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Effect of temporary cessation of milking and estradiol combination on the antimicrobial components in goat milk

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ABSTRACT

A temporary cessation of milking is widely used in Japan to treat mastitis in dairy cows. Exogenous administration of estradiol (E2) is known to inhibit milk production in dairy cows. Therefore, we aimed to evaluate the effects of the temporary cessation of milking in combination with E2 administration on the antimicrobial components of goat milk. Twelve goats, divided into two groups—with and without E2 injection (E2 and control group, respectively), were subjected to cessation of milking in both udder halves for 3 d (day 0–2). Milk yield in the E2 group was significantly lower than that in the control group on days 7 to 10. The concentrations of cathelicidin-2, IgA, and lactoferrin in the E2 group were significantly higher than those in the control group. These results suggest that the temporary cessation of milking with simultaneous E2 administration leads to a higher concentration of certain antimicrobial components in milk than that observed after using cessation of milking alone. Thus, this combination may contribute to a stronger innate immune system and a faster recovery from mastitis, and might prove to be an alternative to antibiotic treatment upon further research.

1. Introduction

Mastitis, an inflammatory response of the udder tissue in the mammary gland, has a high worldwide prevalence and is considered the most common disease leading to economic loss in the dairy industry (Gomes and Henriques, 2016). Previous studies have shown that clinical and subclinical mastitis is caused by approximately 10 bacterial species or species groups (Katholm et al., 2012; Rysanek et al., 2009). Due to the predominance of bacterial causes, the treatment of mastitis accounts for the majority of antibiotics administered to dairy cows (Pol and Ruegg, 2007; Saini et al., 2012). However, several of the available drugs have limited application against the varied pathogens recovered from cases of mastitis because of the diverse etiologies of the disease (Erskine et al., 2003). Furthermore, the introduction of antibiotics has led to the emergence and dissemination of antimicrobial resistance (Saini et al., 2012).

Cessation of milking occurs when normal daily milking is terminated on a set day (Gott et al., 2017). A temporary 3-day cessation of milking combined with antibiotic administration has been introduced as a new approach toward treating bovine mastitis in Japan. This combination was found to be successful in the treatment of mastitis caused by Streptococci and coagulase-negative Staphylococcus in dairy cows (Kondo et al., 2013). We investigated the effects of a temporary 3-day cessation of milking on the innate immune components in goat milk in a previous study and found that it increased the somatic cell count (SCC) and concentrations of several innate immune components in milk, such as goat beta-defensin 1 (DEFB1), S100A7, cathelicidin-2 and -7 (CATHL-2 and -7), IgA, and lactoferrin (LF), which may be involved in the prevention and treatment of mastitis (Purba et al., 2021). This increase in innate immune components was partially due to a decrease in milk yield.

Kuwahara et al. (2017) reported that E2 intramuscular injection in goats for 3 d resulted in a dramatic decrease in milk yield, which caused an increase in innate immune components. Similarly, exogenous E2 administration decreases milk production in dairy cows (Athie et al., 1996; Delbecchi et al., 2005). Hence, there is a possibility that the combination of temporary cessation of milking with E2 administration will increase the levels of antimicrobial components in milk, which are beneficial for the mammary gland's immune response against mastitis. Moreover, E2 modulates the innate immune response and creates an anti-inflammatory environment in the epithelium of the female reproductive tract (Turner et al., 2014; Wen et al., 2012). The anti-inflammatory and antimicrobial properties of E2 have also been

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demonstrated in the epithelial cells of the bovine mammary glands (Medina-Estrada et al., 2016). Accordingly, at low E2 concentrations during the postpartum period, cows experience increased susceptibility to inflammatory disorders in the mammary gland and uterus. This condition has been correlated with decreased functionality of the innate immune response due to abrupt changes in the levels of E2 (Lamote et al., 2006). As reported previously by Lavon et al. (2010), cows with subclinical mastitis exhibit low circulating E2 levels.

Taken together, E2 has two functions: to decrease milk yield, which causes upregulation of innate immune components, and to directly improve innate immune function. Therefore, in the present study, we aimed to elucidate the effects of a combination of temporary cessation of milking and E2 administration on the antimicrobial components in goat milk.

