The protective effect of chlorogenic acid on bovine mammary epithelial cells and neutrophil function

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ABSTRACT

Chlorogenic acid (CGA) is the ester of caffeic acid and quinic acid and plays an important role in antibacterial activity and anti-inflammatory properties. The objective of this study was to examine the effects of CGA on the growth of *Staphylococcus aureus* and the mRNA levels of the genes encoding the inflammatory response cytokines, κ -casein, and neutrophil function in bovine mammary epithelial cells (BMEC) exposed to *S. aureus*. Chlorogenic acid has important antibacterial, antioxidant, and anti-inflammatory functions; however, the effect of CGA on BMEC and neutrophils exposed to *S. aureus* has not been investigated previously. Our results demonstrated that 10, 20, and 30 $\mu\text{g/mL}$ CGA had no cytotoxic effects on BMEC in culture, and that 20 $\mu\text{g/mL}$ CGA enhanced the viability of BMEC exposed to *S. aureus*, whereas 30 $\mu\text{g/mL}$ CGA reduced *S. aureus* growth after 9 h compared with controls. The rate of *S. aureus* invasion into BMEC was also attenuated by 30 $\mu\text{g/mL}$ CGA compared with controls, whereas this treatment led to reduced abundance of *IL6*, *IL8*, and *TLR2* mRNA in *S. aureus*-exposed BMEC. Migration of bovine polymorphonuclear leukocytes was significantly decreased in *S. aureus*-exposed BMEC with 10 and 20 $\mu\text{g/mL}$ CGA treatment when compared with *S. aureus* treatment alone. In addition, incubation with 20 or 30 $\mu\text{g/mL}$ CGA enhanced the phagocytic ability of polymorphonuclear leukocytes compared with the control group. Importantly, levels of κ -casein were enhanced by treatment of *S. aureus*-exposed BMEC with CGA. Our results suggest that the use of CGA may be a potent therapeutic tool against bovine mastitis caused by *S. aureus*.

Key words: chlorogenic acid, *Staphylococcus aureus*, bovine mammary epithelial cells, inflammatory response

INTRODUCTION

Mastitis, an inflammation of the mammary gland, is a serious infectious disease triggered in cattle by infection with bacteria, such as *Staphylococcus aureus* or *Escherichia coli* (Thompson-Crispi et al., 2014; Gao et al., 2017), and results in decreased milk production and quality (Bar et al., 2008). The inflammatory response to bovine mastitis caused by *S. aureus* infection is often slow, and the infection frequently becomes persistent (Schukken et al., 2011; Petzl et al., 2018). The high pathogenicity of *S. aureus* is mediated via pathways that allow it to evade host innate immune defenses (Spaan et al., 2013; Graves et al., 2010; Xu et al., 2013), which consist of 3 major effectors mechanisms: antimicrobial peptides and enzymes, the complement system, and phagocytes (Medzhitov and Janeway, 2000; Rooijakkers et al., 2005). Furthermore, *S. aureus* virulence factors can affect the levels of specific immune-modulating proteins (Rooijakkers et al., 2005). Moreover, infection with methicillin-resistant *S. aureus*, which is resistant to the majority of antimicrobial agents, is a serious health hazard (Li et al., 2012), and methicillin-resistant *S. aureus* infections have been observed in cases of bovine mastitis (Devriese et al., 1972). Hence, alternative approaches aimed at resolution of bovine mastitis caused by *S. aureus* are required.

Previous research efforts have focused on the extensive development of antimicrobial agents to prevent bovine mastitis caused by *S. aureus* and *E. coli* (Dias et al., 2013); however, consumer concerns about the development of resistance to antimicrobial agents are growing (Bhattacharyya et al., 2016). In addition, the treatment of bovine mastitis using antimicrobial agents is an increasing threat to human health due to the presence of residues of these agents in milk. Hence, the development of novel, alternative therapies for mastitis caused by *S. aureus* also has the potential to improve milk production and quality.

