Betaine protects bovine mammary epithelial cells against LPS-induced inflammatory response and oxidative damage via modulating NF-κB and Nrf2 signalling pathway

Nannan Zhao, Yuhang Yang, Haixu Xu, Lulu Li, Yun Hu, Enqi Liu and Jue Cui

School of Food and Biology Engineering, Xuzhou University of Technology, Xuzhou, People’s Republic of China; College of Animal Science and Technology, Yangzhou University, Yangzhou, People’s Republic of China

ABSTRACT
Bovine mastitis is among the most serious disease in the dairy industry and brings huge economic losses due to the decrease in milk quality and quantity. Betaine, a naturally occurring compound, possesses several pharmacological activities including anti-inflammatory and anti-oxidant ability, but whether betaine has protective effects on bovine mastitis is unknown. The aim of this study is to investigate the effect of betaine on mastitis and further discover its feasible molecular mechanism in lipopolysaccharide (LPS)-stimulated bovine mammary epithelial cells (BMECs). BMECs were pre-treated with or without betaine or LPS. Cell viability was measured with CCK-8 to examine the cytotoxicity. The levels of pro-inflammatory cytokines were measured by ELISA kits. Western blotting was used to explore the regulation of genes associated with inflammatory and oxidative stress genes. The results showed that LPS treatment significantly increased the production of pro-inflammation (IL-1β, IL-6 and TNFα), enhanced malondialdehyde (MDA) content, reduced superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity, and markedly up-regulated the protein expression of COX2 and iNOS (p < 0.05). However, betaine pre-treatment remarkably restored the above phenomenon compared with the LPS group. Additionally, we also observed that betaine exposure significantly restricted LPS-induced the phosphorylation of IκB and NF-κB p65 (p < 0.05). Moreover, pre-treatment of BMECs with betaine abolished LPS-induced the increase of Nrf2 and HO-1 protein levels (p < 0.05). These results confirm that betaine can alleviate LPS-induced inflammatory response and oxidative damage by modulating NF-κB and Nrf2 signalling pathways.

HIGHLIGHTS
• Betaine alleviated the production of pro-inflammation cytokines (IL-1β, IL-6 and TNFα) in BMECs after LPS stimulation.
• Betaine restored LPS-elicited nuclear factor kappa-B (NF-κB) signalling pathway activity.
• Betaine recovered LPS-induced the activity of SOD and GSH-Px and MDA content.
• Betaine inhibited the increase of protein expression of Nrf2 and HO-1 challenged with LPS.

Introduction
Mastitis is a well-recognized dairy cow disease with the highest incidence and leads to considerable economic losses (Malcata et al. 2020). It is an inflammatory reaction that occurs when the mammary gland is attacked by various pathogenic bacteria and stimulated by physical and chemical factors. In addition, the long-term abuse of antibiotics will lead to pathogen resistance and antibiotic residue problems, which seriously endanger animal welfare and human health (Cheng et al. 2019). At present, about 33% of dairy cows are worldwide suffering from mastitis, resulting in a decrease in both the quality and quantity of milk and inducing metabolic disorders (Derakhshani et al. 2018). Therefore, new mastitis therapies have gradually entered the public’s field of vision, such as breed improvement, nutritional regulation, feeding management and so on (Donadeu et al. 2020). Now, growing pieces of evidence have shown that nutritional regulation is the most effective way to prevent or control the occurrence of mastitis, such as organic selenium (Grossi et al. 2021; Yang Y et al. 2021), choline (Yang M et al. 2021) and curcumin (Li et al. 2021). Therefore, how to effectively control bovine mastitis from the
aspect of nutritional regulation, has important theoretical and practical significance for the healthy breeding of dairy cattle.

Betaine, also known as trimethylglycine, is widely distributed in animals, plants and microorganisms (Cholewa et al. 2014). In the process of livestock and poultry production, it is mainly manifested in promoting the growth and development of animals and improving meat quality (Sahebi-Ala et al. 2021). In recent years, it has been found that the addition of betaine can enhance the production performance, nutrient digestion, milk production and immunity of dairy cows, reduce the number of somatic cells in the milk (Wang et al. 2019, 2020), and improve the oxidative damage in bovine mammary epithelial cells (BMECs) challenged with heat stress (Li et al. 2019). However, the mechanism of betaine on lipopolysaccharide (LPS)-induced damage in BMECs has not been revealed yet.

