Tilmicosin modulates the innate immune response and preserves casein production in bovine mammary alveolar cells during *Staphylococcus aureus* infection¹

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ABSTRACT: Tilmicosin is an antimicrobial agent used to treat intramammary infections against *Staphylococcus aureus* and has clinical anti-inflammatory effects. However, the mechanism by which it modulates the inflammatory process in the mammary gland is unknown. We evaluated the effect of tilmicosin treatment on the modulation of the mammary innate immune response after *S. aureus* infection and its effect on casein production in mammary epithelial cells. To achieve this goal, we used immortalized mammary epithelial cells (MAC-T), pretreated for 12 h or treated with tilmicosin after infection with *S. aureus* (ATCC 27543). Our data showed that tilmicosin decreases intracellular infection ($P < 0.01$) and had a protective effect on MAC-T reducing apoptosis after infection by 80% ($P < 0.01$). Furthermore, tilmicosin reduced reactive oxygen species (ROS) ($P < 0.01$), IL-1 β ($P < 0.01$), IL-6

($P < 0.01$), and TNF- α ($P < 0.05$) production. In an attempt to investigate the signaling pathways involved in the immunomodulatory effect of tilmicosin, mitogen-activated protein kinase (MAPK) phosphorylation was measured by fluorescent-activated cell sorting. Pretreatment with tilmicosin increased ERK1/2 ($P < 0.05$) but decreased P38 phosphorylation ($P < 0.01$). In addition, the anti-inflammatory effect of tilmicosin helped to preserve casein synthesis in mammary epithelial cells ($P < 0.01$). This result indicates that tilmicosin could be an effective modulator inflammation in the mammary gland. Through regulation of MAPK phosphorylation, ROS production and pro-inflammatory cytokine secretion tilmicosin can provide protection from cellular damage due to *S. aureus* infection and help to maintain normal physiological functions of the bovine mammary epithelial cell.

Keywords: inflammation, innate immunity, mammary epithelial cells, *S. aureus*, tilmicosin

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INTRODUCTION

Staphylococcus aureus is the major pathogen causing clinical and subclinical mastitis, which usually turns into chronic infection (Reshi et al., 2015; Scali et al., 2015). Chronic infection is one

of the mechanisms of antibacterial resistance and is related to the ability of the pathogen to internalize into professional and nonprofessional phagocytic cells like bovine mammary epithelial cells (bMECs) (Scali et al., 2015; Zheng et al., 2016).

The bMECs are cuboid epithelial cells responsible for the synthesis, package, and export of milk components including caseins, lipids, vitamins, and minerals. In addition, bMECs function as innate immune cells because they can sense pathogen-associated molecular patterns and activate early mechanisms to prevent mastitis (Yang et al., 2008; Akers and Nickerson, 2011; Ezzat Alnakip et al., 2014). Toll-like receptors (TLR2 and TLR8) on the surface of bMECs recognize *S. aureus* or its remnants, inducing the activation of an inflammatory process through a series of activation pathways including the production of reactive oxygen species (ROS) (Menzies and Ingham, 2006; Whelehan et al., 2011; Ezzat Alnakip et al., 2014; Bergström et al., 2015). Excessive accumulation of ROS is responsible for the oxidative stress and the activation of mitogen-activated protein kinases (MAPK) (P38 and ERK1/2), with the consequent production of inflammatory cytokines and chemokines (Akira et al., 2006; Lutzow et al., 2008; Alva-Murillo et al., 2015).

The complex oxidative processes induced by ROS accumulation and the activation of MAPK in the mammary gland alter cell metabolism, protein synthesis, cellular apoptosis, reduce the resistance to invasion of pathogens, lead to disruption of cell polarity and determine the cell fate (proliferation or death) (Simone Reuter, 2011; Son et al., 2013; Bae et al., 2017). Excessive ROS production also promotes monocyte infiltration, exacerbating the inflammatory process (Moloney and Cotter, 2018). Furthermore, excessive oxidative stress decreases immortalized mammary epithelial cells (MAC-T) viability and alters their physiological functions, including a decrease of casein (CSN1 and CSN2) production (Paape et al., 1995; Akers and Nickerson, 2011).