Chlorogenic acid (CGA) is the ester of caffeic acid and quinic acid and is a natural chemical, present in numerous plants, including tobacco (Wang et al., 2008). The chemical exhibits strong antibacterial activity, can reduce the reactive oxygen species (ROS), and

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has immunoprotective and anti-inflammatory properties (Rice-Evans et al., 1996; Kim et al., 2003; Perrone et al., 2008). A previous study demonstrated that CGA has antioxidant properties and protective effects against ischemia-reperfusion injury in the rat small intestine (Sato et al., 2011). Furthermore, CGA is protective against acute hepatotoxicity triggered by LPS in mice, through reduction of NF- κ B p65 phosphorylation (Xu et al., 2010). Hence, various studies have evaluated the antioxidant and anticarcinogenic effects of CGA in humans and mice; however, to date, no studies have investigated the effects of CGA as an alternative strategy for the treatment of bovine mastitis caused by *S. aureus*. The objectives of our study were to investigate the effects of CGA on the growth of *S. aureus*, the expression of genes involved in the inflammatory response, κ -CN levels after exposure of BMEC to *S. aureus*, and the responses of bovine neutrophils to CGA.

MATERIALS AND METHODS

Cell Culture

Bovine mammary epithelial cells (BMEC) of 3 mid-lactation Holstein cows were obtained from the Institute of Animal Culture Collection and Application, Yangzhou University. These bovine mammary tissues were digested by collagenase type I (Invitrogen, Shanghai, China) for 3 h and cells were filtered by nylon mesh (75 μ m) to obtain the BMEC. The BMEC were seeded in DMEM/F12 medium supplemented with 10% fetal bovine serum, 4 mM glutamine, 1 $\times$ insulin, transferrin, sodium selenite (10 μ g/mL insulin, 5.5 μ g/mL transferrin, 0.0067 μ g/mL sodium selenite; Invitrogen), 15 ng/mL epidermal growth factor (Peprotech, Shanghai, China), 1 μ g/mL hydrocortisone, and 4 μ g/mL prolactin (Sigma-Aldrich, Shanghai, China). Polymorphonuclear leukocytes were obtained as described previously (Garcia et al., 2015) and cultured in RPMI-1640 medium supplemented with 5% inactivated fetal bovine serum and 4 mM glutamine.

Bacterial Strain and Culture

Staphylococcus aureus (ACTT29213) were obtained from American Type Culture Collection and cultured in brain heart infusion (BHI) media at 37°C.

Cytotoxicity Assay

The cytotoxic effects of CGA on BMEC were determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Shanghai, China), according to the manufacturer's protocol (<https://www.dojindo.com/store/p/456-Cell>

[-Counting-Kit-8.html](#)). The BMEC (5×10^3 cells/well) were seeded into 96-well plates. After 12 h, cells were incubated for further 48 h with 0 (control group), 10, 20, 30, 40 or 50 μ g/mL CGA ($n = 3$). Subsequently, 10 μ L of CCK-8 reagent was added to cells, followed by incubation at 37°C, 5% CO₂ for 4 h. Absorbance at 450 nm was then measured in each well using an auto-microplate reader (Thermo Scientific, Shanghai, China).

Cell Viability Assay

Bovine mammary epithelial cells were seeded into 96-well plates (5×10^3 cells/well) and *S. aureus* was added after 12 h (5×10^3 cfu/well). Cells were then cultured in DMEM/F12 medium in the absence of *S. aureus* and CGA (control group), in the presence of *S. aureus* and absence of CGA (*S. aureus* group), and in the presence of *S. aureus* and 10, 20, or 30 μ g/mL CGA (CGA-treated groups) for 6 h ($n = 3$). After incubation, cells were vigorously washed 5 times with 200 μ L of sterile water and incubated with 100 μ L of DMEM/F12 supplemented with 10 μ L of CCK-8 at 37°C and 5% CO₂ for 4 h. Absorbance at 450 nm was then measured in each well using an auto-microplate reader. Cell viability (%) was calculated as (treatment OD – blank OD)/(control OD – blank OD, where OD = optical density).

S. aureus Growth Assay

Staphylococcus aureus was seeded into 96-well plates (1×10^5 cfu/well) in BHI medium supplemented with 0 (control group), 10, 20, or 30 μ g/mL CGA ($n = 4$) at 37°C, 5% CO₂. Absorbance at 600 nm was then measured for 3, 6, 9, 12, and 24 h using an auto-microplate reader.

