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SHORT COMMUNICATION

Lipoteichoic Acid Disrupts Mammary Epithelial Barrier Integrity by Altering Expression of Occludin and Zonula Occluden (ZO)-1

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ABSTRACT

This study aimed to investigate the effect of the cell wall component lipoteichoic acid (LTA) of *Staphylococcus aureus* on mammary alveolar tight junctions (TJs) proteins including claudin-1, occludin and zonula occluden (ZO)-1. Primary bovine mammary epithelial cells were grown on Transwell inserts for 108 hours. The integrity and tightness of the growing epithelial cell layer were evaluated by measuring transepithelial electrical resistance. The permeability of bovine mammary epithelial cells (BMECs) was assessed by measuring horseradish peroxidase transmission. The mRNA levels of BMECs TJ components were measured with quantitative real-time polymerase chain reaction. In LTA-induced mastitis of mouse model, the protein expression of TJs (claudin-1, occludin and ZO-1) were determined by western blot analysis. Our results showed that LTA increased barrier permeability of BMECs. Treatment with LTA decreased mRNA and protein levels of occludin and ZO-1 in vivo and in vitro, however, the expression of claudin-1 did not change. The results suggested that disruption of mammary epithelial barrier integrity is caused by the alteration of occludin and ZO-1.

Keywords: Lipoteichoic acid, Mammary, Tight junctions

INTRODUCTION

Cow mastitis is an inflammation of the mammary gland commonly caused by bacterial infection [1]. *Staphylococcus aureus* is one of the predominant pathogens of clinical and subclinical bovine mastitis [2]. Chronic mastitis caused by *S. aureus* leads to reduced milk quantity and quality, and even leads to culling of cows, resulting in serious economic losses [3]. The major immunogenic cell wall component, lipoteichoic acid (LTA), deriving from *S. aureus*, are commonly infused in the bovine mammary gland to elicit experimental mastitis [2].

The blood-milk barrier is mainly composed of mammary epithelial cells. The blood-milk barrier has several functions, such as resisting pathogen invasion and preventing leakage between milk and blood components [4]. Disruption of the blood-milk barrier leads to the aggravation of bacterial infections and the development of inflammation [5]. Tight junctions (TJs) are important for the establishment of the blood-milk barrier [6]. TJs are made of multiprotein complexes such as claudins, occludin, zonula occludens (ZO) and junctional adhesion molecules (JAMs) [7]. The tight junctions act as a barrier to prevent leakage of milk and blood components [8]. TJs function as a barrier to control the paracellular passage and selectively allow solutes and water as well as some microorganisms to pass through. Some bacterial pathogens and toxins can break barrier function by disruption of TJs, which leads to facilitate bacterial entry and spread [9].

Mastitis is able to disrupt the tight junction barrier and it has been reported that LPS disrupts the blood-milk barrier by regulating the expression of claudins and occludin proteins [10,11]. LTA has been reported in the literature to affect bovine mammary epithelial barrier integrity [12]. However, the effect of LTA-induced mastitis on mammary tight junction proteins remains unclear. In this study, we determined permeability of bovine mammary epithelial cells (BMECs) and the differential expression of genes of claudin-1, occludin and ZO-1 in BMECs stimulated with purified LTA. We injected LTA into the mouse mammary glands to induce experimental mastitis and investigated TJ proteins of claudin-1, occludin and ZO-1.

Materials and Methods

Ethical Approval

All the procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Inner Mongolia Agricultural University (IMAU) (License No. SYXK, Inner Mongolia, 2014–0008) with adherence to IMAU guidelines.

Primary BMEC Culture and Stimulation

BMECs were isolated from lactating cows as previously described, with slight modifications [13]. The mammary tissue pieces were washed twice with phosphate buffered saline before digestion. The tissue samples were minced using surgical scissors. Minced samples were incubated in aseptic Hank's balanced salt solution for 1 h at 37°C. The prepared tissue was digested with collagenase I (Sigma-Aldrich, St. Louis, MO) for 3 h at 37°C. The digest was filtered through a nylon mesh (BD Bioscience, 100 µm) and the filtrate centrifuged for 10 min/1.250 rpm. The cell inoculum was transferred into 25 cm^2 cell culture flasks (Corning, Corning, NY, USA) and cultured in Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12 (DMEM/F12; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, USA), and 100 U/mL penicillin and streptomycin (Invitrogen). Fibroblasts-free cultures were obtained by differential trypsinization. BMECs were identified as previously described [14]. For stimulation of BMECs with LTA, the medium was removed, and 0, 0.01, 0.1, 1.0, 10 µg/mL LTA in stimulation medium were added at the desired concentration in 1 mL of stimulation medium. After incubation for 24 h, cell culture supernatant was removed and BMECs were harvested for RNA extraction.