Macrolides are drugs with a macrocyclic lactone ring of 12 or more elements that are widely used in human and veterinary medicine to treat different kinds of pathologies and also bovine mastitis (Rubin, 2004; López-Boado and Rubin, 2008; Ou et al., 2008). The ability of an antibiotic to enter into cells enables it to be effective against susceptible intracellular organisms. Although their effectiveness depends on the particular antibiotic and cell type, macrolides can have intracellular activity. Tilmicosin is a 16-member semi-synthetic macrolide antimicrobial agent obtained from tylosin

with broad activity against Gram-positive and Gram-negative bacteria and frequently used in the treatment of pulmonary infection in calves, sheep, and lately to treat mastitis (Cao et al., 2006; Buret, 2010). Tilmicosin accumulates inside different types of cells including phagocytic, kidney, colon epithelial (Stuart et al., 2007; Buret, 2010), and (although at lower concentrations) also mammary gland epithelial cells (Scorneaux and Shryock, 1999).

Tilmicosin can modulate the inflammatory process in respiratory tissue in pigs and cattle (López-Boado and Rubin, 2008; Ou et al., 2008) and exerts an important anti-inflammatory effect in macrophages and mouse peripheral blood mononuclear cells stimulated with Lipopolysaccharide (LPS) (Cao et al., 2006). In addition, tilmicosin is effective in the elimination of *S. aureus* from the mammary gland (Dingwell et al., 2003). Through the parenteral administration in lactating cows, tilmicosin reaches therapeutic concentrations in milk against *S. aureus* for 7 d (Dingwell et al., 2003). Using a new long-acting preparation, therapeutic concentrations can last 20 d or more in cows during the dry period (Mendoza et al., 2016). These findings underscore the usefulness of tilmicosin in parenteral dry-cow therapy (Mendoza et al., 2016; Mohammadsadegh, 2018). In this context, tilmicosin showed a potent effect against *S. aureus* infections leading to important anti-inflammatory clinical effects (Mendoza et al., 2016; Mohammadsadegh, 2018). Despite all these well-established benefits, the mechanisms whereby tilmicosin could modulate the inflammatory process in the mammary gland are currently unknown.

The objective of this work was to assess the potential effect of tilmicosin in the regulation of the inflammatory process of bMECs induced by *S. aureus* infection. To achieve this, ROS and pro-inflammatory cytokine production, MAPK phosphorylation and its physiological effect on epithelial cell viability and the casein production in vitro were evaluated.

MATERIALS AND METHODS

Staphylococcus aureus Strain

The American type culture collection (ATCC) *S. aureus* subsp. *Aureus* Rosenbach 27543 strain isolated from a case of bovine clinical mastitis was used in this study. Bacteria were grown at 37 °C overnight in Brain-Heart Infusion (BHI) (BD Bioxon), and the CFUs were adjusted at an optical density of 620 nm (OD 0.4 = 1.5×10^7 CFU/mL).

Bacteria were frozen in BHI 10% glycerol at a concentration of 3.0×10^7 CFU/mL at -70°C .

Culture of Mammary Epithelial Cell Line (MAC-T)

MAC-T cells were kindly donated by Dr. Looor. Cells were cultured in Petri dishes (Corning-Costar) in growth medium (GM) composed of DMEM medium/nutrient mixture F-12 Ham (DMEM/F-12K, Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal calf serum, 10 $\mu\text{g/mL}$ insulin, 5 $\mu\text{g/mL}$ hydrocortisone, 100 U/mL penicillin and streptomycin (100 $\mu\text{g/mL}$), and 1 $\mu\text{g/mL}$ amphotericin B (all from Thermo Fisher Scientific, Waltham, MA). Cells were grown in a 5% CO_2 atmosphere at 37°C .

For casein induction, MAC-T were cultured in lactogenic media composed by Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 5 mg/L insulin, 1 mg/L (50 μmol) hydrocortisone, 5 $\mu\text{mol/L}$ ascorbic acid, 5 mmol/L sodium acetate, 100 U/mL penicillin/streptomycin, 1 $\mu\text{g/mL}$ progesterone, 0.5 mg/mL 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 1 $\mu\text{g/mL}$ amphotericin B (all from Thermo Fisher Scientific), and 2.5 $\mu\text{g/mL}$ prolactin (Sigma-Aldrich). Cells were incubated in a 5% CO_2 atmosphere at 37°C for 48 h.