2. Materials and methods

2.1. Experimental animals

Twelve Tokara goats (body weight, 20–25 kg; parity, 1–4; mid-lactation stage; milk yield, 300–800 mL/day) were used in this study. Goats were fed 0.6 kg of hay cubes and 0.2 kg of barley per day with free access to water and trace mineral salt blocks. Feed was provided twice daily at 08:00 and 15:00 h.

2.2. Experimental design

The goats were divided into two groups ($n = 6$), the E2 group and the control group. In the E2 group, goats were injected with 0.5 mL/day of 1 mg/mL estradiol benzoate (ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) intramuscularly for 3 d beginning at the time of cessation of milking from both udder halves. In the control group, goats were only subjected to cessation of milking from both udder halves for 3 d without E2 injection. After 3 d of cessation, milking was resumed once daily for both groups. The day of onset of cessation of milking was considered day 0. All experiments in this study were approved by the Hiroshima University Animal Research Committee and conducted according to their guidelines (C19–4).

2.3. Collection of milk samples

Milk samples were collected from 3 d before the temporary cessation of milking until 7 d after the resumption of hand milking. During the 3 d of cessation of the milking period, only 4 mL of milk was collected gently without whole milking for SCC measurement and enzyme immunoassay analyses. Milk was then centrifuged at 1900 $\times g$ for 5 min at 4 °C. Skim milk was stored at −20 °C for enzyme immunoassay, and precipitates were resuspended in PBS to measure SCC using a Countess Automated Cell Counter (Life Technologies Japan Ltd., Tokyo, Japan), as reported previously (Suzuki et al., 2020).

2.4. Enzyme immunoassay

An enzyme immunoassay was conducted to measure the concentrations of DEFB1, S100A7, CATHL-2, CATHL-7, IgA, and LF in goat milk. Antibodies against DEFB1, S100A7, CATHL-2, and CATHL-7 were generated in rabbits as described previously (Isobe et al., 2020; Kuwahara et al., 2017; Nishikawa et al., 2018). These antibodies were used for competitive enzyme immunoassays as described previously (Nishikawa et al., 2018; Zhang et al., 2014). Milk samples were diluted to 50, 5000, 20, and 20 times for DEFB1, S100A7, CATHL-2, and CATHL-7 measurements, respectively. The concentrations of IgA in goat milk were measured using a goat IgA measurement kit (A50–106; Bethyl Laboratories, Montgomery, TX, USA) after skim milk was diluted 1000 times. Milk LF concentration was measured as described by Kuwahara et al. (2017) using anti-LF antibody (Life Laboratories, Yamagata, Japan). Milk samples for LF measurements were diluted 5000 times. Optical density was measured using a microplate reader (Thermo Scientific Multiskan FC Microplate type 357; Thermo Fisher Scientific Co. Ltd., Tokyo, Japan).

2.5. Statistical analyses

Data were analyzed statistically using JMP Pro14 (SAS Institute Inc., Cary, NC, US). The Kolmogorov–Smirnov test was used to check the normal distribution of the data. A non-parametric Kruskal–Wallis test followed by the Steel–Dwass test was used to compare the data on different days. One-way ANOVA followed by the Tukey multiplex analysis was used to compare SCC on different days. The results are reported as least squares means (LSMs) and standard errors of LSMs (SEM). Differences were considered statistically significant at $P < 0.05$.

3. Results

In this experiment, milking was stopped in both the udder halves for 3 d. During the temporary cessation of milking, an intramuscular injection of E2 was administered to the E2 group but not to the control group. Milk yield on days 5 and 6 was significantly lower than that on day 0 in both groups (Fig. 1). Additionally, milk yield in the E2 group was significantly lower on days 7 to 10 compared with that in the control group ($P < 0.05$). The mean SCC was significantly higher on day 5 in the control group and on days 5 and 6 in the E2 group compared with that on day 0 (Fig. 2). Furthermore, on days 7 and 10, the SCCs in the E2 group were significantly higher than those in the control group ($P < 0.05$).