Lipopolysaccharide (LPS), a well-known key component of the outer membrane of Gram-negative bacteria, causes an inflammatory reaction in BMECs, which are major components of the mammary alveolus for milk production (Dessauge et al. 2011) and constitute the first line of defense against invading pathogens (Swanson et al. 2009). It is important to note that in order to obtain higher milk production, farmers usually feed high-concentration diets, which generally alter the structure and function of microbiota in the rumen or intestines (Mu et al. 2021). It is widely known that LPS could activate the nuclear transcription factor-kappa B (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway, which plays a vital role in modulating the inflammatory response and oxidative stress, respectively. Therefore, it is necessary to study the protective effect of betaine on LPS-challenged in BMECs from the aspect of the NF-κB and Nrf2 signalling pathways.

In this project, BMECs were used to explore the protective effect of betaine under LPS exposure and clarified the underlying mechanism by which betaine modulates the activation of NF-κB and Nrf2.

Material and methods

Cell culture

The bovine mammary epithelial cells (BMECs) in our study were from Yangzhou University in Yangzhou, China, and were cultured as previously described (Chen et al. 2019). Briefly, BMECs were cultured in DMEM/F12 medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. The resuscitated BMECs were cultured at 37°C, 5% CO₂, and suitable humidity. The medium was changed once every other 48 h.

Cell viability assay

BMECs (1 x 10⁵) were seeded into a 96-well plate. After incubation for 24 h, the culture medium was replaced. The BMECs were subjected to different concentrations of LPS (10, 50, 100, 200, 500 µg/mL) or betaine (5, 25, 50, 100, 200 mM) for 24 h, respectively, which were purchased from Sigma-Aldrich. Then 10 µL cell counting kit-8 (CCK8, C0037, Beyotime Biotechnology, Shanghai, China) solutions were added to each well for 1 h. Finally, the absorbance was measured at 450 nm. Finally, LPS was chosen as a suitable dose for establishing a cellular inflammatory model, which had no cytotoxic effect on BMECs.

Measurement of antioxidative enzyme activity and nitric oxide content

After cell treatment, commercial kits, purchased from Nanjing Jiancheng Biotechnology, were used to determine the activities of glutathione peroxidase (GSH-Px) and total superoxide dismutase (T-SOD), and malondialdehyde (MDA) content in BMECs according to the manufacturer instructions. Additionally, nitric oxide (NO) content was detected using a commercial kit (Beyotime Biotechnology, Shanghai, China).

Determination of cytokine levels

After cell treatment, the supernatant of BMECs were collected and then measured the levels of pro-inflammatory cytokines including interleukin-1β, interleukin-6 and tumour necrosis factor α by commercial ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to the manufacturers’ instruction.

Western blot analysis

The total protein of the cells was extracted as previously described (Jiang et al. 2020). Briefly, the BMECs were lysed with RIPA buffer containing phosphatase and protease inhibitor and then measured protein concentrations using the BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer’s instructions. Protein was
subjected to electrophoresis on a 10% SDS-PAGE gel and transferred onto a nitrocellulose (NC) membrane. Next, 3% milk was used to block the membranes for 2 h at room temperature. After blocking, the NC membranes were incubated with the primary antibodies at 4 °C overnight, including iNOS (18985-1-AP, Proteintech, USA), COX2 (12375-1-AP, Proteintech, USA), NF-κB p65 (10745-1-AP, Proteintech, USA), NF-κB p-p65 (AF2006, Affinity Biosciences), IκB (AF5002, Affinity Biosciences), p-IκB (AF2002, Affinity Biosciences), Nrf2 (16396-1-AP, Proteintech, USA) and HO-1 (10701-1-AP, Proteintech, USA). The next day, the NC membranes were washed with TBST buffer three times for 10 min and then incubated with secondary antibodies. Finally, the NC membranes were washed three times for 10 min in TBST buffer and exposed to chemiluminescence. The bands were detected by Image J software. The β-actin (66009-1-Ig, Proteintech, USA) was selected as the internal control.