Measurement of Transepithelial/Transendothelial Electrical Resistance

Cells were seeded on the upper surface of polycarbonate membrane with 3 µm pores in Transwell inserts in 12-well cell culture plates (Corning). Cells were grown in 1.5 and 0.5 mL of growth medium on the basolateral and apical sides, respectively, in the presence of 5% CO_2 at 37°C. Medium was changed every 48 h, Cells were seeded in Transwell plates and recorded as 0 h. Transepithelial/transendothelial electrical resistance (TEER) was measured at 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, 96 h and 108 h using an EVOMX ohmmeter with a STX2 Chopstickelectrode set (World Precision Instruments, Sarasota, Fla., USA). TEER values ($\Omega \times cm^2$) were obtained by subtracting the resistance of blank filters without cells from the resistance of filters with cells. The results were then multiplied by the area of the membrane from the filter insert.

HRP Flux

Horseradish peroxidase (HRP, Sigma-Aldrich) transmissivity was measured in order to determine the *in vitro* permeability of BMECs. At 24 h after LTA exposure, the culture medium was replaced with DMEM/F12 without phenol red. In order to maintain the culture supernatant level, 0.5 mL of medium containing 50 ng HRP was added into the insert, and 1 mL of medium was added into the well. A total of 50 µL of medium was collected from each well at 1 h, which was replaced with 50 µL of fresh medium after each collection in order to maintain the liquid level on both sides. The collected samples were stored at 4°C until processing, while 50 µL of peroxidase substrate containing tetramethyl benzidine and hydrogen peroxide was added to each sample and incubated for 10 min. The reaction was terminated by adding 50 µL of 1 M sulphuric acid. The optical density was measured at 450 nm, and the HRP transmissivity was assayed from the standard curve according to the following equation: $P_{HRP\%} = [(C_{HRPo} \times V_o / C_{HRPi} \times V_i) \times 100\%]$, where C_{HRPo} is the HRP concentration in the well, C_{HRPi} is the HRP concentration in the insert, V_o is the medium volume in the well, and V_i is the medium volume in the insert.

Reverse Transcription and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from treated BMECs using TRIzol reagent (Invitrogen). Total RNA was reverse transcribed with the PrimeScript RT reagent kit (Takara Bio, Dalian, Japan) according to the manufacturer's instructions. The sequences of primers were as follows:
claudin-1: F:AAGACGACGAGGCACAGAAGA
and R:GAAGGTGCTGGCTTGGGATAG;
occludin: F:CAGCAGCAGTGGTAACTTGGA
and R:CCGGTCGTGTAGTCTGTTTCAT;
ZO-1: F:GCGAAATGAGAAACAAGCACC
and R:ATGAGTTGAGTTGGGCAGGAC;
GAPDH: F:GTTTGTGATGGGCGTGAACC and
R:CAGTCTTCTGGGTGGCAGTGAT.

Quantitative RT-PCR was performed with a SYBY Green Master Mix (Takara Bio, Dalian, Japan) using Mx3000P Real-Time QPCR System (Stratagene, USA). PCR cycles consisted of an initial denaturation step at 95°C for 30 s, followed by 95°C for 5 s, and 60°C for 30 s. All values were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as the change relative to GAPDH mRNA expression.

Animals

Our experimental protocols were conducted in accordance with the NIH guidelines outlined in the Guide for the Care and Use of Laboratory Animals and were approved by a local ethics committee. Pregnant BALB/c mice (10 to 12 weeks old, 25 to 30 g weight) were purchased from Changchun Institute of Biological Products. After parturition, the lactating mouse was kept with suckling neonatal pups. LTA that originated from *S. aureus* (InvivoGen, San Diego, CA, USA) was solubilized

in physiological saline. Doses of 0, 0.01, 0.1, 1.0 and 10 μg were injected into the fourth inguinal mammary gland via teat canal on day 7 of lactation under anesthesia with pentobarbital. No symptoms were observed in the mice. 24 h after LTA injection, the mice were decapitated, and the mammary glands were collected.

