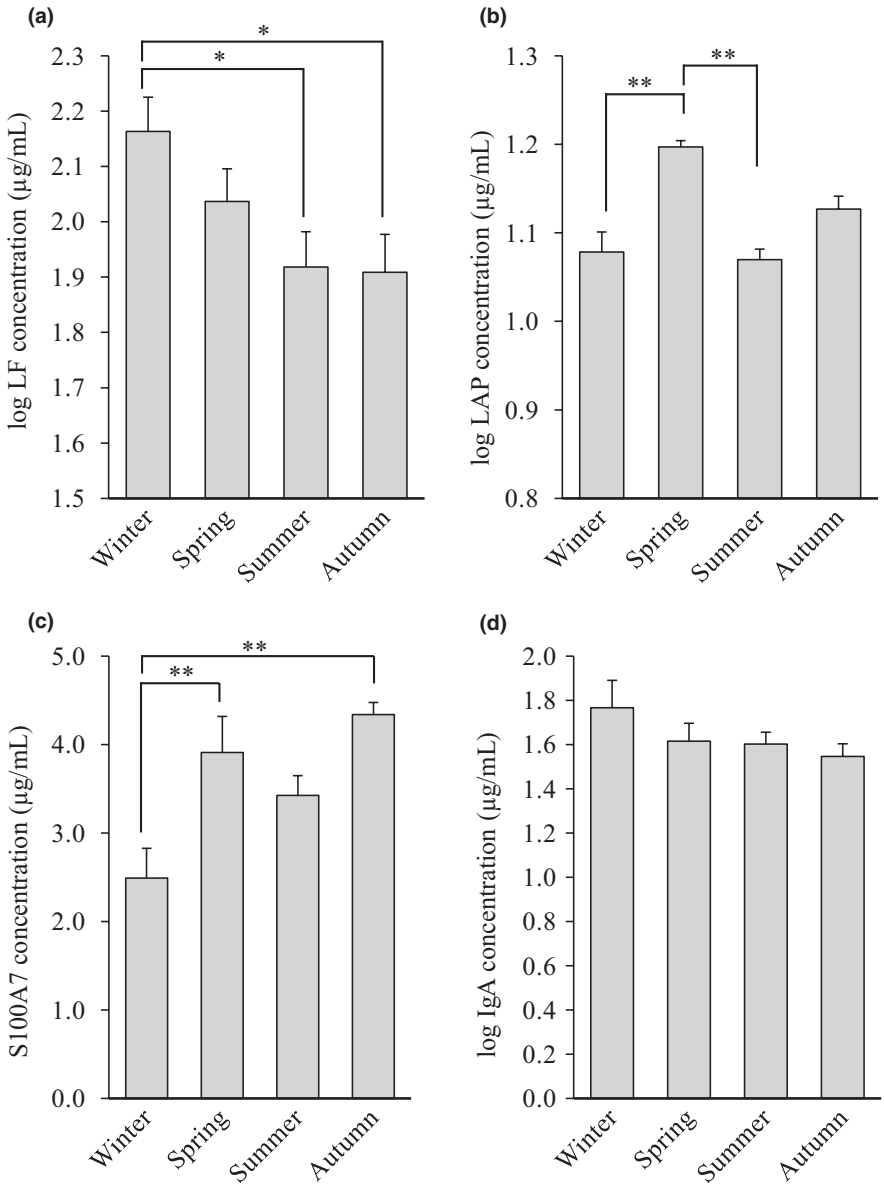

FIGURE 3 Seasonal differences in the mean concentrations of lactoferrin (log LF; a), lingual antimicrobial peptide (log LAP; b), S100A7 (c), and IgA (log IgA; d) in quarter milk samples with SCC < 300,000 cells/ml ($n = 75$). Results are presented as mean ± *SEM*. * and **indicate significant difference between groups ($p < .05$ and .01, respectively)

TABLE 2 Correlation coefficients between the innate immune components and somatic cell counts (SCC) in quarter milk samples with SCC < 300,000 cells/ml (n = 75)

Item	SCC	LF	LAP	S100A7	IgA
SCC	—				
LF	0.089	—			
LAP	0.090	0.015	—		
S100A7	−0.068	−0.28*	−0.33	—	
IgA	0.22	0.53**	−0.055	−0.18	-

Note: SCC: common logarithm of SCC (cells/ml), LF: common logarithm of lactoferrin concentration (μg/ml) in milk, LAP: common logarithm of lingual antimicrobial peptide concentration (μg/ml) in milk, S100A7: S100A7 concentration (μg/ml) in milk, IgA: common logarithm of immunoglobulin A concentration (μg/ml) in milk.