**Statistical analysis**

All experiments were performed with three replicates and the data were presented as means ± SEM. All results were analysed by one-way analysis of variance (ANOVA) using the mixed procedure of SPSS, version 20.0 (Chicago, IL). A p-value < 0.05 was considered statistically significant.

**Results**

**Betaine had no cytotoxic effect on BMECs at low concentrations**

CCK-8 assay was used to investigate the cytotoxic effect of betaine on BMECs. These results showed that betaine had no cytotoxic effect on BMECs at concentrations ranging from 2.5 to 50 mM. However, it significantly increased the cell viability at 100 and 200 mM concentrations (Figure 1).

**LPS was chosen as a suitable dose for establishing cellular inflammatory model**

CCK-8 and Western blot analyses were used to verify the acceptable dose of LPS in this study. We detected the cell viability on BMECs induced by LPS at various concentrations (2, 10, 50, 100, 200 and 500 μg/mL) for 24 h, as shown in Figure 2(A). The results showed that LPS had no cytotoxic effect on BMECs at concentrations ranging from 2 to 100 μg/mL. In contrast, it dramatically decreased the cell viability at 200 and 500 μg/mL concentrations. Moreover, NO production in BMECs was significantly increased after LPS treatment at different doses (Figure 2(B), p < 0.05). Additionally, COX2 and iNOS protein levels were dramatically decreased after LPS stimulation (Figure 2(C), p < 0.05). Therefore, the 100 μg/mL dose of LPS was chosen as a suitable treatment dose in the subsequent experiments.

**Betaine treatment abolished the LPS-induced production of NO, COX2 and iNOS in BMECs**

The effect of betaine on LPS-induced production of NO and protein expression of COX2 and iNOS in BMECs were determined by Griess reaction and Western blot analysis, respectively. The results demonstrated that betaine markedly declined LPS-induced NO production (Figure 3(A), p < 0.05). Besides, LPS significantly upregulated the protein level of COX2 and iNOS, while betaine downregulated the increase of protein levels, as shown in Figure 3(C–D).

**Betaine alleviated LPS-stimulated the production of pro-inflammation cytokines in BMECs**

The effect of betaine on LPS-induced production of pro-inflammation cytokines in BMECs was measured by ELISA kits. As shown in Figure 4(A–C), compared with the control group, the LPS challenge markedly evaluated the production of IL-1β, IL-6 and TNFα. However, betaine treatment diminished the increase of LPS-induced the production of pro-inflammation cytokines (p < 0.05).
Figure 2. The effect of lipopolysaccharide (LPS) on bovine mammary epithelial cells (BMECs). The BMECs were subjected to various concentrations of LPS for 24 h. (A) Cell viability of LPS on BMECs. (B) Production of NO. (C) Protein level of COX2 and iNOS. Values represent means ± SEM, *p < 0.05 compared with the control group.

Figure 3. Betaine supplementation suppressed the inflammatory factors in bovine mammary epithelial cells (BMECs) after lipopolysaccharide (LPS) stimulation. BMECs were pre-treated with betaine (5, 25, 50 and 100 mM) for 2 h followed by LPS (100 µg/mL) for another 12 h. (A) Production of NO. (B) Representative Western blot bands for COX2 and iNOS proteins. (C) Quantitation of COX2 protein. (D) Quantitation of iNOS protein. Values represent means ± SEM, *p < 0.05 compared with the control group, #p < 0.05 compared with the LPS group.
Betaine restored LPS-elicited nuclear factor kappa-B (NF-κB) signalling pathway activity

To further investigate the anti-inflammatory effect of betaine in BMECs, we examine the protein expression of IκB and p65. These results suggested betaine inhibited the increase of LPS-elicited IκB and phosphorylation of IκB (Figure 5(B,C)). Furthermore, betaine also ameliorated LPS-induced the increase of NF-κB p65 and p-p65 (Figure 5(D,E)). The results indicated that betaine might alleviate the inflammatory response in BMECs challenged with LPS by regulating NF-κB signalling pathway.