Western Blot Analysis

Frozen mammary glands (0.1 g) were homogenized and lysed in RIPA buffer (Beyotime, China) supplemented with protease inhibitor cocktail (Sigma). The homogenized lysate was centrifuged at 12.000xg at 4°C for 15min, and the supernatants were collected and determined with BCA protein assay kits (Beyotime). Equal volumes of protein (30 μg) were loaded onto SDS-PAGE gels and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, MA, USA) using a Mini Trans-Blot cell (Bio-Rad, CA, USA). The membranes were blocked for 2 h with 5% milk in TBST (0.1% Tween-20 in tris-buffered saline) at room temperature and incubated overnight at 4°C with primary antibodies against claudin-1 (1:200 dilution, santa cruz), occludin (1:200 dilution, santa cruz), ZO-1(1:200 dilution, santa cruz) and GAPDH (1:10000 dilution, abcam). The membranes were further incubated with horseradish peroxidase-(HRP-) conjugated anti-rabbit or anti-mouse IgG for 1 h at room temperature and finally developed with an electrochemiluminescence system (ECL; Solarbio, Beijing, China). To quantify relative protein expression levels, the intensity of specific protein bands was quantified using Quantity One software and then normalized normalized to β-actin for each lane.

Statistical Analysis

Standard statistical analysis was completed using SPSS16.0 (SPSS, Chicago, IL, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA). $P<0.05$ was considered significant.

Results

TEER of BMECs Monolayer

Before the transepithelial permeability study, TEER measurement was used to assess the tightness of the BMECs monolayer. The TEER of the cells gradually increased in a time-dependent manner *(Fig. 1)*. TEER increased from 24 h (6.8±1.0Ω•cm^2) to 108 h (260.3±13.00Ω•cm^2). After seeding, an obvious increase in the TEER value could be observed from 24 h to 96 h. The TEER reached a plateau after 96 h.

LTA Increased Barrier Permeability of BMECs

The permeability of the BMECs monolayer was assessed by Horseradish Peroxidase (HRP) flux assay after LTA treatment. As shown in *Fig. 2*, compared to the untreated group, the HRP permeability significantly increased at 24 h after treatment with 0.1, 1.0 and 10 μg/mL LTA ($P<0.05$). When LTA concentration was 0.01 μg/mL, HRP permeability was unchanged.

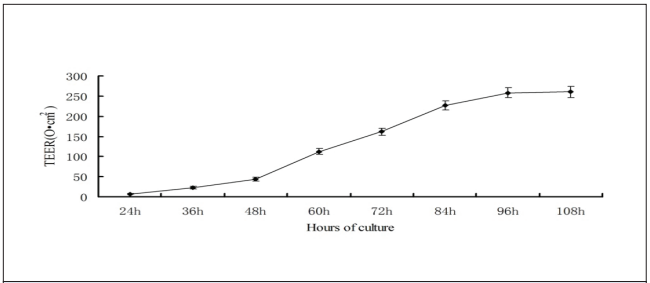

Fig 1. TEER values of BMECs monolayers on various hours in cultures. Each value represents the mean±S.D. (n=3)

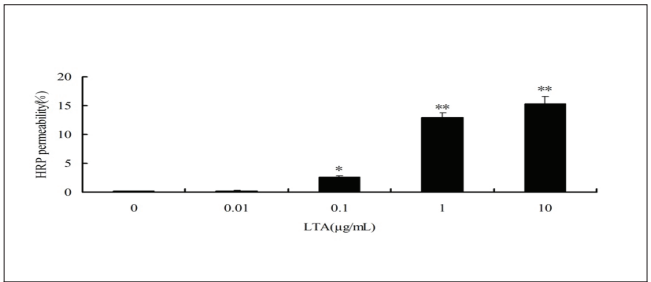

Fig 2. HRP permeability increased in BMECs monolayers challenged with LTA. Each data represents the mean±S.D. (n=5). *P<0.05, **P<0.01, compared with corresponding sham-treated controls

LTA Decreased Occludin and ZO-1 mRNA in BMECs

To examine the effects of LTA on the TJ protein expression levels, BMECs were exposed to different concentrations of LTA (0, 0.01, 0.1, 1.0 and 10 μg/mL) for 24 h, LTA treatment significantly decreased occludin and ZO-1 mRNA expression at concentrations of 0.1, 1.0 and 10 μg/mL ($P<0.05$). 0.01 μg/mL LTA had no effect on the expression of occludin and ZO-1 mRNA in BMECs. However, LTA treatment had no effect on expression of claudin-1 *(Fig. 3)*.