Effect of Tilmicosin on S. aureus Growth

To analyze the anti-microbial effect of tilmicosin on *S. aureus*, 2.0×10^8 CFU/mL were cultured at 37°C in BHI supplemented with 10 $\mu\text{g/mL}$ of tilmicosin or gentamicin and the bacterial growth was measured by OD (620 nm) in a multiskan GO plate reader (Thermo Fisher Scientific) at different time points up to 24 h.

Effect of Tilmicosin on Internalization of S. aureus in MAC-T

For the infection assay, 5×10^5 MAC-T cells per well were cultured on plates (Sigma-Aldrich) with maintenance media for 12 h. When cells reached confluence, they were treated with tilmicosin (10 $\mu\text{g/mL}$) as follows: pre-treatment for 12 h (pre-TIL), treatment for 2 h post-infection (Tx-TIL) or control without treatment. Internalization assays (gentamicin protection assay) were carried out as described (Ochoa-Zarzosa et al., 2009).

Cells were infected with a multiplicity of infection (MOI) of 50:1 bacteria per cell. MAC-T

monolayers were then washed three times with PBS, pH 7.4, inoculated with 1,650 μL of bacterial suspensions at a density of 1.5×10^7 CFU/mL and incubated for 2 h in 5% CO_2 at 37°C .

After infection, MAC-T monolayers were washed three times with PBS (Gibco, Waltham, MA) and incubated in GM without serum, supplemented with 50 $\mu\text{g/mL}$ gentamicin for 1 h at 37°C and 5% CO_2 to eliminate extracellular bacteria. Then the Tx-TIL group was treated with tilmicosin (10 $\mu\text{g/mL}$) for 2 h. Next, MAC-T monolayers were detached with trypsin-EDTA (Sigma-Aldrich) and lysed with 250 μL of sterile distilled water. MAC-T lysates were diluted 100-fold, plated on LB agar in triplicate and incubated overnight at 37°C . The total number of CFU was determined by the standard colony counting technique. Data are presented as the percentage of internalization in relation to untreated MAC-T cultures.

Determination of ROS Production

ROS production was determined via the nitro blue tetrazolium (NBT) reduction as described previously (Campillo-Navarro et al., 2017). For this assay, 2.0×10^5 MAC-T per well were plated in a 24-well plates. Tilmicosin treatments and infection were done as described above. Subsequently, 20 μL of NBT (Sigma Aldrich) (1 mg/mL) were added to every well and incubated for 20 min in 5% CO_2 at 37°C . Finally, a mix of 54 μL of 2 M KOH and 46 μL of dimethyl sulfoxide (Sigma-Aldrich) were added to dissolve formazan crystals and the formazan solution was transferred to a 96-well plate. OD was measured with a microplate spectrophotometer at 620 nm (multiskan GO plate reader, Thermo Fisher Scientific).

Effect of Tilmicosin on MAC-T Viability

MAC-T viability was determined by seeding of 2.5×10^5 cells per well in a 24-well plate. Tilmicosin treatments and infection assay were done as described above. Cells were incubated with 30 μL of Zombie Nir Fixable Viability Kit (BioLegend, San Diego, CA) (1/200) for 20 min at room temperature followed by fixation with Fix/Perm kit (Tonbo Biosciences, San Diego, CA) for 1 h. Cells were then blocked with 10% goat serum in PBS for 40 min at 4°C . Samples were acquired in a fluorescent-activated cell sorting (FACS) Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific).

Determination of Cytokines Production

Pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) secreted by MAC-T into culture medium were evaluated via ELISA from 2.5×10^5 cells per well that were plated in 48-well dishes, treated and infected as described above. For this, medium was collected at different points and the concentrations of cytokines measured using the bovine IL-1 β and IL-6 (Thermo Fisher Scientific) and TNF- α (Kingfisher Biotech, Inc., St Paul, MN) ELISA kits according to the manufacturer's instructions.