S. aureus Biofilm Formation Assay

Biofilm formation was assessed as described previously (Federman et al., 2016). Briefly, *S. aureus* was seeded into 96-well plates (2×10^6 cfu/well) and incubated at 37°C, 5% CO₂ for 24 h. Supernatants were then removed, BHI medium supplemented with 0 (control group), or 10, 20, or 30 μ g/mL CGA ($n = 4$) added, and plates wrapped with Parafilm (Solarbio, Beijing, China) and incubated at 37°C, 5% CO₂ for 36 h. Next, the plates were rinsed 5 times with sterile distilled water, *S. aureus* fixed with 100 μ L of methyl alcohol for 15 min, and the supernatant removed. The plates were allowed to dry naturally at room temperature and then treated with 200 μ L of 0.1% crystal violet at room temperature for 10 to 15 min. Plates were then rinsed

5 times, allowed to air dry overnight, and 30% acetic acid in sterile distilled water (200 μ L) was then added to each well. Plates were incubated with shaking for 1 h, and the solution in each well transferred to a new 96-well plate. To examine *S. aureus* biofilm formation, absorbance at 540 nm was measured using an auto-microplate reader.

***S. aureus* Invasion Assay**

To determine the number of *S. aureus* invading BMEC under different conditions, we followed a previously described method (Díaz-Murillo et al., 2016). The BMEC grown in 24-well plates were infected using DMEM/F12 medium containing *S. aureus* (1×10^5 cfu/well) at a multiplicity of infection of 1, and treated with 0 (control group), 10, 20, or 30 μ g/mL CGA ($n = 3$) at 37°C, 5% CO₂ for 3 h. Cells were then washed 5 times with sterile PBS. Next, 250 μ g/mL gentamicin was added, samples were incubated at 37°C, 5% CO₂ for 1 h, and then washed 5 times with PBS to remove the gentamicin. Cells were lysed with 0.5% Triton X-100 (Solarbio) for 15 min. The remaining live bacteria were then serially diluted, seeded in BHI agar plates, and colony-forming units were counted. Invasion rate (%) was calculated as (number of intracellular *S. aureus*/number of added *S. aureus*) $\times$ 100.

PMNL Chemotaxis Assay

The BMEC were seeded into 12-well plates (1×10^6 cells/well). After 12 h, the supernatant was removed and cells washed 3 times with PBS. The *S. aureus* (1×10^6 cfu/well) was then added and incubated with BMEC for 6 h. Supernatants were then filtered through 0.22- μ m sterile filters to collect media containing high levels of the chemoattractant, IL-8. The PMNL suspension (600 μ L) was seeded into 12-well plates (3×10^6 cells/well) and cells pretreated with RPMI-1640 medium containing 0 (control group), 10, 20, or 30 μ g/mL CGA ($n = 3$) at 37°C, 5% CO₂ for 2 h. Next, 500 μ L of medium containing IL8 was added to the bottom wells of 24-well plates. Chambers (pore size, 5.0 μ m; diameter, 6.5 mm; Corning Inc., Corning, NY) were placed in the top of wells and 100 μ L of PMNL suspension (5×10^5 cells/chamber) pretreated with different concentrations of CGA was seeded into the chambers. Samples were incubated at 37°C, 5% CO₂ for 1.5 h. Subsequently, chambers were rinsed vigorously 3 times with 100 μ L of calcium and magnesium-free Hanks' balanced salt solution, and PMNL that had migrated to the bottom wells counted. In addition, another experiment was also performed to evaluate PMNL migration using supernatant

obtained from *S. aureus*-exposed BMEC with 0 (control group), 10, 20, or 30 μ g/mL CGA treatment ($n = 3$).