The mean concentration of DEFB1 was significantly higher on day 5 than that on day 0 in the E2 group ($P < 0.05$; Fig. 3), whereas S100A7 was significantly higher on day 4 than that on day 0 in both groups ($P < 0.05$; Fig. 4). Compared with that on day 0, the mean concentration of CATHL-2 was significantly higher on days 4 and 5 in the E2 group, and on days 5 and 6 in the control group (Fig. 5). The mean concentration of CATHL-2 in the E2 group was significantly higher on days 8 to 10 than that in the control group ($P < 0.05$). Compared with that on day 0, the mean concentration of CATHL-7 was significantly higher on days 5 and 7

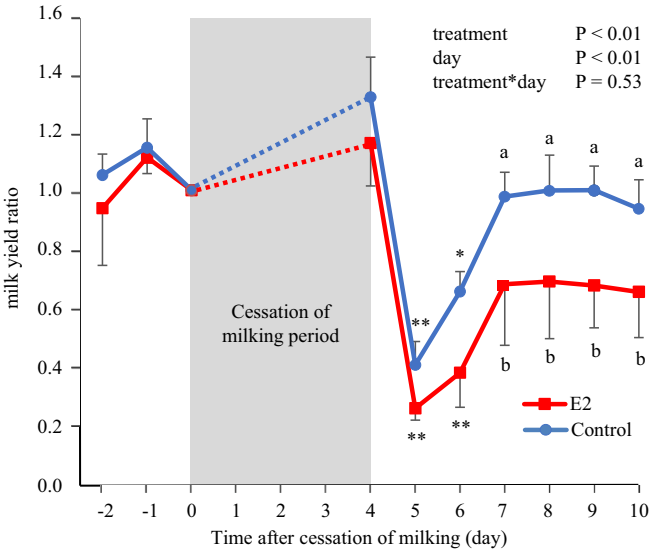

Fig. 1. Changes in goat milk yield after a 3-d cessation of milking from both udder halves ($n = 6$). The vertical axis displays the ratio wherein day 0 (first day of cessation of milking) is set as 1. Results are represented as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ indicate significant difference compared with day 0. a, b indicate a significant difference between the control and E2 groups on the same day ($P < 0.05$).

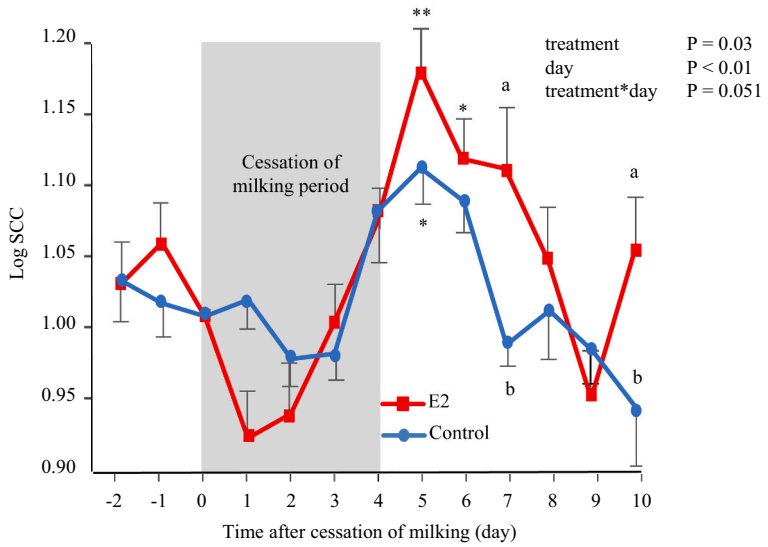

Fig. 2. Changes in Log somatic cell count (Log SCC) in goat milk after a 3-d cessation of milking of both udder halves (n = 6). The vertical axis displays the ratio where day 0 (first day of cessation of milking) is set as 1. Results are represented as mean ± SEM. * $P < 0.05$; ** $P < 0.01$ indicate significant difference compared with day 0. a, b indicate a significant difference between the control group and E2 group on the same day ($P < 0.05$).