MAPK Activation Assay

Phospho-p38 MAPK (p-P38) and phospho p44/42 MAPK (p-ERK 1/2) were determined only in pretreated (pre-TIL) cells after starving with DMEM 0.2% FBS for 6 and 24 h, respectively. Cells were pre-treated with tilmicosin (10 μ g/mL) for 12 h and stimulated with *S. aureus* (MOI 50:1). The levels of p-ERK and p-P38 were measured at 5 min after stimulation with *S. aureus*. As positive control, cells were treated with pervanadate (1 mM) for 20 min. After stimulation, cells were fixed and lysed with Lyse/Fix Buffer (Phosphoflow, BD Biosciences, San Jose, CA) for 10 min at room temperature followed by incubation with Perm Buffer II (BD Phosphoflow) for 20 min at room temperature. Then, cells were washed with FACS buffer solution, centrifuged and suspended in 30 μ L of purified anti-mouse CD16/32 antibody (Fc block) for 30 min at 4 °C. After washing twice with FACS buffer solution, the pellet was resuspended in the desired antibody as follows, 1/200 for phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology) and 1/800 for phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling Technology) for 30 min at 4 °C. Cells were then incubated with secondary antibody (Alexa Fluor 488 Goat Anti-Mouse IgG H&L) for 15 min at room temperature in the dark; finally, cells were resuspended in 600 μ L of 0.5% paraformaldehyde. MAPK activation levels were evaluated by flow cytometry in FACS Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific).

Analysis of Casein Expression

MAC-T were cultured in lactogenic media for 24 h, and after that period cells were washed with PBS and incubated with GM. Tilmicosin treatments and infection were performed as previously described for casein detection MAC-T were fixed with 300 μ L

of BD Phosphoflow Lyse/Fix 1 $\times$ with incubation at 37 °C for 10 min following by blocking with 10% goat serum to 4 °C for 40 min and permeabilization with 0.1% Triton X-100 for 20 min. MAC-T were stained with anti-cytokeratin 18 antibody and one anti casein antibody (both from Abcam, Cambridge, UK) for 30 min at 4 °C. Finally, cells were incubated with Alexa fluor 488 F(ab')₂ goat anti-Alexa Fluor 488 (1/1,000) and Alexa Fluor 647 goat anti-rabbit Ig H+L (Thermo Fisher Scientific) (1/300) for 25 min at 4 °C in the dark. Cytokeratin and casein expression were evaluated by flow cytometry in a FACS Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific).

Fluorescence Microscopy

A total of 2.0×10^5 MAC-T were adhered to 20 mm glass coverslips treated with poly-L-lysine solution (0.01%) and cultured in 5% CO₂ at 37 °C for 24 h with LM. After that period, cells were washed with PBS and changed for GM. Tilmicosin treatments and infection were done as previously described. Then, cells were washed three times with PBS, fixed with 4% paraformaldehyde for 1 h at room temperature followed by blocking with 10% goat serum at 4 °C for 40 min and permeabilized with 0.1% Triton X-100 for 20 min. Cells were stained with anti-bovine casein antibody (Abcam, Cambridge, UK) for 30 min at 4 °C. Finally, cells were incubated with Alexa Fluor 647 goat anti-rabbit (H+L) IgG (Thermo Fisher Scientific) (1/300) for 25 min at 4 °C in the dark. Images were captured with an inverted microscope (Olympus IX71).

Statistical Analysis

Data were obtained from three independent experiments each performed in triplicate, analyzed by ANOVA test and compared between groups via Tukey test. All statistical analyses were performed using Prism 6.0 statistical software (Graphpad Software Inc., San Diego, CA). The results are reported as SEM. Data with $P < 0.05$ were considered as statistically significant.

RESULTS

Antimicrobial Activity of Tilmicosin Against S. aureus

To investigate the potential antibacterial activity of tilmicosin on *S. aureus* growth, we performed a minimal inhibitory concentration (MIC) assay

at a concentration of 10 $\mu\text{g/mL}$. This concentration was determined by performing dose–response experiments (Supplementary Figure 1). We used gentamicin at the same dose as a control. This is the reference antimicrobial agent used in protection assays (Klein et al., 2017). Compared with the untreated control, tilmicosin had a bacteriostatic activity against *S. aureus* from 2 up to the 24 h evaluated ($P < 0.01$). Gentamicin inhibited bacterial growth from 12 h of incubation ($P < 0.01$) (Figure 1).