*p < .05; **p < .01 (statistically significant correlation between items).

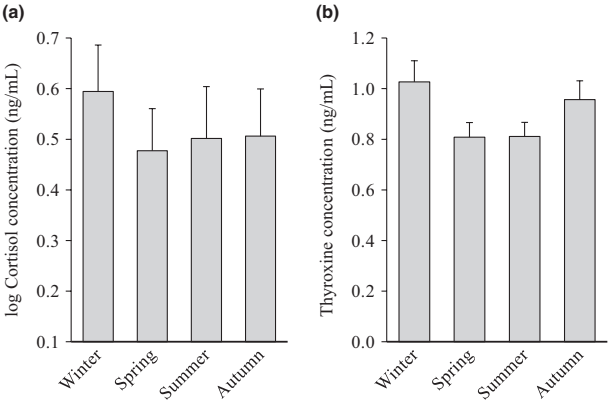

FIGURE 5 Seasonal differences in the concentrations of cortisol (log cortisol; a) and thyroxine (b) in the plasma of dairy cows (n = 92). Results are presented as mean ± *SEM*

correlation between LF and S100A7 (p = .02), and significant positive correlation between LF and IgA (p < .01) were observed.

Seasonal differences in mean rectal temperature and plasma d-ROMs are depicted in Figure 4. Rectal temperature varied significantly across the seasons (p < .01), and it was significantly higher in summer than in other seasons (p < .01). Conversely, plasma d-ROMs did not exhibit any seasonal variation (p = .41).

Furthermore, plasma cortisol and thyroxine concentrations did not exhibit any seasonal variation (p = .52 for both, Figure 5).

4 | DISCUSSION

The THI in Hiroshima Prefecture was low in January, and had increased significantly by August. Similarly, the mean BMSCC was significantly higher in August (mid-summer in Japan) than in January (mid-winter), April, May, and June (spring to early summer). These results indicate that high THI results in an increase in the BMSCC of dairy cows. Similar results have been reported in other countries, where the highest BMSCC was recorded in the summer season (Norman, Miller, Wright, & Wiggans, 2000; Olde Riekerink et al., 2007).

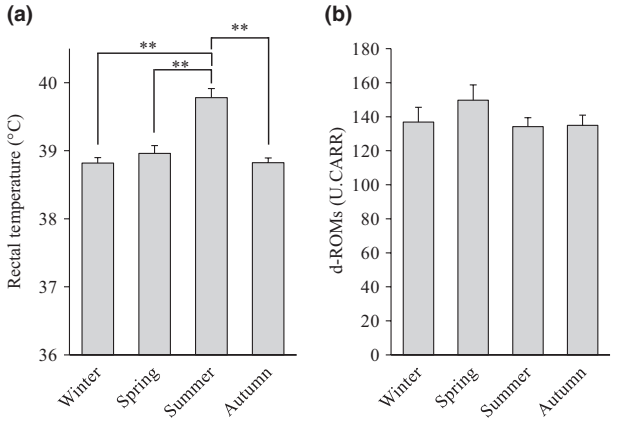

FIGURE 4 Seasonal differences in the rectal temperature (n = 72; a) and derivatives of reactive oxygen metabolites (d-ROMs) in the serum of dairy cows (n = 48, b). Results are presented as mean ± *SEM*. **indicate significant difference between groups (p < .01)

It was likely that dairy cows were influenced by heat stress in summer, because the rectal temperatures recorded in summer were significantly higher than in other seasons. Plasma d-ROMs and cortisol and thyroxine concentrations were measured to confirm if the cows were experiencing heat stress. D-ROMs have previously been employed as an indicator of oxidative stress in dairy cows (Fiore, Spissu, Sechi, & Cocco, 2019), and are measured by detecting the hydroperoxide metabolized by the oxidative reaction. However, d-ROMs in plasma did not exhibit any seasonal variation, which was comparable to the results of a previous report (Mirzad et al., 2018). In addition, it has been reported that heat stress can increase cortisol concentration (Wise, Armstrong, Huber, Hunter, & Wiersma, 1988) and decrease the thyroxine concentration (Chen et al., 2018) in dairy cows. From these results, we can conclude that the high ambient temperature prevalent in summer is not sufficient to cause any significant changes in the heat stress parameters, such as plasma d-ROMs, cortisol, and thyroxine.