LTA Decreased Occludin and ZO-1 Protein Expression in Mouse Mammary Glands

We further investigated whether LTA exerted a prohibitive effect by regulating expression of TJ proteins in mouse mammary glands. Using immunoblotting, 0.01 μg LTA treatment had no effect on the expression of occludin and ZO-1 protein. LTA treatment with 0.1, 1.0 and 10 μg significantly decreased occludin and ZO-1 protein expression in mouse mammary glands ($P<0.05$). However, claudin-1 showed no change 24 h after LTA injection *(Fig. 4)*.

Discussion

TEER is a method to measure the integrity of TJs in cell

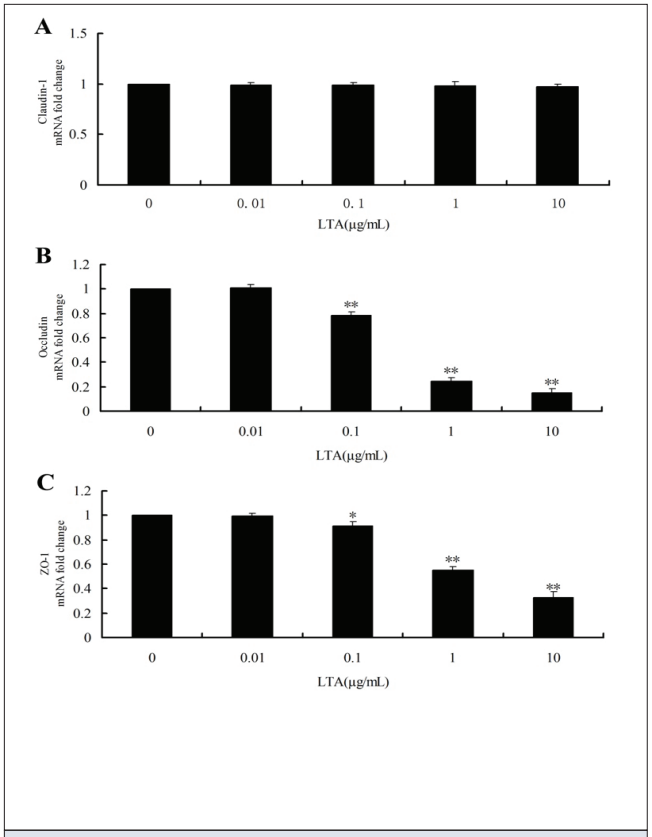

Fig 3. Effect of LTA on mRNA expression of TJ proteins in BMECs.Real-time PCR was performed to determine mRNA expression of (A) claudin-1, (B) occludin and (C) ZO-1 at 24 h after LTA challenge, respectively. Data represent means ± SD (n = 3) and were analyzed using one-way ANOVA followed by the Duncan test for multiple comparisons. *P<0.05, **P<0.01, compared with corresponding sham-treated controls