Effect of Tilmicosin on the Internalization of *S. aureus* into MAC-T

We used a model of immortalized bMECs (MAC-T) to evaluate the protective effect of tilmicosin against *S. aureus* invasion. Gentamicin protection assays were carried out to eliminate the bacteria adhered to the cellular membrane or residing in the medium and assessed the effect of tilmicosin in the internalization of *S. aureus* into MAC-T. Cells were pre-treated with 10 $\mu\text{g/mL}$ of tilmicosin for 12 h previous to challenge with *S. aureus* (pre-TIL) or treated after infection for 2 h with the same concentration of tilmicosin (Tx-TIL). By microscopic evaluation, we observed that *S. aureus* was internalized by MAC-T after 2 h of incubation (Figure 2A). Based on the number of CFU recovered, we observed that MAC-T treated with tilmicosin (pre-TIL and Tx-TIL) had a reduction in CFU of *S. aureus* ($P < 0.01$) at the time points evaluated (Figure 2B).

Figure 1. Tilmicosin exerts antimicrobial activity against *Staphylococcus aureus* faster than gentamicin. *S. aureus* (2×10^8 bacteria) were challenged with 10 $\mu\text{g/mL}$ of tilmicosin or gentamicin for different times and the optical density (OD) was measured at 620 nm. *** $P < 0.01$ denotes statistical differences compared with cultures infected *S. aureus* without treatment.

Tilmicosin Maintains MAC-T Viability During *S. aureus* Infection

The cytotoxic potential of tilmicosin (10 $\mu\text{g/mL}$) on cell viability was evaluated after Zombie NIR staining and FACS analysis after incubation of MAC-T with tilmicosin. The results indicated that cell viability was not affected by tilmicosin alone (control) ($P = 0.5$) (Figure 3A), ensuring that the effects of tilmicosin on MAC-T were not attributable to cytotoxic effects caused by the drug.

After infection of MAC-T with *S. aureus*, viability of cells at 6 h in the pre-TIL and Tx-TIL cultures was higher than that of MAC-T that had not been treated with tilmicosin ($P < 0.001$) up to 24 h postinfection ($P < 0.01$ (pre-Til) and $P < 0.05$ (Tx-Til)) (Figure 3A).

Figure 2. Tilmicosin diminishes *Staphylococcus aureus* load in bovine mammary epithelial cells (MAC-T). (A) Representative image of infected MAC-T (arrows indicate *S. aureus* inside the cells). (B) MAC-T were pre-treated with tilmicosin (10 $\mu\text{g/mL}$) for 12 h (pre-TIL), treated for 2 h postinfection (Tx-TIL) or left untreated. Cells were infected with *S. aureus* (MOI 50:1) for 2 h at 37 °C under 5% CO_2 and incubated for 0, 6, 12, and 24 h. CFUs were determined for each condition. *** $P < 0.001$ denotes statistical differences compared with *S. aureus*.

Tilmicosin Modulates ROS Production During *S. aureus* Infection

To determine whether tilmicosin modulates the inflammatory process in MAC-T, ROS production was analyzed under the same experimental conditions. Compared with uninfected MAC-T, when MAC-T cells were challenged only with *S. aureus*, we observed high amounts of ROS production

Figure 3. Tilmicosin diminishes ROS production and prevents death of bovine mammary epithelial cells (MAC-T) infected with *Staphylococcus aureus*. MAC-T were either pre-treated for 12 h (pre-TIL), treated for 2 h postinfection (Tx-TIL) or without treatment with tilmicosin (10 µg/mL). Cells were infected with *S. aureus* (MOI 50:1) for 2 h and incubated for 0, 6, 12, and 24 h and ROS production was determined for each condition. (A) Representative image of ROS production in MAC-T infected with *S. aureus* and treated with Tilmicosin. (B) Cellular viability after Zombie Nir staining and analysis by FACS. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ show statistical differences compared with *S. aureus*-infected untreated cells.

Figure 4. Tilmicosin decreases proinflammatory cytokine production in bovine mammary epithelial cells (MAC-T) infected with *Staphylococcus aureus*. MAC-T were pre-treated for 12 h (pre-TIL), treated for 2 h postinfection (Tx-TIL) or without treatment with tilmicosin (10 µg/mL). Cells were infected with *S. aureus* (MOI 50:1) for 2 h and the supernatant was collected to determine IL-6, IL-1β, and TNF-α by ELISA technique. N.I., cells without infection. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ indicate statistical differences compared with *S. aureus*-infected untreated cells.