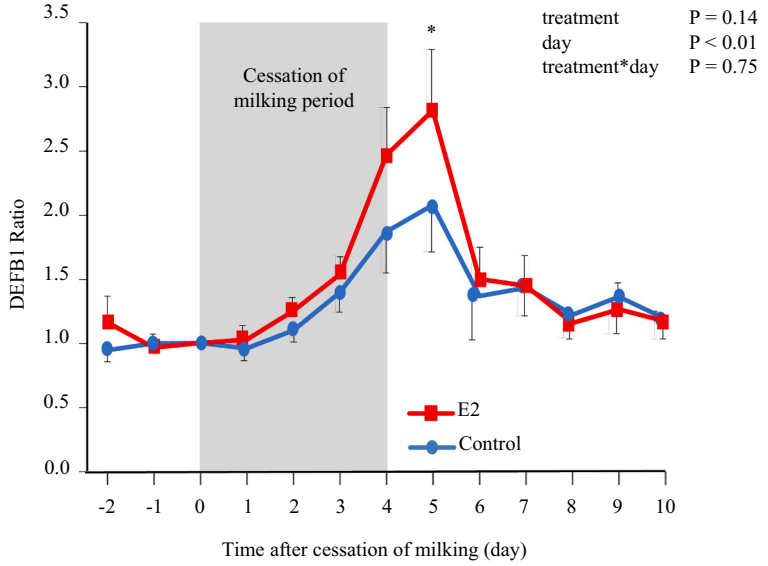

Fig. 3. Changes in concentration of beta-defensin 1 (DEFB1) in goat milk after a 3-d cessation of milking of both udder halves (n = 6). The vertical axis displays the ratio where day 0 (first day of cessation of milking) is set as 1. Results are represented as mean ± SEM. * $P < 0.05$ indicate significant difference compared with day 0.

in the control group and on days 5 to 7 in the E2 group ($P < 0.05$; Fig. 6).

The mean concentration of IgA was significantly higher on day 5 than on day 0 in both groups. Furthermore, the IgA concentration in the E2 group was significantly higher on day 2 than that in the control group ($P < 0.05$; Fig. 7). The mean LF concentration was significantly higher on days 5 to 7 than on day 0 in both groups. The LF concentration in the E2 group was significantly higher on days 4, 6, 7, 9, and 10 than that in the control group ($P < 0.05$; Fig. 8).

4. Discussion

Goats have been widely used in lactation studies because of their high availability, convenience in size and handling, and economic considerations. There are differences in the size and arrangement of mammary glands between dairy goats and dairy cows that affect milk composition, reflecting possible differences in the synthetic and transfer mechanisms during lactation. In general, however, the similarities in metabolism between the two species far outweigh the differences, which in most cases may be only slightly different (Larson, 1978). In this study, we used dairy goat as a model to demonstrate the effect of temporary cessation of milking and E2 administration on the antimicrobial components in milk.

In our previous study, temporary cessation of milking decreased milk yield and increased the concentration of some antimicrobial components in goat milk (Purba et al., 2021). In the present study, we hypothesized that the administration of E2 enhanced the effect of temporary cessation of milking by increasing the innate immune system, which may be beneficial against mastitis.

In the present experiment, the milk yield was significantly lower on days 5 and 6 than on day 0 in both groups. Cessation of milking is the most commonly used method for drying off dairy cows in the dairy industry (Barkema et al., 2015; Blowey and Edmondson, 2010), and is highly associated with a decrease in milk yield (Gott et al., 2017; Stelwagen et al., 2013). Once milking is stopped, numerous modifications