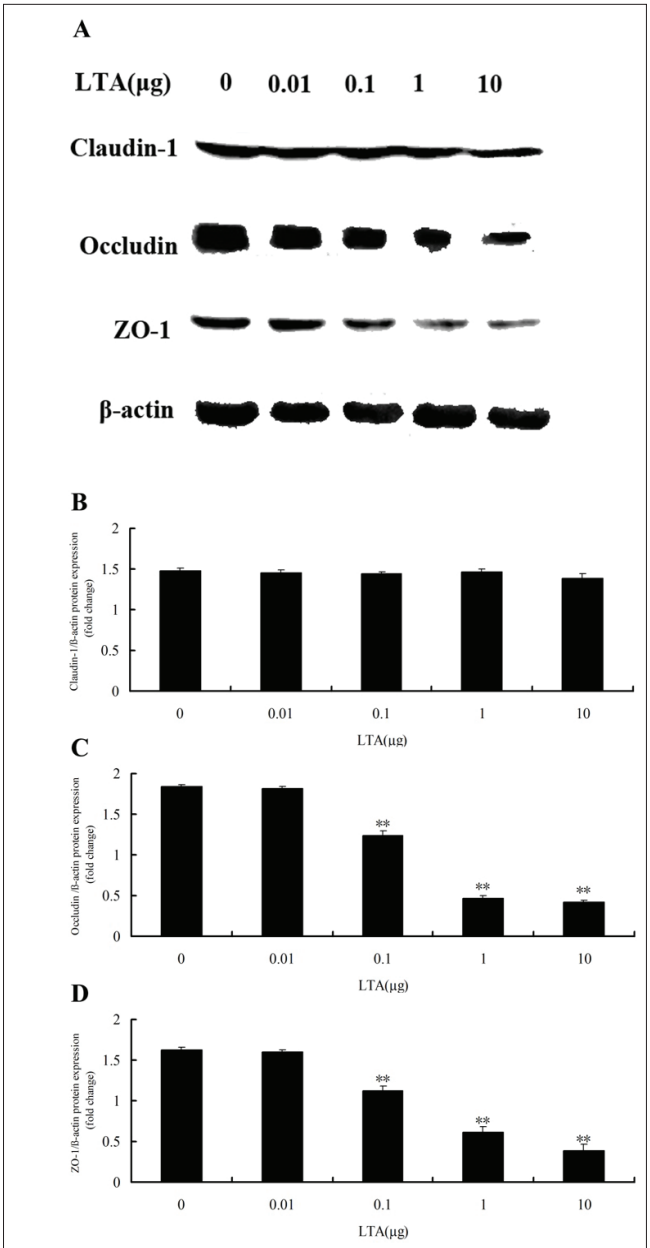

Fig 4. Effects of LTA on protein expression of TJ proteins challenged mouse mammary glands. (A) Results of a western blot analysis of claudin-1, occludin, ZO-1 and β-actin in the mammary glands 24 h after LTA injection. The relative expression levels of (B) claudin-1, (C) occludin and (D) ZO-1 were analyzed by densitometry. β-actin was used as a normalization control. Data represent mean means ± SD (n = 3). *P<0.05, **P<0.01, compared with corresponding sham-treated controls

culture models of endothelial and epithelial monolayers and reflects the integrity of the cellular barriers [15]. It has been demonstrated that primary bovine mammary epithelial cells cultured on Transwell inserts were grown to confluence by measurement of TEER [16]. In our study, continuous enhancement of TEER values indicated that cultured epithelial cells were able to form a tight barrier. In this experiment, the TEER value of primary BMECs reached a plateau at 96 hours, which was similar to the result that the TEER value of bovine mammary alveolar cell line (MAC-T) almost reached a plateau at 4 days [17].

HRP has been used to study macromolecule diffusion across epithelial/endothelial monolayers by tracking supernatant HRP activity [15]. The challenge of BMECs with 0.1, 1.0 and 10 μg/mL LTA resulted in a significant increase of BMECs permeability. Another study demonstrated that LPS or LTA can increase BMECs permeability by diffusion of Lucifer yellow [12]. In accordance with *in vitro* study, *in vivo* study showed that intramammary administration of LPS or LTA could cause impairment in the permeability of the blood-milk barrier leading to transfer of blood and milk components [18]. It has been reported that BMECs were treated with LPS of 10 μg/mL, and mastitis was induced by LPS of 10 μg in mice [19,20]. In this study, the doses of 0, 0.01, 0.1, 1.0, 10 μg/mL LTA were selected to treat BMECs according to LPS, and the doses of 0, 0.01, 0.1, 1.0 and 10 μg were used to induce mastitis in mice.

A decrease in the TJ protein mRNA and protein levels has been shown to damage the barrier's architecture, resulting in an increased leaking of the barrier [21]. LTA is recognized by TLR2 as a pathogen-associated molecular pattern and

induces inflammation and inflammatory cytokines via TLR2/NF-κB [22]. Therefore, we infer that LTA induced mastitis causes BMECs increased permeability is likely to be due to the decrease of TJ protein expression. This study showed that LTA decreased mRNA levels of TJ proteins occludin and ZO-1 but not claudin-1 in BMECs. It has been demonstrated that LPS or LTA disrupted epithelial/endothelial tight junction *in vitro* by suppressing expression of TJ proteins [23]. Different from LTA, the transcription levels and protein expression levels of occludin, ZO-1 and claudin-1 in BMECs treated with LPS were decreased [20]. We speculate that this difference may be related to the difference in the levels of inflammatory cytokines induced by LTA and LPS in BMECs.