($P < 0.001$) (Figure 3B). Compared with MAC-T infected with *S. aureus* and not treated with tilmicosin, pre-TIL or Tx-TIL cultures had a decrease in ROS production at 2, 6, and 24 h postinfection ($P < 0.001$) (Figure 3B).

Tilmicosin Reduces the Production of Pro-inflammatory Cytokines in *S. aureus*-Infected MAC-T

As we previously found a reduction in ROS production by tilmicosin effect, we next evaluated whether tilmicosin was able to modulate the production of pro-inflammatory cytokines in these cells after *S. aureus* infection. Compared with uninfected cells, infection with *S. aureus* induced a high production of IL-1β, IL-6 ($P < 0.001$), and TNFα ($P < 0.01$) in MAC-T (Figure 4). Tx-TIL treatment of infected cells decreased IL-1β, IL-6 ($P < 0.001$), and TNF-α ($P < 0.05$) production compared with infection alone (Figure 4A–C). Interestingly, the pre-TIL treatment further decreased the production of IL-6 in MAC-T compared to Tx-TIL cultures, while pre-TIL did not significantly decreased TNF production.

Tilmicosin Modulates MAPK Phosphorylation in MAC-T Stimulated with *S. aureus*

To analyze the mechanism by which tilmicosin could reduce inflammatory cytokine production, we evaluated the levels of phospho-ERK1/2 (p-ERK1/2) and phospho-P38 (p-P38) in MAC-T pre-treated with tilmicosin and after stimulation with *S. aureus* for 5 min. Compared with untreated cells, tilmicosin pre-treated cells had an increase of p-ERK1/2 levels ($P < 0.05$) (Figure 5A). In contrast to p-ERK1/2, compared to untreated cells,

tilmicosin decreased p-P38 levels ($P < 0.001$) in MAC-T after *S. aureus* stimulation (Figure 5B).

Tilmicosin Maintains the Production of Casein in MAC-T During Infection with S. aureus

One of the consequences of a chronic *S. aureus* infection in the mammary gland is the reduction of casein production. Therefore, we next evaluated the effect of tilmicosin on the expression of intracellular caseins after *S. aureus* infection of MAC-T. Compared with untreated cells, after infection with *S. aureus* tilmicosin allowed for sustained production of caseins ($P < 0.001$) (Figure 6). Casein production was better sustained up to 24 h after infection in pre-treated cells (pre-Til), than in cells treated after infection (Tx-Til) ($P < 0.001$). Interestingly, only tilmicosin pre-treatment rescued the casein production to the same level as non-infected cells (N.I.).

DISCUSSION

Bovine mastitis is a serious prevalent disease with high economic impact to the dairy industry worldwide (Zhao and Lacasse, 2008; Viguier et al., 2009). *Staphylococcus aureus* infection of the bovine mammary gland is commonly characterized by a persistent, recurrent, and subclinical mastitis that can turn into chronic infection (Matsunaga et al., 1993; Seegers et al., 2003) due to the ability of the bacteria to internalize into mammary epithelial cells. Bacterial internalization into epithelial cells is one of the most important pathogenic mechanisms that bacteria possess for the establishment of mastitis. This process can be modulated by different factors such as environment, hormonal factors (prolactin, estradiol), nutrients (fatty acids), or drugs (DeGo et al., 2002; Gutiérrez-Barroso et al., 2008).

It has been reported that some macrolides have anti-inflammatory properties in several pathologies, including respiratory diseases and mastitis (Buret, 2010; Mohammadsadegh, 2018; Ou et al., 2008). However, to date, there is no evidence of the immunomodulatory properties of a 16-member macrolide such as tilmicosin (Rubin, 2004; Er and Yazar, 2012). In the present study, we show that tilmicosin has antimicrobial activity against *S. aureus*. Our results demonstrate that tilmicosin has an anti-bacterial effect on bacterial growth under the conditions evaluated. Moreover, we showed that it did not inhibit bacterial internalization but helped to control the infection in MAC-T, and regulated the inflammatory process through a reduction of ROS