PMNL Phagocytosis Assay

Staphylococcus aureus was preloaded with 5 μ g/mL fluorescein isothiocyanate (FITC) at 37°C, 5% CO₂ for 2 h. After preloading, samples were washed 3 times with PBS and stored, protected from light, at 4°C. Subsequently, PMNL (2×10^5 cells/well) were seeded into 12-well plates and treated with RPMI-1640 medium containing 0 (control group), 10, 20, or 30 μ g/mL CGA ($n = 3$) at 37°C, 5% CO₂ for 2 h. The *S. aureus* preloaded with FITC (2×10^5 cfu/well) was then incubated with the PMNL at 37°C, 5% CO₂ for 20 min. The mixture was then washed 3 times with PBS and examined by flow cytometry. The PMNL populations were gated by forward and side scatter, and FSC and FL1 channel detection was performed immediately to analyze the phagocytosis of FITC-labeled *S. aureus*. Data in the gate set for bovine PMNL were collected for 20,000 events per sample, and the percentage fluorescence used as a quantitative index of PMNL response. Intracellular bacteria were distinguished by quenching the extracellular fluorescence with 0.4% Trypan blue (Solarbio).

Quantitative Real-Time PCR

For mRNA expression analysis, BMEC were seeded in 6-well plates (1×10^6 cells/well) and grown at 37°C, 5% CO₂. Cells were divided into 4 experimental groups: (1) control, DMEM/F12 medium ($n = 3$); (2) CGA treatment, DMEM/F12 medium containing 30 μ g/mL CGA ($n = 3$); (3) *S. aureus* treatment, DMEM/F12 medium supplemented with 1×10^6 cfu of *S. aureus* ($n = 3$); and (4) *S. aureus* with CGA treatment, DMEM/F12 medium supplemented with both 1×10^6 cfu of *S. aureus* and 30 μ g/mL CGA ($n = 3$). The *S. aureus* and *S. aureus* with CGA treatment groups were pretreated with *S. aureus* for 6 h. Medium supernatant was removed and cells were washed 3 times with PBS. The control and *S. aureus* treatment groups were further incubated with DMEM/F12 medium for 6 h. The CGA treatment and *S. aureus* with CGA treatment group were further incubated with 30 μ g/mL CGA for 6 h. After 6 h, total RNA was isolated from the cultured cells using a TRIzol kit (Tiangen, Beijing, China). Reverse transcription (RT) was performed using an RT Kit (Takara, Beijing, China). The RT reaction mixtures contained 1 μ g of total RNA and 1 $\times$ PrimeScript RT Master Mix (Takara) in a final volume of 20 μ L and reactions were performed for 15 min at 37°C. Reverse transcriptase was inactivated by heating to 85°C for 5

Table 1 Primers for real-time PCR analyses

Gene	Primer sequence, ¹ 5' to 3'	Accession number	Size (bp)	Efficiencies ² (%)
<i>GAPDH</i>	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	NM_001034034	176	112.96
<i>IL6</i>	F: TCCTTGCTGCTTTTCACACTC R: CACCCAGGCAGACTACTTC	NM_173923.2	129	94.73
<i>IL1β</i>	F: CAGTGCCTACGCACATGTCT R: AGAGGAGGTGGAGAGCCTTC	NM_174093.1	209	103.09
<i>TNFA</i>	F: GCCCTCTGGTTCAGACACTC R: AGATGAGGTAAAGCCCGTCA	NM_173966.3	192	110.23
<i>TLR2</i>	F: CAGGCTTCTTCTCTGTCTTGT R: CTGTTGCCGACATAGGTGATA	NM_174197.2	140	108.78
<i>IL8</i>	F: TGGGCCACACTGTGAAAAT R: TCATGGATCTTGCTTCTCAGC	NM_173925.2	136	84.94
<i>CSN3</i>	F: CCAGGAGCAAAACCAAGAAC R: TGCAACTGGTTTCTGTTGTT	NM_174294.2	148	109.47

¹F = forward; R = reverse.²Efficiency is calculated as $[10^{(-1/\text{Slope})} - 1]$.

s. The quantitative (q) RT-PCR assays were performed using SYBR Premix Ex Taq II Kit (Takara). The qRT-PCR reaction mixture contained 1× SYBR Premix Ex Taq II, 0.4 μM each forward and reverse primers, and 120 ng of cDNA templates in a final volume of 20 μL. Reactions were performed as initial denaturation at 95°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Before qRT-PCR for samples, the amplification efficiencies of all primers were determined by using standard dilution series. The primers used are listed in Table 1. The relative expression of target genes was normalized to that of *GAPDH* and calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

All data were presented as means ± standard error from at least 3 independent experiments. In addition to the results of the invasion, IL-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-8, all results were distribution of normality and performed by one-way ANOVA, followed by the least significant difference (LSD) for post hoc multiple comparisons of treatment means, using SPSS 19.0 software (SPSS Inc., Chicago, IL). *P*-values of <0.05 were considered significant.