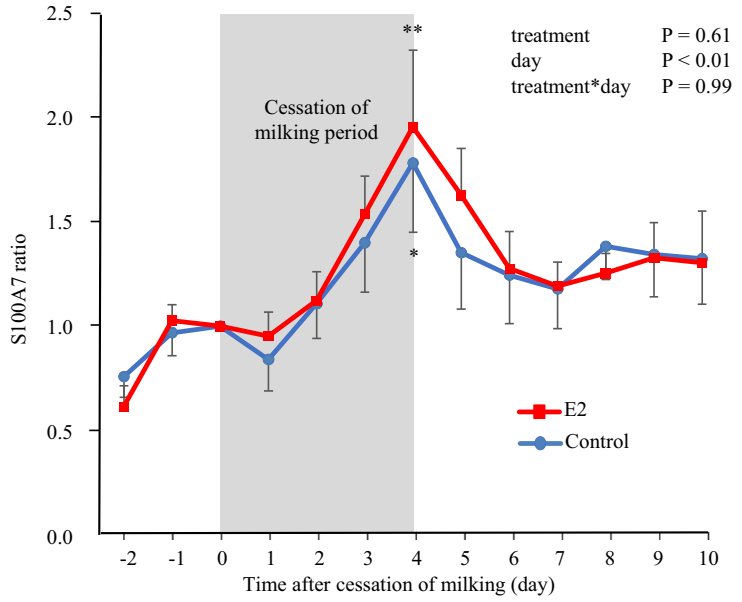

Fig. 4. Changes in concentration of S100A7 in goat milk after a 3-d cessation of milking of both udder halves (n = 6). The vertical axis displays the ratio where day 0 (first day of cessation of milking) is set as 1. Results are represented as mean ± SEM. * $P < 0.05$; ** $P < 0.01$ indicate significant difference compared with day 0.

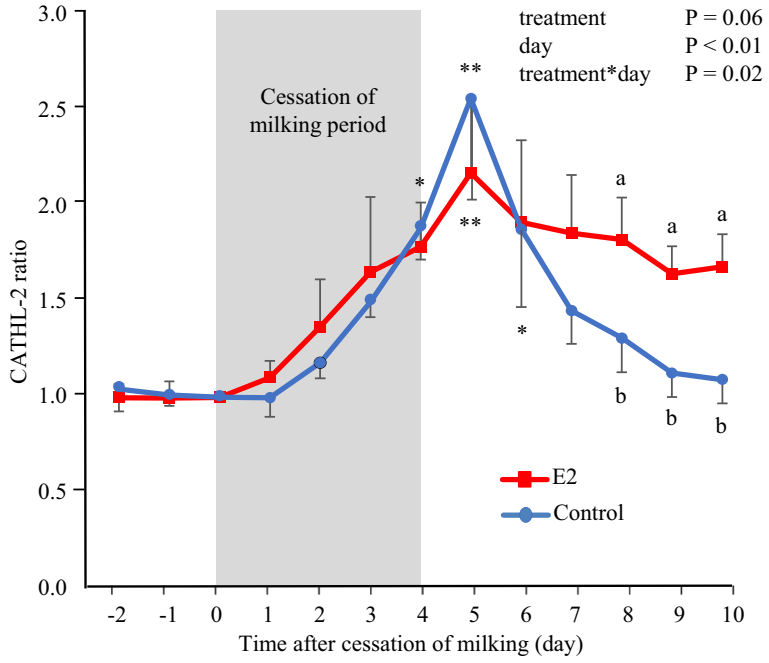

Fig. 5. Changes in concentration of cathelicidin-2 (CATHL-2) in goat milk after a 3-d cessation of milking of both udder halves (n = 6). The vertical axis displays the ratio where day 0 (first day of cessation of milking) is set as 1. Results are represented as mean ± SEM. * $P < 0.05$ and ** $P < 0.01$ indicate significant difference compared with day 0. a, b indicate a significant difference between the control group and E2 group on the same day ($P < 0.05$).

occur in the composition of the mammary tissue and the ultrastructure of the epithelial cells, leading to a decrease in mammary epithelial cell activity (Zhao et al., 2019). Previous studies have demonstrated that prolonging milking intervals leads to impaired mammary tight junction integrity, which subsequently decreases milk yield (Lakic et al., 2011; Stelwagen et al., 1994a). However, this process seems to be reversible, as milk yield began to recover by day 7, particularly in the control group. Conversely, frequent milking, five times per day, increases the milk yield in goats (Tsugami et al., 2022).

Milk yield in the E2 group was significantly lower on days 7 to 10 than that in the control group. E2 induces a reduction in milk yield (Kuwahara et al., 2017). Furthermore, estrogen significantly increases the activity of the apoptotic enzyme caspase-3 in mammary epithelial cells (Zielniok et al., 2014). Therefore, our results suggest that, in addition to the decrease in mammary epithelial cell activity after the cessation of milking, E2 administration may induce apoptosis in the mammary epithelial cells, resulting in the severe reduction in milk yield.