Betaine prevented BMECs from LPS-induced oxidative stress

The activity of SOD and GSH-Px and MDA content were detected. As shown in Figure 6(A–C), compared with the control group, LPS stimulation significantly increased the SOD and GSH-Px activities and reduced the MDA content after 12 h. However, compared with the LPS group, pre-treatment with betaine led to an increase in SOD and GSH-Px activities and a reduction in MDA content, respectively.

Betaine resumed the LPS-induced Nrf2 signalling pathway in BMECs

In order to further investigate the anti-oxidative damage, we detected the protein levels of Nrf2 and its downstream target gene HO-1. The protein expressions of both Nrf2 and HO-1 were significantly up-regulated compared with the control group, while betaine pre-treatment inhibited the phenomenon mentioned before (Figure 7(A–C), p < 0.05). The results indicated that betaine exposure plays a critical role in regulating oxidative stress.

Discussion

Bovine mastitis is among the most serious disease in animal husbandry and brings about a decrease in milk quality and quantity, which finally results in huge...
economic losses (Malcata et al. 2020). Under normal conditions, the inflammatory reaction is necessary to provide protection from infection and strengthen the body’s immunity (Abdulkhaleq et al. 2018). However, the long-term inflammatory is harmful to the dairy cows. Therefore, some effective methods are needed to attach great importance to controlling bovine mastitis. Betaine is an important nutrient derived from either dietary intake or endogenous synthesis from choline (Arumugam et al. 2021). On one hand, betaine could act as an osmolyte to maintain cell volume and stabilise protein conformations (Khan et al. 2010; Al-Abdullatif et al. 2021). Recent studies have shown that betaine supplementation can enhance ruminal fermentation under thermal and osmotic stress (Mahmood et al. 2020) and restore the affinity of amylase and trypsin to counteract the inhibitory effect of hyperosmolarity (Sisi et al. 2019). On the other hand, betaine contains three methyl groups and thereby exerts epigenetic modification. Previously we have shown that maternal betaine exposure could regulate the lipid metabolism in rats (Zhao et al. 2018), piglets (Cai et al. 2014) and chicken (Hu et al. 2018) through DNA methylation. However, the underlying mechanism of betaine on LPS-induced BMECs has not been clarified.

LPS, an endotoxin derived from the outer membrane of Gram-negative bacteria, is widely applied to establish animal or cellular inflammatory models. Moreover, BMECs, sever as the first line of defense against invading pathogens, which could be of considerable significance to prevent and control mastitis. In the present study, we found that LPS had no cytotoxic effect on BMECs at concentrations ranging from 2 to 100 μg/mL. Thus, a 100 μg/mL dose of LPS was chosen as a suitable treatment dose, which is consistent with previous studies (Li et al. 2021). Nevertheless, in other experiments, treatment of BMECs with 1 μg/mL (Jeong et al. 2017; Kan et al. 2021), 5 μg/mL (Jiang et al. 2020), 50 μg/mL (Liu et al. 2021), and 200 μg/mL (Chen et al. 2020) have often employed to the induction of inflammatory response. These inconsistent findings may be due to the types of LPS. According to its serotype, it can be divided into Escherichia coli O111:B4, Escherichia coli O26:B6, Escherichia coli O55:B5, Escherichia coli O127:B8 etc. Herein, Escherichia coli O55:B5 was chosen to construct the inflammatory models in our study.

Accumulating pieces of evidence have demonstrated that the mammary glands of dairy cows are activated and liberate numerous pro-inflammatory cytokines, which are key mediums in the inflammatory response.

Figure 5. Betaine restored lipopolysaccharide (LPS)-elicited nuclear factor kappa-B (NF-κB) signalling pathway activity. Bovine mammary epithelial cells (BMECs) were pre-treated with betaine (5, 25, 50 and 100 mM) for 2 h before treated with LPS (100 μg/mL) for 12 h. (A) Representative Western blot bands for IκB, p-IκB, p65 and p-p65 proteins. (B–E) Quantitation of IκB, p-IκB, p65 and p-p65 proteins, respectively. The data are presented as means ± SEM, *p < 0.05 compared with the control group, #p < 0.05 compared with the LPS group.
response when challenged with LPS. In the study, we found that the increase of production of NO, IL-1β, IL-6 and TNFα induced by LPS were remarkably decreased by betaine treatment, which was in line with previous studies in animals (Rasineni et al. 2020) and cellular models (Wu et al. 2020). Moreover, COX2 and iNOS are major inflammatory mediators and are extremely expressed in LPS-stimulated cells. Our results showed that betaine downregulated the protein expression of COX2 and iNOS in LPS-challenged BMECs. Similarly, previous reports showed that the LPS-induced increase of COX2 was counteracted by betaine in the Kupffer cell (Weik et al. 1998) and RAW264.7 cell (Kim et al. 2014). Our data suggested that betaine is an effective anti-inflammatory agent in BMECs.