The TJ proteins ZO-1 and occludin are two key proteins that maintain the integrity of epithelial cell TJ in mammary glands [24]. To assess the *in vivo* effects of LTA, we also developed an *in vivo* mouse mammary model of LTA modulation of expression levels of claudin-1, occludin and ZO-1. Our results demonstrate that LTA (0.1 μg, 1.0 μg, and 10 μg) obviously decreases occludin and ZO-1 protein levels. However, in our study LTA treatment had no effect on protein expression of claudin-1. Recently, it has been reported that LPS-induced mastitis in mice reduces the protein expression of occludin, ZO-1 and claudin-1 [19]. We hypothesized that LPS-induced mastitis may result from more intense production of inflammatory cytokines than LTA.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

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Competing Interests

The author declared that there is no competing interests.

Ethical Approval

All the procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Inner Mongolia Agricultural University (IMAU) (License No. SYXK, Inner Mongolia, 2014-0008) with adherence to IMAU guidelines.

References

1. Deplanche M, Alekseeva L, Semenovskaya K, Fu CL, Dessauge F, Finot L, Petzl W, Zerbe H, Le Loir Y, Rainard P, Smith DGE, Germon P, Otto M, Berkova N: *Staphylococcus aureus* phenol-soluble modulins impair interleukin expression in bovine mammary epithelial cells. *Infect Immun,* 84 (6): 1682-1692, 2016. DOI: 10.1128/IAI.01330-15

2. Giovannini AEJ, van den Borne BHP, Wall SK, Wellnitz O, Bruckmaier RM, Spadavecchia C: Experimentally induced subclinical mastitis: Are lipopolysaccharide and lipoteichoic acid eliciting similar pain responses? *Acta Vet Scand,* 59 (1): 40, 2017. DOI: 10.1186/s13028-017-0306-z

3. Caceres ME, Ledesma MM, Lombarte Serrat A, Vay C, Sordelli DO, Giacomodonato MN, Buzzola FR: Growth conditions affect biofilms of *Staphylococcus aureus* producing mastitis: Contribution of MALDI-TOF-MS to strain characterization. *Curr Res Microb Sci,* 2:100073, 2021. DOI: 10.1016/j.crmicr.2021.100073

4. Wellnitz O, Bruckmaier RM: Invited review: The role of the blood-milk barrier and its manipulation for the efficacy of the mammary immune response and milk production. *J Dairy Sci,* 104 (6): 6376-6388, 2021. DOI: 10.3168/jds.2020-20029

5. Guo W, Liu B, Yin Y, Kan X, Gong Q, Li Y, Cao Y, Wang J, Xu D, Ma H, Fu S, Liu J: Licochalcone A protects the blood-milk barrier integrity and relieves the inflammatory response in LPS-Induced mastitis. *Front Immunol,* 10:287, 2019. DOI: 10.3389/fimmu.2019.00287

6. Zhu H, Jia Q, Zhang Y, Liu D, Yang D, Han L, Chen J, Ding Y: Regulation of tight junctions by sex hormones in goat mammary epithelial cells. *Animals (Basel),* 12 (11):1404, 2022. DOI: 10.3390/ani12111404

7. Heinemann U, Schuetz A: Structural features of tight-junction proteins. *Int J Mol Sci,* 20 (23):6020, 2019. DOI: 10.3390/ijms20236020

8. Kobayashi K: Culture models to investigate mechanisms of milk production and blood-milk barrier in mammary epithelial cells: A review and a protocol. *J Mammary Gland Biol Neoplasia,* 28 (1):8, 2023. DOI: 10.1007/s10911-023-09536-y

9. Lu RY, Yang WX, Hu YJ: The role of epithelial tight junctions involved in pathogen infections. *Mol Biol Rep,* 41 (10): 6591-6610, 2014. DOI: 10.1007/s11033-014-3543-5

10. Kobayashi K, Oyama S, Numata A, Rahman MM, Kumura H: Lipopolysaccharide disrupts the milk-blood barrier by modulating claudins in mammary alveolar tight junctions. *PLoS One,* 8 (4):e62187, 2013. DOI: 10.1371/journal.pone.0062187