RESULTS

Assessment of the Cytotoxic Effects of CGA on BMEC

As shown in Figure 1, the results of cytotoxicity assays demonstrated that treatment with 10 μg/mL CGA enhanced the viability of BMEC compared with the untreated control group (*P* = 0.047). In addition, treatment with 20 (*P* = 0.57) and 30 μg/mL (*P* = 0.76) of CGA did not exert significant toxic effects on BMEC,

whereas 40 (*P* = 0.21) and 50 μg/mL (*P* = 0.07) of CGA inhibited cell viability compared with controls; however, the differences were not significant.

BMEC Viability After Exposure to *S. aureus*

Compared with controls, BMEC exposed to *S. aureus* exhibited (*P* = 0.002) reduced viability (Figure 2). The BMEC exposed to *S. aureus* with 10 or 30 μg/mL CGA treatment enhanced the BMEC viability compared with *S. aureus* treatment alone had not significantly enhanced viability (*P* = 0.87 and 0.14, respectively). However, the viability of BMEC exposed to *S. aureus*

Figure 1. Evaluation of the cytotoxic effects of chlorogenic acid (CGA) on bovine mammary epithelial cells (BMEC) incubated with 0 (control group), 10, 20, 30, 40, or 50 μg/mL CGA. Data are presented as the means ± SEM (n = 3). Means at the different concentrations of CGA with different letters (a-c) differ significantly for treatment effect.

Figure 2. Viability of bovine mammary epithelial cells (BMEC) exposed to *Staphylococcus aureus* with or without chlorogenic acid (CGA) treatment; BMEC were cultured in DMEM/F12 medium in the absence of *S. aureus* and CGA (control group), in the presence of *S. aureus* and absence of CGA (*S. aureus* group), and in the presence of *S. aureus* and 10, 20, or 30 µg/mL CGA (CGA-treated groups) for 6 h. Data are presented as the means $\pm$ SEM (n = 3). Means at the different treatment groups with different letters (a–c) differ significantly for treatment effect.

was enhanced ($P = 0.03$) by treatment with 20 µg/mL CGA compared with *S. aureus* alone (Figure 2).

Effect of CGA on *S. aureus* Growth

Addition of 20 µg/mL CGA inhibited *S. aureus* growth in 12 ($P = 0.02$) and 24 h ($P = 0.04$), compared with the control group (Figure 3). Notably, addition of 30 µg/mL CGA inhibited *S. aureus* growth in 9 ($P = 0.03$), 12 ($P = 0.01$), and 24 h ($P = 0.01$) compared with the untreated control group (Figure 3).

Effect of CGA on *S. aureus* Biofilm Formation

The *S. aureus* biofilm formation tended to be decreased by addition of 10 ($P = 0.69$), 20 ($P = 0.2$), and 30 µg/mL ($P = 0.43$) of CGA compared with *S. aureus* alone (Figure 4).

Effect of CGA on *S. aureus* Invasion of BMEC

Compared with controls, the invasion of BMEC by *S. aureus* was not affected by addition of 10 ($P = 0.25$) and 20 µg/mL ($P = 0.21$) of CGA, whereas at a concentration of 30 µg/mL CGA strongly inhibited ($P = 0.004$) *S. aureus* invasion of BMEC (Figure 5).

Effect of CGA on PMNL Chemotaxis

Pretreatment of PMNL with 10, 20, and 30 µg/mL CGA did not affect their chemotactic ability compared with untreated control groups (Figure 6).

Figure 3. Effect of chlorogenic acid (CGA) on *Staphylococcus aureus* growth; cultures were incubated with 0 (control group), 10, 20, or 30 µg/mL CGA. Absorbance at 600 nm was measured after 0, 3, 6, 9, 12, and 24 h. Data are presented as the means $\pm$ SEM (n = 4). Means at the same time (h) point with different letters (a,b) differ significantly for treatment effect.