In order to clarify the underlying mechanism by which betaine blocked the production of pro-inflammatory cytokines, we detected IκB and NF-κB protein by Western blot. Under normal conditions, NF-κB (p50/p65) heterodimers combine with IκB and are in a ‘resting’ state. When LPS stimulation, NF-κB p65 can be rapidly released from the cytoplasmic complex and translocated to nuclear, thereby regulating its downstream pro-inflammatory genes expression. The present study showed that betaine abolished LPS-triggered the phosphorylation of IκB and NF-κB p65, which is consistent with previous reports in LPS-stimulated N9 Cells (Shi et al. 2019) and AOM/DSS-induced colon tumorigenesis (Kim et al. 2014). These results suggested that betaine may exert potent anti-inflammatory effects by modifying IκB/NF-κB signalling pathway.

It is well-known that there has a cross-talk between inflammatory response and oxidative stress to defend against the invasion of pathogenic microorganisms (Dandekar et al. 2015). The Nrf2 serves as an important transcription factor responsible for regulating downstream antioxidant proteins and enzymes, such as SOD, GSH-Px and HO-1, to protect cells from oxidative stress damage (He et al. 2021). As shown in the present study, betaine pre-treatment significantly restored the levels of SOD, GSH-Px and MDA, which are signs of oxidative markers. Similarly, previous findings showed that betaine supplementation could enhance the activities of total antioxidative capacity (T-AOC), SOD and GSH-Px in bovine (Shah et al. 2020) and BMECs (Li et al. 2019) induced by heat stress.

Figure 6. Betaine prevented lipopolysaccharide (LPS)-induced oxidative stress in bovine mammary epithelial cells (BMECs). BMECs were pre-treated with betaine (5, 25, 50 and 100 mM) for 2 h before treated with LPS (100 μg/mL) for 12 h. (A) Superoxide dismutase (SOD) activity. (B) Glutathione peroxidase (GSH-Px) activity. (C) malondialdehyde (MDA) content. The data are presented as means ± SEM, *p < 0.05 compared with the control group, #p < 0.05 compared with the LPS group.
Figure 7. Antioxidative effect of betaine in bovine mammary epithelial cells (BMECs) challenged with lipopolysaccharide (LPS). The expression of Nrf2 signalling pathway-related proteins were detected by Western blots in BMECs pre-treated with different doses of betaine for 2 h challenged with LPS for another 12 h. (A) Representative Western blot bands for HO-1 and Nrf2 proteins. (B) Quantitation of HO-1 protein. (C) Quantitation of Nrf2 protein. The data are presented as means ± SEM, *p < 0.05 compared with the control group, #p < 0.05 compared with the LPS group.

Figure 8. Scheme to summarise the protective effect of betaine on lipopolysaccharide (LPS-induced mastitis and its possible mechanisms.
further elucidate whether Nrf2/HO-1 signalling pathway involves in oxidative stress, the protein expression of Nrf2 and HO-1 were examined. Interestingly, LPS-challenged markedly up-regulated the expression of Nrf2 and HO-1, which is in line with the results of heat stress in BMECs (Li et al. 2019; Yang M et al. 2021). However, pre-treatment with betaine reduced the above increase compared with the LPS group. These findings were consistent with a previous report (Li et al. 2019). Additionally, choline (a precursor of betaine) attenuates heat stress-induced accumulation of Nrf2 and HO-1 in BMECs (Yang M et al. 2021). It is noteworthy that Nrf2 is usually activated when challenged with LPS or other antioxidants (Xu et al. 2020; Liu et al. 2021), which is different from our results. We speculated that hypermethylation on these antioxidant enzymes or Nrf2 genes promoter causes downregulation of mRNA expression due to epigenetic modification of betaine, which thereby contributes to scavenging excess reactive oxygen species to protect from oxidative stress damage.