No seasonal variation could be detected in the mean SCC of quarter milk in the present study. However, when the SCC was categorized as <300,000, 300,000–1,000,000, and >1,000,000 cells/ml, the proportion of quarters with SCC < 300,000 cells/ml was significantly lower in summer than in autumn. This suggests that the SCC of milk is higher in summer. However, the quarter milk samples analyzed in this study were not collected from udders with clinical mastitis. Therefore, the high SCC observed in summer may be attributable to the occurrence of subclinical mastitis. Alternatively, it may be due to the decreased milk yield under heat stress (Tao et al., 2018), because SCC is known to increase under decreased milk yield (Purba et al., 2020).

It was possible that the high incidence of mastitis was due to weak innate immune functions of the affected cows (Isobe, 2017). Therefore, LF, LAP, and S100A7 were measured, representing the milk antimicrobial components. As shown in Figure 3, LAP and LF concentrations in milk were significantly lower in summer than in

winter and spring, respectively. These results suggested that low concentrations of LAP and LF in summer may be associated with high incidence of bacterial infection, resulting in high SCC. Milk LF and LAP concentrations increase in response to bacterial components, such as LPS (Huang et al., 2012). Therefore, only quarter milk samples with SCC < 300,000 cells/ml were included in the analyses (Khatun et al., 2019). No correlations could be detected between SCC and LAP or LF concentrations. LAP and LF are secreted from mammary epithelial cells (Huang et al., 2012; Hurley & Rejman, 1993; Isobe, Hosoda, et al., 2009). Therefore, these results suggest that the seasonal differences observed in the antimicrobial factors in milk are due to the ability of mammary epithelial cells to secrete these components under healthy conditions.

On the other hand, concentration of S100A7 was lower in winter than in spring, whereas it was comparable in summer and spring. S100A7 is a highly-expressed antimicrobial peptide in teat epithelium (Zhang et al., 2014), and is the major constituent of bovine teat canal lining along with keratin, protecting the skin against pathogenic invasion (Smolenski, Cursons, Hine, & Wheeler, 2015). The low concentration of S100A7 may be related to the decreased ability of S100A7 synthesis in winter, due to rough and dry teat skin caused by the extremely low humidity characteristic of Japanese winter. In addition, the antimicrobial activity of the recombinant bovine S100A7 is limited to *E. coli* (Regenhard et al., 2009), suggesting that low S100A7 concentration in teat skin may allow *E. coli* to easily invade the mammary glands in winter. Conversely, the LF concentration was higher in winter than in summer and autumn. LF, which has high affinity to iron molecules (Hurley & Rejman, 1993), is considered an important factor preventing *E. coli* infection in mammary glands, because mammary pathogenic *E. coli* require iron for successful infection. The ferric citrate transport system (Fec system) of *E. coli*, which plays crucial roles in binding, translocating, and internalizing iron (Braun & Mahren, 2005), is more highly expressed in mammary pathogenic *E. coli* than in other environmental *E. coli* (Blum et al., 2018; Olson, Siebach, Griffitts, Wilson, & Erickson, 2018). Additionally, in comparison with the wild type strain, the *fecA* (the gene encoding ferric dicitrate transport system) deletion variant of *E. coli* exhibited significantly less growth in bovine and mouse milk (Olson et al., 2018). Thus, it is suggested that bacterial multiplication is prevented by LF, to compensate for lower S100A7 concentration in winter, which was further reflected by the inverse correlation between LF and S100A7 concentration observed in the present study (Table 2).

In contrast to LF, LAP, and S100A7, the milk IgA concentration did not exhibit any seasonal variation. This may be due to the differences in their secreting cells. It is known that IgA in bovine milk is secreted from plasma cells via homing of immune cells through CCL10/CCL28 chemokine interactions (Pallister et al., 2015), whereas LF, LAP, and S100A7 in milk are mainly secreted by mammary or teat epithelium (Isobe, 2017). This indicates that sensitivity to heat stress may be different between plasma and epithelial cells.

In conclusion, the concentrations of antimicrobial components, such as LF and LAP decreased in summer season, which may explain the frequent occurrence of mastitis in this season. Further studies are required to reveal a more direct relationship between milk antimicrobial components and high incidence of mastitis in summer.

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