SCC increased significantly on day 5 compared with that on day 0 in both groups. Cessation of milking is often associated with an increase in SCC in milk (Lakic et al., 2009). Although mastitis is the main cause of increased SCC (Sordillo et al., 1997), reducing milking frequency also results in the same (Stelwagen and Lacy-Hulbert, 1996). Furthermore,

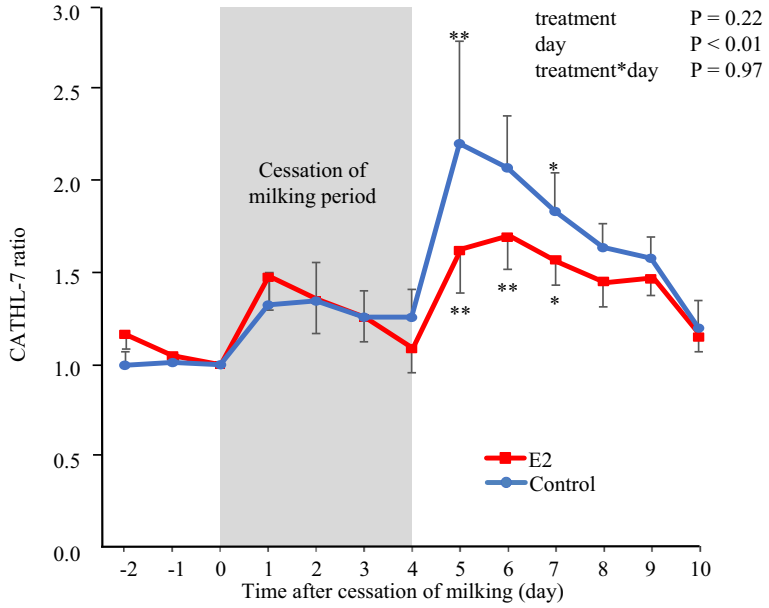

Fig. 6. Changes in concentration of cathelicidin-7 (CATHL-7) in goat milk after a 3-d cessation of milking of both udder halves (n = 6). The vertical axis displays the ratio where day 0 (first day of cessation of milking) is set as 1. Results are represented as mean ± SEM. * $P < 0.05$ and ** $P < 0.01$ indicate significant difference compared with day 0.

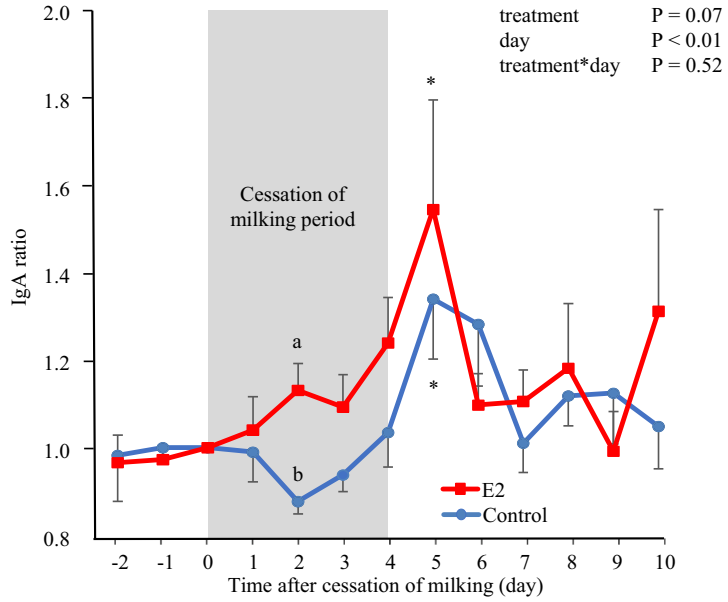

Fig. 7. Changes in concentration of IgA in goat milk after a 3-d cessation of milking of both udder halves (n = 6). The vertical axis displays the ratio where day 0 (first day of cessation of milking) is set as 1. Results are represented as mean ± SEM. * $P < 0.05$ indicates significant difference compared with day 0. a, b indicate a significant difference between the control group and E2 group on the same day ($P < 0.05$).

during once-daily milking, tight junctions between mammary epithelial cells become leaky, allowing greater movement of substances from milk into blood and vice versa (Stelwagen et al., 1994b). Therefore, leaking tight junctions may allow some polymorphonuclear cells in the blood to enter the mammary gland, contributing to the increase in SCC in milk.