There are several limitations of this research that should be noted due to test conditions. The limitations of this study are as follows: (1) our research lacks animal experiments, which could better clarify the anti-inflammatory effect of betaine against LPS-induced inflammatory response. (2) our research lacks a rescue experiment (knock out or overexpression), which may better reveal the mechanism of betaine alleviating bovine mastitis. (3) in our study, we do not explain the correlation between NF-κB and Nrf2 signalling pathway. Therefore, it will be considered in our future study.

Conclusion
In summary, our study demonstrates that pre-treatment of BMECs with betaine effectively alleviates LPS-triggered inflammation response and oxidative stress damage by modulating NF-κB and Nrf2 signalling pathway (Figure 8). Collectively, these findings provide strong evidence that betaine can be used as a potential additive for bovine mastitis.

Ethical approval
All procedures were approved by the Animal Care and Use Committee of the Xuzhou University of Technology, Xuzhou, China.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
The present study was supported by the Natural Science Foundation of Jiangsu Province [BK20210077] and the Research Starting Fund of Xuzhou University of Technology [02900192].

Data availability statement
The original data of the paper are available upon request from the corresponding author.

References
Abdulkhaleq LA, Assi MA, Abdullah R, Zamri-Saad M, Taufiq-Yap YH, Hezmee MNM. 2018. The crucial roles of inflammatory mediators in inflammation: a review. Vet World. 11(5):627–635.
Al-Abdullatif AA, Al-Sagan AA, Hussein EOS, Saadeldin IM, Suliman GM, Azzam MM, Al-Mafarrej SI, Alhotan RA. 2021. Betaine could help ameliorate transport associated water deprivation stress in broilers by reducing the expression of stress-related transcripts and modulating water channel activity. Ital J Anim Sci. 20(1):14–25.
Arunugam MK, Paal MC, Donohue TM, Jr., Ganesan M, Osna NA, Kharbanda KK. 2021. Beneficial effects of betaine: a comprehensive review. Biology. 10(6):456.
Cai D, Jia Y, Lu J, Yuan M, Sui S, Song H, Zhao R. 2014. Maternal dietary betaine supplementation modifies hepatic expression of cholesterol metabolic genes via epigenetic mechanisms in newborn piglets. Br J Nutr. 112(9):1459–1468.
Chen Z, Chu S, Wang X, Sun Y, Xu T, Mao Y, Loor JJ, Yang Z. 2019. MiR-16a regulates milk fat metabolism by targeting large tumor suppressor kinase 1 (LATS1) in bovine mammary epithelial cells. J Agric Food Chem. 67(40):11167–11178.
Chen Z, Zhang Y, Zhou J, Lu L, Wang X, Liang Y, Loor JJ, Gou D, Xu H, Yang Z. 2020. Tea tree oil prevents mastitis-associated inflammation in lipopolysaccharide-stimulated bovine mammary epithelial cells. Front Vet Sci. 7:496.
Cheng J, Qu W, Barkema HW, Nobrega DB, Gao J, Liu G, De Buck J, Kastelic JP, Sun H, Han B. 2019. Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. J Dairy Sci. 102(3):2416–2426.
Cholewa JM, Guimaraes-Ferreira L, Zanchi NE. 2014. Effects of betaine on performance and body composition: a review of recent findings and potential mechanisms. Amino Acids. 46(8):1785–1793.
Dandekar A, Mendez R, Zhang K. 2015. Cross talk between ER stress, oxidative stress, and inflammation in health and disease. Methods Mol Biol. 1292:205–214.
Derakhshani H, Fehr KB, Sepehri S, Francoz D, De Buck J, Barkema HW, Plazier JC, Khaipour E. 2018. Invited review: microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. J Dairy Sci. 101(12):10605–10625.
Dessauge F, Lollivier V, Ponchon B, Bruckmaier R, Finot L, Wiart S, Cutillic E, Disenhaus C, Barbey S, Boutinaud M.
2011. Effects of nutrient restriction on mammary cell turnover and mammary gland remodeling in lactating dairy cows. J Dairy Sci. 94(9):4623–4635.