11. Wang JJ, Wei ZK, Zhang X, Wang YN, Fu YH, Yang ZT: Butyrate protects against disruption of the blood-milk barrier and moderates inflammatory responses in a model of mastitis induced by lipopolysaccharide. *Br J Pharmacol,* 174 (21): 3811-3822, 2017. DOI: 10.1111/bph.13976

12. Wellnitz O, Zbinden C, Huang X, Bruckmaier RM: Short communication: Differential loss of bovine mammary epithelial barrier integrity in response to lipopolysaccharide and lipoteichoic acid. *J Dairy Sci,* 99 (6): 4851-4856, 2016. DOI: 10.3168/jds.2016-10927

13. Jedrzejczak M, Szatkowska I: Bovine mammary epithelial cell cultures for the study of mammary gland functions. *In Vitro Cell Dev Biol Anim,* 50 (5): 389-398, 2014. DOI: 10.1007/s11626-013-9711-4

14. Xu DD, Wang G, He XJ, Wang JF, Yang B, Sun ZP, Sun DB, He QY, Zhang X, Wu R: 17beta-Estradiol and progesterone decrease MDP induced NOD2 expression in bovine mammary epithelial cells. *Vet Immunol Immunopathol,* 188, 59-64, 2017. DOI: 10.1016/j.vetimm.2017.04.010

15. Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ: TEER measurement techniques for *in vitro* barrier model systems. *J Lab Autom,* 20 (2): 107-126, 2015. DOI: 10.1177/2211068214561025

16. Tsugami Y, Suzuki N, Kawahara M, Suzuki T, Nishimura T, Kobayashi K: Establishment of an *in vitro* culture model to study milk production and the blood-milk barrier with bovine mammary epithelial cells. *Anim Sci J,* 91 (1):e13355, 2020. DOI: 10.1111/asj.13355

17. Zhao X, Sun P, Liu M, Liu S, Huo L, Ding Z, Wang S, Lv C, Wu H, Yang L, Liang A: Deoxynivalenol exposure inhibits biosynthesis of milk fat and protein by impairing tight junction in bovine mammary epithelial cells. *Ecotoxicol Environ Saf,* 237:113504, 2022. DOI: 10.1016/j.ecoenv.2022.113504

18. Wall SK, Wellnitz O, Hernandez-Castellano LE, Ahmadpour A, Bruckmaier RM: Supraphysiological oxytocin increases the transfer of immunoglobulins and other blood components to milk during lipopolysaccharide- and lipoteichoic acid-induced mastitis in dairy cows. *J Dairy Sci,* 99 (11): 9165-9173, 2016. DOI: 10.3168/jds.2016-11548

19. Liu Y, Jiang Y, Yang Y, Wang H, Ye J, Liu D, Chen Y, Lian C, Wang R, Gao Y, Meng Y, Gao L: Houttuynia essential oil and its self-microemulsion

preparation protect against LPS-induced murine mastitis by restoring the blood-milk barrier and inhibiting inflammation. *Front Immunol,* 13:842189, 2022. DOI: 10.3389/fimmu.2022.842189

20. Song J, Hu Y, Wang L, Ao C: Ethanol extract of artemisia annua prevents LPS-induced inflammation and blood-milk barrier disruption in bovine mammary epithelial cells. *Animals (Basel),* 12 (10), 2022. DOI: 10.3390/ani12101228

21. He C, Deng J, Hu X, Zhou S, Wu J, Xiao D, Darko KO, Huang Y, Tao T, Peng M, Wang Z, Yang X: Vitamin A inhibits the action of LPS on the intestinal epithelial barrier function and tight junction proteins. *Food Funct,* 10 (2): 1235-1242, 2019. DOI: 10.1039/c8fo01123k

22. Chandler CE, Ernst RK: Bacterial lipids: Powerful modifiers of the innate immune response. *F1000Res,* 6:F1000, 2017. DOI: 10.12688/f1000research.11388.1

23. Singh AK, Jiang Y, Gupta S: Effects of bacterial toxins on endothelial tight junction *in vitro:* A mechanism-based investigation. *Toxicol Mech Methods,* 17 (6): 331-347, 2007. DOI: 10.1080/15376510601077029

24. Zhang C, Zhai S, Wu L, Bai Y, Jia J, Zhang Y, Zhang B, Yan B: Induction of size-dependent breakdown of blood-milk barrier in lactating mice by TiO_2 nanoparticles. *PLoS One,* 10 (4):e0122591, 2015. DOI: 10.1371/journal.pone.0122591