In the E2 group, milk SCC was significantly higher on days 7 and 10 than in the control group. Also, E2 induces an increase in SCC in the milk of uninfected cows (Kuwahara et al., 2017). Some authors (McDougall and Voermans, 2002; Moroni et al., 2007; Paape et al., 2001; Yamasaki et al., 2017) have reported that SCC was elevated during the preovulatory and estrous periods (high E2 period). However, SCC seems to increase with a decrease in milk yield. This result suggests that the increase in SCC must be at least partially due to the decrease in milk volume.

In our previous study, the temporary cessation of milking significantly increased the levels of some antimicrobial components in milk, such as goat DEFB1, S100A7, CATHL-2, CATHL-7, IgA, and LF (Purba et al., 2021). In the present study, the concentrations of these antimicrobial components also increased significantly after the cessation of milking in both groups. The concentrations of IgA and LF were significantly higher in the E2 group than in the control group within the cessation of milking period. Furthermore, the concentration of CATHL-2 was significantly higher after milking was resumed in the E2 group than that in the control group. Estrogen has both anti-inflammatory and pro-

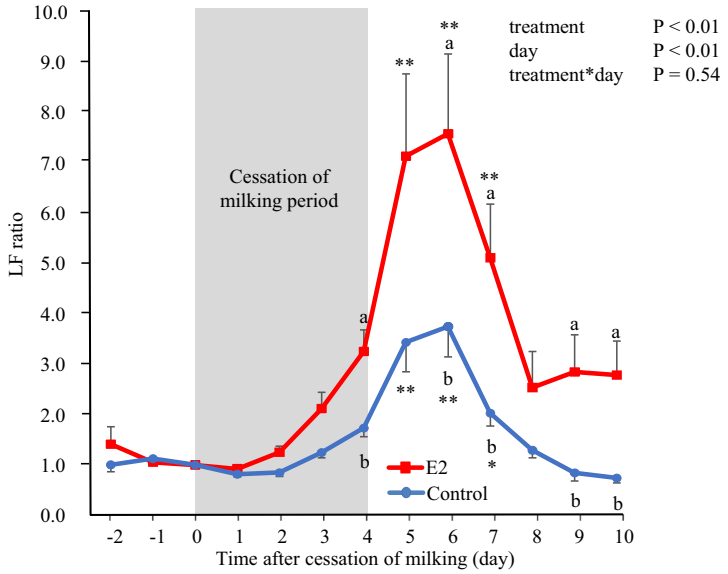

Fig. 8. Changes in concentration of lactoferrin (LF) in goat milk after a 3-d cessation of milking of both udder halves (n = 6). The vertical axis displays the ratio where day 0 (first day of cessation of milking) is set as 1. Results are represented as mean ± SEM. * $P < 0.05$ and ** $P < 0.01$ indicate significant difference compared with day 0. a, b indicate a significant difference between the control group and E2 group on the same day ($P < 0.05$).

inflammatory functions, and its role in the modulation of the innate immune response of epithelial cells during infection has been documented (Fahey et al., 2008).

The concentration of IgA in both groups was significantly higher on day 5 than on day 0. It plays a dominant role in mammary gland defenses against bacterial pathogens (Ezzat Alnakip et al., 2014), particularly, in toxin neutralization and bacterial agglutination (Korhonen et al., 2000). Furthermore, IgA is found at much lower concentrations in healthy bovine mammary glands (Butler, 1983), indicating its important role during infection.

The concentration of IgA in the E2 group was significantly higher on day 2 than that in the control group. In rats, the secretion of IgA is high during pro-estrus when the estrogen concentration is high (Wira and Sandoe, 1977). E2 treatment has been reported to increase the number of IgA-positive uterine cells and the uterine IgA content (Wira and Sullivan, 1985). Furthermore, 17 beta-estradiol benzoate increased the frequency of IgA-producing B cells in mice (Lagerquist et al., 2008). Therefore, the present results suggest that E2 enhances the secretion of IgA from the mammary gland cells into the milk.