Effect of CGA on PMNL Phagocytosis

In comparison with the control group, the treatment of PMNL with 20 ($P = 0.03$) and 30 µg/mL ($P = 0.007$) of CGA enhanced the phagocytic ability of PMNL compared with the control group (Figure 7).

Effect of CGA on the Inflammatory Response and κ -Casein Expression

The levels of mRNA encoding the cytokines, IL-1 β , IL-6, IL-8, and TNF- α , encoding κ -CN were determined by qRT-PCR. Compared with *S. aureus* treatment alone, addition of 30 µg/mL CGA decreased the levels of mRNA encoding IL-1 β ($P = 0.73$), IL-6 ($P = 0.57$),

Figure 4. Effect of chlorogenic acid (CGA) on *Staphylococcus aureus* biofilm formation. The *S. aureus* cultures were incubated with 0 (control group), 10, 20, or 30 µg/mL CGA and absorbance at 540 nm was measured. Data are presented as the means $\pm$ SEM (n = 4).

Figure 5. Effect of chlorogenic acid (CGA) on *Staphylococcus aureus* invasion of bovine mammary epithelial cells (BMEC). The BMEC were incubated in DMEM/F12 medium containing *S. aureus* and 0 (control group), 10, 20, or 30 µg/mL CGA. Data are presented as the means $\pm$ SEM (n = 3). Means at the different concentration of CGA with different letters (a,b) differ significantly for treatment effect.

Figure 7. Effect of chlorogenic acid (CGA) on phagocytosis by PMNL. Bovine PMNL were pretreated with 0 (control group), 10, 20, or 30 µg/mL CGA for 2 h, and then *Staphylococcus aureus* preloaded with fluorescein isothiocyanate (2×10^5 cfu/well) were incubated with the PMNL for 20 min. Data are presented as the means $\pm$ SEM (n = 3). Means at the different concentration of CGA with different letters (a,b) differ significantly for treatment effect.

and IL-8 ($P = 0.79$) in *S. aureus*-exposed BMEC (Table 2); however, the differences were not significant. Notably, 30 µg/mL CGA strongly decreased ($P = 0.001$) the levels of toll-like receptor-2 mRNA in *S. aureus*-exposed BMEC compared with *S. aureus* treatment alone (Table 2). The chemotactic ability of PMNL in *S. aureus*-exposed BMEC was decreased by adding 10 ($P = 0.005$) and 20 µg/mL ($P = 0.02$) of CGA when BMEC were incubated with *S. aureus* treatment alone (Figure 8). In addition, addition of 30 µg/mL CGA

enhanced the abundance of κ -CN mRNA [(Cq (quantification cycle) = 29.7] in *S. aureus*-exposed BMEC when compared with *S. aureus* treatment alone (Table 2), although statistical significance was not reached ($P = 0.11$).

DISCUSSION

Bovine mastitis, an inflammatory response of the mammary gland, is characterized by a mass recruitment of PMNL to local the mammary tissue, triggering a multistep adhesion cascade and defense responses against pathogenic bacterial infection (Sordillo and Streicher, 2002; Mayadas et al., 2014). The BMEC are the first line of defense during pathogen invasion, and are vital in the early response to limit the severity of infections. The BMEC infected with pathogen trigger the release of inflammatory mediators, enhancing the activation of innate immune responses and stimulating the expression of antimicrobial genes (Mitterhuemer et al., 2010); however, these cells can be disrupted during the process of defending against infection, leading to the formation of hard mammary tissue, milk clots, and decreased milk production and quality (Alluwaimi et al., 2003; Bar et al., 2008). Antimicrobial agents are useful for treatment of bovine mastitis caused by *S. aureus* and *E. coli*; however, such treatment can lead to unwanted residues in milk and bacterial resistance to treatment. Therefore, the development of alternative, novel therapies for bovine mastitis is necessary.

Figure 6. Effect of chlorogenic acid (CGA) on PMNL chemotaxis. Bovine PMNL were pretreated with 0 (control group), 10, 20, or 30 µg/mL CGA for 2 h, and then the number of migrated PMNL migrating in response to high levels of the chemokine IL-8 were counted. Data are presented as the means $\pm$ SEM (n = 3).