Donadeu FX, Howes NL, Esteves CL, Howes MP, Byrne TJ, Macrae Al. 2020. Farmer and veterinary practices and opinions related to the diagnosis of mastitis and metabolic disease in UK dairy cows. Front Vet Sci. 7:127.

Grossi S, Dell’Anno M, Rossi L, Compani R, Sgoifo Rossi CA. 2021. Supplementation of live yeast, mannan oligosaccharide, and organic selenium during the adaptation phase of newly arrived beef cattle: effects on health status, immune functionality, and growth performance. Antibiotics. 10(9):1114.

He L, Guo C, Peng C, Li Y. 2021. Advances of natural activators for Nrf2 signaling pathway on cholestatic liver injury protection: a review. Eur J Pharmacol. 910:174447.

Hu Y, Sun Q, Hu Y, Hou Z, Zong Y, Omer NA, Abobaker H, Zhao R. 2018. Corticosterone-induced lipogenesis activation and lipophagy inhibition in chicken liver are alleviated by maternal betaine supplementation. J Nutr. 148(3):316–325.

Jeong CH, Cheng WN, Bae H, Lee KW, Han SM, Petriello MC, Lee HG, Seo HG, Han SG. 2017. Bee venom decreases LPS-induced inflammatory responses in bovine mammary epithelial cells. J Microbiol Biotechnol. 27(10):1827–1836.

Jiang L, Wang J, Liu Z, Jiang A, Li S, Wu D, Zhang Y, Zhu X, Zhou E, Wei Z, et al. 2020. Sodium butyrate alleviates lipopolysaccharide-induced inflammatory responses by downregulation of NF-κB, NLRP3 signaling pathway, and activating histone acetylation in bovine macrophages. Front Vet Sci. 7:579674.

Kan X, Liu J, Chen Y, Guo W, Xu D, Cheng J, Cao Y, Yang Z, Fu S. 2021. Protective effect of myricetin on LPS-induced mastitis in mice through ERK1/2 and p38 protein author. Naunyn Schmiedebergs Arch Pharmacol. 394(8):1727–1735.

Khan SH, Ahmad N, Ahmad F, Kumar R. 2010. Naturally occurring organic osmolytes: from cell physiology to disease prevention. IUBMB Life. 62(12):891–895.

Kim DH, Sung B, Kang YJ, Jiang JY, Wang SY, Lee Y, Kim M, Im E, Yoon JH, Kim CM, et al. 2014. Anti-inflammatory effects of betaine on AOM/DSS-induced colon tumorigenesis in ICR male mice. Int J Oncol. 45(3):1250–1256.

Li C, Wang Y, Li L, Han Z, Mao S, Wang G. 2019. Betaine protects against heat exposure-induced oxidative stress and apoptosis in bovine mammary epithelial cells via regulation of ROS production. Cell Stress Chaperones. 24(2):453–460.

Li R, Fang H, Shen J, Yin J, Zhao Y, Wang R, Fu Y, Tian Y, Yu H, Zhang J. 2021. Curcumin alleviates LPS-induced oxidative stress, inflammation and apoptosis in bovine mammary epithelial cells via the NFE2L2 signaling pathway. Toxins. 13(3):208.

Liu M, Zhang C, Xu X, Zhao X, Han Z, Liu D, Bo R, Li J, Liu Z. 2021. Ferulic acid inhibits LPS-induced apoptosis in bovine mammary epithelial cells by regulating the NF-κB and Nrf2 signalling pathways to restore mitochondrial dynamics and ROS generation. Vet Res. 52(1):104.

Mahmood M, Petri RM, Gavrâu A, Zebeli Q, Khiaosa-Ard R. 2020. Betaine addition as a potent ruminal fermentation modulator under hyperthermal and hypersomotic conditions in vitro. J Sci Food Agric. 100(5):2261–2271.

Malcata FB, Pepler PT, O’Reilly EL, Brady N, Eckersall PD, Zadoks RN, Viora L. 2020. Point-of-care tests for bovine clinical mastitis: what do we have and what do we need? J Dairy Res. 87(5):60–66.