Figure 5. Tilmicosin differentially modulates mitogen-activated protein kinase (MAPK) phosphorylation in MAC-T cells infected with *Staphylococcus aureus*. Time course of MAPK phosphorylation. (A) ERK, (B) P38 of MAC-T cells pretreated for 12 h (pre-TIL) or untreated with tilmicosin (10 μ g/mL) and infected with *S. aureus* (MOI 50:1). After infection, cells were permeabilized and incubated with anti-phospho ERK and anti-phospho-P38 antibodies and the levels of phosphorylated protein were measured by FACS. *** $P < 0.001$, * $P < 0.05$ indicate statistical differences compared with *S. aureus*-infected untreated cells.

and pro-inflammatory cytokine, in part through alteration in MAPK phosphorylation.

Staphylococcus aureus is capable of invading the epithelial cells within the mammary gland and cause subclinical mastitis (Shompole et al., 2003; Alva-Murillo et al., 2017). Especially, when used by parenteral administration for dry-cow therapy, the efficacy of tilmicosin against *Staphylococcus* is mixed and some reports actually addressed high bacterial resistance rates to tilmicosin (Ling, 2016).

Figure 6. Casein production in MAC-T cells infected with *Staphylococcus aureus* can be rescued by treatment with tilmicosin. Casein expression in MAC-T cells pre-treated for 12 h (pre-TIL), treated 2 h post-infection (Tx-TIL), or without treatment with tilmicosin (10 $\mu\text{g/mL}$). Cells were infected with *S. aureus* (MOI 50) for 2 h and incubated for 0, 6, 12, and 24 h, and casein levels were measured by FACS using anti- α and β casein (CSN 1 and 2) antibodies. (A) Representative experiment of casein production at 24 h, (B) time course experiment $n = 3$ independent experiments, *** $P < 0.001$ shows significant differences in casein production in pre-TIL and Tx-TIL groups at all the time points evaluated compared with *S. aureus*-infected untreated cells. (C) Representative confocal images of casein production in MAC-T cells with the different conditions evaluated at 24 h. Nucleus: blue; casein: red.

For this reason, we examined the antimicrobial activity of tilmicosin against *S. aureus* growth. We confirmed that tilmicosin at a concentration of 10 $\mu\text{g/mL}$ has the classic bacteriostatic activity (Van Bambeke et al., 1998) against *S. aureus* growth. This activity is stable up to 24 h and is better than gentamicin at the same concentration although that aminoglycosides have faster bacterial killing activity compared with macrolides (Van Bambeke et al., 1998). The efficacy of tilmicosin plus the postantibiotic leukocyte enhancement effect (PALE) in

microorganisms reported in macrolides is important since those render the bacteria more susceptible to phagocytosis and killing by innate immune cells (Van Bambeke et al., 1998).

As macrolides have the ability to diffuse through cellular membranes, accumulate in phagolysosomes (Van Bambeke et al., 1998) and exert PALE activity, we hypothesized that tilmicosin could increase the microbicidal mechanisms of MAC-T. Compared with TX-TIL, we found that tilmicosin reduced the bacteria load of *S. aureus* invasion

into MAC-T at the times evaluated. Interestingly, pre-TIL was more efficient at decreasing *S. aureus* internalization. This could be explained by the fact that intracellular drugs can modulate factors inside the cells that may increase or decrease the activity of the intracellular antibiotic and, thereby, modify the cellular response against microorganisms (Van Bambeke et al., 1998).

Although the majority of them are not used for treatment of mastitis, different strategies have been evaluated to inhibit *S. aureus* internalization into mammary cells (Alva-Murillo et al., 2015, 2017; Asli et al., 2017). The effect observed with tilmicosin is similar to the effect of estradiol and sodium acetate, which reduce internalization of *S. aureus* by 50% or more (Medina-Estrada et al., 2016; Wei et al., 2017). However, tilmicosin controlled the growth of bacteria up to 24 h postinfection, while the other compounds elicit a shorter time response (2 h).

After long-term administration, the high tissue penetration of macrolides has been shown to cause cell damage after use in animals and can also increase the cellular apoptosis (Van Bambeke et al., 1998). Based on this knowledge, we evaluated the effect of tilmicosin on MAC-T viability. However, none of the treatments elicited toxicity on MAC-T according to the analysis of cell viability. On the contrary, tilmicosin improved cell viability of infected cells at 24 h postinfection up to 50% in comparison with untreated cells. These data indicate that tilmicosin helps prevent death of MAC-T during infection with *S. aureus*.