Figure 8. Effect of *Staphylococcus aureus*-exposed bovine mammary epithelial cells (BMEC) with different concentration of chlorogenic acid (CGA) treatment on PMNL chemotaxis in vitro. The BMEC were incubated with *S. aureus* alone for 6 h (control group) or with *S. aureus* with addition of 10, 20, or 30 µg/mL CGA for 6 h (CGA treatment group), and then the number of migrated PMNL migrating in response to different levels of the chemokine IL-8 was counted. Data are presented as the means $\pm$ SEM ($n = 3$). Means at the different concentrations of CGA with different letters (a,b) differ significantly for treatment effect.

Chlorogenic acid has beneficial anti-inflammatory and antimicrobial effects (dos Santos et al., 2006). We demonstrated that CGA is not cytotoxic to BMEC at low doses (10, 20, and 30 µg/mL), and at the lowest dose tested (10 µg/mL) significantly enhanced BMEC viability. These results indicate that CGA does not impair BMEC physiological functions. Furthermore, treatment of BMEC challenged with *S. aureus* with CGA (20 µg/mL) significantly enhanced BMEC viability and, notably, CGA (30 µg/mL) markedly inhibited *S. aureus* growth. These findings are in agreement with those of another study demonstrating that CGA has antimicrobial activity against *S. aureus* and *E. coli* (Bar

et al., 2008). We also evaluated the effect of CGA on *S. aureus* biofilm formation in our study. *Staphylococcus aureus* biofilms are important in chronic and persistent mastitis infections and can cause bacterial resistance to treatment with antimicrobial agents (Melchior et al., 2006). A previous study demonstrated that CGA could significantly repress biofilm formation in the context of persistent *Klebsiella pneumoniae* infection (Rajasekharan et al., 2017). Although statistical significance was not reached, our results suggest a trend for the reduction of biofilm with CGA treatment in BMEC cultures challenged with *S. aureus*. Another study demonstrated an inhibitory effect of CGA against *Aspergillus fumigatus* biofilm formation (Kong et al., 2017). Our results indicate that CGA has the potential role in preventing and controlling biofilm formation strategy in the context of bovine mastitis caused by *S. aureus*.

We also examined the effect of CGA on *S. aureus* invasion of BMEC. The adhesion of *S. aureus* to epithelial cells may involve in nonspecific or specific interactions between bacterial cell-associated ligands and host cell surface receptors, and evidence exists that invasion of mammary epithelial cells by this bacterium may involve an endocytic process requiring binding of bacteria to host cells via surface proteins (Kerro Dego et al., 2002). Persistent mastitis caused by *S. aureus* is a serious problem, which may result from the invasion of mammary epithelial cells by *S. aureus*, as *S. aureus* within cells is protected from antimicrobial agents (Barkema et al., 2006). Our study showed that treatment with CGA (30 µg/mL) significantly inhibited the invasion of BMEC by *S. aureus*, indicating that CGA may contribute to the destruction of extracellular bacteria.

During bovine mastitis, mass recruitment of PMNL to the mammary gland tissue is induced as part of the immune defense response against pathogens; hence, PMNL are a key to the resolution of mastitis. The process of PMNL migration to the bovine mammary gland is often accompanied by the expression of high levels

Table 2. Expression of genes in bovine mammary epithelial cells (BMEC) incubated with 0 (control) or 30 µg/mL chlorogenic acid (CGA) or *Staphylococcus aureus* or *S. aureus* with 30 µg/mL CGA in vitro

Symbol	Gene name	Treatment ¹				SEM	P-value
		Control	CGA	<i>S. aureus</i>	<i>S. aureus</i> with CGA		
<i>IL1β</i>	Interleukin-1β	1.01 ^c	1.20 ^b	97.47 ^a	69.89 ^{ab}	13.62	0.36
<i>IL6</i>	Interleukin-6	1.02 ^b	1.21 ^b	15.87 ^a	11.99 ^{ab}	2.04	0.34
<i>TNFA</i>	Tumor necrosis factor-α	1.01 ^{ab}	0.83 ^b	22.77 ^a	26.38 ^a	4.02	0.03
<i>IL8</i>	Interleukin-8	1.02 ^b	1.28 ^b	24.18 ^a	16.29 ^{ab}	3.16	0.03
<i>TLR2</i>	Toll-like receptor 2	1.03 ^b	0.98 ^b	2.56 ^a	1.39 ^b	0.20	0.001
<i>CSN3</i>	Kappa-casein	1.00	0.93	0.79	1.10	0.05	0.11

^{a-c}Means in the same row with different superscripts differ significantly for treatment effect ($P < 0.05$).