The concentration of LF in both groups was significantly higher on days 5 to 7 than on day 0. Accordingly, the suspension of milking in dairy cows causes a transient decrease in milk yield, subsequently increasing the concentration of LF (Davis and South, 2015). It is mainly recognized as a bacteriostatic and bactericidal protein (Chaneton et al., 2008; Jahani et al., 2015). In bovine milk, LF is mainly produced by the secretory epithelium and, to a lesser extent, by the polymorphonuclear cells (Huang et al., 2012; Persson et al., 1992). The LF concentration in milk increases during intramammary infections (Hagiwara et al., 2003; Kawai et al., 1999; Molenaar et al., 1996; Raj et al., 2021).

The LF concentration was significantly higher in the E2 group than in the control group on days 4, 6, 7, 9, and 10. This result suggests that LF secretion was elevated by E2 administration. The concentration of LF in milk increases under the influence of E2 administration in goats stimulated by *Staphylococcus aureus* infusion into the mammary gland (Kuwahara et al., 2017). In humans, LF gene expression in the endometrium increased during the proliferative phase, whereas in monkeys, the immunoreactive LF protein in the endometrium was elevated by E2 treatment (Teng, 2002). Similarly, our results show that LF was upregulated by E2 administration and plays an important role in the innate defense of the mammary gland.

The concentration of CATHL-2 was significantly higher after the cessation of milking in both groups. Cathelicidin is a peptide with broad-spectrum antimicrobial properties (Scott et al., 2011). CATHL-2 is secreted by polymorphonuclear cells, and its concentration is elevated by the intramammary infusion of lipopolysaccharides (Zhang et al., 2014). In the E2 group, the concentrations of CATHL-2 on days 8 to 10 were significantly higher than those in the control group. No study has reported that E2 upregulates the secretion of CATHL-2. However, as CATHL-2 is secreted by polymorphonuclear cells, it may be associated with an increase in SCC. It has been reported that CATHL-2 is secreted by leukocytes even without lipopolysaccharide stimulation (Srisaikham et al., 2016).

During the 3-d milking cessation period, the concentrations of goat DEFB1, S100A7, and CATHL-2 tended to increase in both groups. Cessation of milking during the lactation period causes an increase in intramammary pressure and milk stasis. Milk stasis that occurs during the cessation of milking could lead to the accumulation of factors that inhibit milk synthesis and secretion in the mammary gland (Zhao et al., 2019). It is possible that these antimicrobial components accumulated in milk during the treatment and subsequently showed increased concentration.

After cessation of milking, the concentrations of milk DEFB1, S100A7, CATHL-2, CATHL-7, IgA, and LF increased significantly in conjunction with a decrease in milk yield. The concentrations of these antimicrobial components decreased along with the recovery of milk yield by day 7 of the experiment. This result suggests that the increase in antimicrobial components after the milking cessation period may be associated with a decrease in milk volume. However, as the bovine mammary gland does not regress to the same extent as the rodent mammary gland during the cessation of milking (Zhao et al., 2019), it is also possible that mammary epithelial cells can maintain the secretion of these antimicrobial components even after the cessation period. Furthermore, some antimicrobial components, such as cathelicidins and LF, are also produced by leukocytes (Srisaikham et al., 2016). Therefore, the increase in the concentration of these antimicrobial components might be partially due to the increase in SCC.

In conclusion, our results indicate that cessation of milking increases the concentration of some antimicrobial components that are beneficial for the protection of the mammary gland against infection. However, this treatment appeared to be only temporarily effective. The

administration of E2 during the cessation of milking enhanced the secretion of some important antimicrobial components such as CATHL-2, IgA, and LF during and after treatment. Therefore, the administration of exogenous E2, in combination with the cessation of milking during infection, offers the opportunity to augment the normal immune response of the mammary gland. In combination with the cessation of milking, E2 must be considered to replace antibiotics in mastitis treatment. Further research is required to confirm whether this combination is effective in the treatment of mastitic udders.

Declaration of Competing Interest

None.

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