Mu YY, Qi WP, Zhang T, Zhang JY, Mao SY. 2021. Gene function adjustment for carbohydrate metabolism and enrichment of rumen microbiota with antibiotic resistance genes during subacute rumen acidosis induced by a high-grain diet in lactating dairy cows. J Dairy Sci. 104(2):2087–2105.

Rasineni K, Lee SML, McVicker BL, Osna NA, Casey CA, Kharbanda KK. 2020. Susceptibility of asialoglycoprotein receptor-deficient mice to Lps/galactosamine liver injury and protection by betaine administration. Biology. 10(1):19.

Sisi L, Haicho W, Jie F. 2019. 95 Betaine improves growth performance by increasing digestive enzymes activities, and ameliorating intestinal structure of piglets. J Anim Sci. 97(3):80.

Sahebi-Ala F, Hassanabadi A, Golan A. 2021. Effect of replacement different methionine levels and sources with betaine on blood metabolites, breast muscle morphology and immune response in heat-stressed broiler chickens. Ital J Anim Sci. 20(1):33–45.

Shah AM, Ma J, Wang Z, Zou H, Hu R, Peng Q. 2020. Betaine supplementation improves the production performance, rumen fermentation, and antioxidant profile of dairy cows in heat stress. Animals. 10(4):634.

Shi H, Wang XL, Quan HF, Yan L, Pei XY, Wang R, Peng XD. 2019. Effects of betaine on LPS-stimulated activation of microglial M1/M2 phenotypes by suppressing TLR4/NF-kappaB pathways in N9 cells. Molecules. 24(2):367.

Swanson KM, Stelwagen K, Dobson J, Henderson HV, Davis SR, Farr VC, Singh K. 2009. Transcriptome profiling of Streptococcus uberis-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. J Dairy Sci. 92(1):117–129.

Wang B, Wang C, Guan R, Shi K, Wei Z, Liu J, Liu H. 2019. Effects of dietary rumen-protected betaine supplementation on performance of postpartum dairy cows and immunity of newborn calves. Animals. 9(4):167.

Wang C, Liu C, Zhang GW, Du HS, Wu ZZ, Liu Q, Guo G, Huo WJ, Zhang J, Pei CX, et al. 2020. Effects of rumen-protected folic acid and betaine supplementation on growth performance, nutrient digestion, rumen fermentation and blood metabolites in Angus bulls. Br J Nutr. 123(10):1109–1116.

Weik C, Warskulat U, Bode J, Peters-Regehr T, Haussinger D. 1998. Compatible organic osmolytes in rat liver sinusoidal endothelial cells. Hepatology. 27(2):569–575.

Wu J, He C, Bu J, Luo Y, Yang S, Ye C, Yu S, He B, Yin Y, Yang X. 2020. Betaine attenuates LPS-induced downregulation of Occludin and Claudin-1 and restores intestinal barrier function. BMC Vet Res. 16(1):75.
Xu D, Liu J, Ma H, Guo W, Wang J, Kan X, Li Y, Gong Q, Cao Y, Cheng J, et al. 2020. Schisandrin A protects against lipopolysaccharide-induced mastitis through activating Nrf2 signaling pathway and inducing autophagy. Int Immunopharmacol. 78:105983.

Yang M, Kuang M, Wang G, Ali I, Tang Y, Yang C, Li Y, Li L. 2021. Choline attenuates heat stress-induced oxidative injury and apoptosis in bovine mammary epithelial cells by modulating PERK/Nrf-2 signaling pathway. Mol Immunol. 135:388–397.

Yang Y, Lv S, Wang Z, Liu J. 2021. Selenium ameliorates S. aureus-induced inflammation in bovine mammary epithelial cells by regulating ROS-induced NLRP3 inflammasome. Biol Trace Elem Res.DOI: 10.1007/s12011-021-02924-7

Zhao N, Yang S, Jia Y, Sun B, He B, Zhao R. 2018. Maternal betaine supplementation attenuates glucocorticoid-induced hepatic lipid accumulation through epigenetic modification in adult offspring rats. J Nutr Biochem. 54:105–112.