¹BMEC were cultured in DMEM/F12 medium in the absence of *S. aureus* and CGA (control group), or in the absence of *S. aureus* and presence of CGA (CGA-treated group), or in the presence of *S. aureus* and absence of CGA (*S. aureus* group), or in the presence of *S. aureus* and 30 µg/mL CGA (*S. aureus* with CGA treatment group).

of chemoattractants. In the present study, we observed that the chemotaxis response of bovine PMNL was improved when they were incubated with CGA. This finding suggests that CGA may enhance PMNL chemotaxis, which would be predicted to enhance the rapid immune response against pathogens. Phagocytosis is a fundamental function of PMNL. To date, no studies have examined the effect of CGA on bovine PMNL phagocytosis function. Our data indicate that the addition of CGA (20 or 30 $\mu\text{g/mL}$) markedly increased phagocytic killing of pathogens by PMNL.

Host cells infected with pathogens produce a cascade of pro-inflammatory cytokines, including IL-1 β , IL-6, and TNF- α (Gao et al., 2015), which are key in promoting and regulating the immune response against bacterial pathogens (Lapointe et al., 2010). Interleukin-1 β and TNF- α are defined as early proinflammatory cytokines (Dinarello, 2000), whereas IL-6 is a third master proinflammatory cytokine and a vital mediator of the acute-phase response in inflammation (Heinrich et al., 2003). Our study demonstrates that CGA can attenuate the expression levels of mRNA encoding IL-1 β , IL-6, and IL-8 in *S. aureus*-exposed BMEC; however, the differences were not significant. The downregulation of TNF- α was not observed. In addition, levels of *TLR2* mRNA were markedly downregulated in *S. aureus*-exposed BMEC by adding the CGA. Toll-like receptor 2 principally mediates responses to gram-positive bacteria (Prince et al., 2011) and is crucial in immune responses during *S. aureus* infection. Toll-like receptor 2 is responsible for the regulation of neutrophil functions, including adhesion, generation of reactive oxygen species, release of chemokines, and activation of proinflammatory signaling pathways (Sabroe et al., 2005). Our findings indicate that CGA causes a decrease in proinflammatory cytokines that could relieve inflammation injury caused by *S. aureus* in bovine mastitis.

To evaluate the safety of CGA for application in bovine in vivo, measurement of casein mRNA level was performed to evaluate the cytotoxic effects of CGA on BMEC. Our results demonstrate that the abundance of κ -CN mRNA was downregulated in *S. aureus*-exposed BMEC compared with controls. Notably, addition of CGA (30 $\mu\text{g/mL}$) enhanced κ -CN mRNA levels in *S. aureus*-exposed BMEC compared with *S. aureus* treatment alone, but we found no significant differences. The results indicated that dietary supplementation of CGA in cattle may be beneficial for the milk protein synthesis and prophylactic mastitis and be anti-inflammatory. However, subsequent studies must be carried out to determine CGA effects on milk yield, SCC, and cytokine production by the addition of different concentration of CGA to the diet in vivo. Oral administration of CGA to dairy cow may be metabolized by the rumen mi-

crobiota; therefore, the rumen-protected CGA product should be used to deliver to the intestinal absorptive sites. These results strongly suggest that CGA has no cytotoxic effects on BMEC and is likely to be safe for in vivo use in cattle.

CONCLUSIONS

The present study indicates that CGA can relieve the inflammatory responses triggered by *S. aureus*. Furthermore, CGA inhibited *S. aureus* growth and invasion and enhanced the phagocytic ability of PMNL. Importantly, CGA also enhanced the expression levels of κ -CN in *S. aureus*-exposed BMEC. Therefore, CGA should be considered for use as a novel therapy for mastitis caused by *S. aureus* to improve milk production and quality. The effects to CGA and bovine mastitis, including antibacterial, anti-inflammatory, SCC, milk production, and quality, must be evaluated in vivo.

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