Milk production in dairy cattle depends of the number and activity of bMEC (Singh et al., 2010) and cellular death and parenchymal fibrosis after infection can reduce synthetic capacity (Nickerson, 2009; Akers and Nickerson, 2011); increased cell viability is a desired physiological outcome in dairy cattle as a means to preserve high levels of milk production and milk quality (Nickerson, 2009; Sharma and Jeong, 2013). To our knowledge, there are very few studies showing an improvement of more than 50% of postmastitis structural damage of the mammary gland (Sharma and Jeong, 2013) as that of tilmicosin. Tilmicosin could be used to protect epithelial cells against the inflammatory damage after infection of *S. aureus*.

ROS are molecules involved in the inflammatory process triggered after infection and are important in the elimination of intracellular microorganisms. However, excessive ROS production can induce toxicity and cell death (Son et al., 2013; Moloney and Cotter, 2018). Compared with uninfected cells, our results showed that tilmicosin decreased ROS

production in MAC-T in response to *S. aureus* infection, which correlated with increased cell viability. Other macrolides have been shown to diminish oxidative stress through inhibition of inducible Nitric Oxide synthase (iNOS) expression and nitric oxide production in macrophages (Ianaro et al., 2000). In addition, tilmicosin was shown to modulate the expression of COX-2 and iNOs gene abundance in mouse macrophages and monocytes stimulated with LPS (Cao et al., 2006). However, it was unknown whether tilmicosin had an effect on ROS production in bovine mammary cells. ROS induce oxidative damage in DNA, lipids, proteins, and other cellular components, and this damage is sufficient to induce malignant transformation and chronic inflammation. This can be accompanied by increased production of tissue ROS that can promote an inflammatory response of the tissue (Li et al., 2017). Our results demonstrate that tilmicosin downregulates ROS production in MAC-T at 6 and 24 h after infection with *S. aureus*, which could explain the increased viability observed in MAC-T treated with tilmicosin.

The modulation of ROS production in mammary epithelial cells by tilmicosin is consistent with the effect observed with AA like *N*-acetyl cysteine that modulate ROS production and inhibit NF- κ B nuclear translocation in MAC-T cells (Bae et al., 2017). Similarly, taurine treatment reduces ROS production, iNOs mRNA expression and pro-inflammatory cytokines in MAC-T after *S. aureus* challenge (Zheng et al., 2016). Tilmicosin could act as an ROS scavenger during *S. aureus* infection.

The effect of *S. aureus* on pro-inflammatory cytokines is controversial, as some authors have reported an inhibitory effect of *S. aureus* on IL-1 β and IL-6 production (Alva-Murillo et al., 2015, 2017). We found that tilmicosin inhibited IL-1 β , IL-6, and TNF- α production after infection, similar to a previous report (Cao et al., 2006) with murine macrophages and monocytes stimulated with LPS and treated with tilmicosin. Fatty acids like sodium butyrate and octanoate also modulate cytokine production after the inhibition of *S. aureus* invasion of bMEC (Alva-Murillo et al., 2015, 2017).

The inactivation of MAPK in MAC-T can inhibit the inflammation after treatment with antioxidant reagents that serves as an ROS scavenger (Bae et al., 2017). We investigated the molecular basis underlying the effect of tilmicosin on MAPK phosphorylation after *S. aureus* infection of MAC-T cells. Tilmicosin pretreatment resulted in increased ERK phosphorylation after *S. aureus* stimulation, similarly to a previous report (Shinkai et al., 2007).

in which clarithromycin treatment of human bronchial epithelial cells infected with *Pseudomonas aeruginosa* led to higher levels of p-ERK. High levels of p-ERK are related with cellular proliferation and survival, and the increase or decrease of cell differentiation (Shinkai M et al., 2006; Moloney and Cotter, 2018). In this context, we found that p-ERK stimulation in pretreated cells correlated with the increased in cell viability observed at 6 h after *S. aureus* infection.

It is known that constitutive ERK activation decreases pro-inflammatory cytokine production over time. On the other hand the activation of p38 culminates in increased production IL-1 β , TNF- α , and IL-6 through an MK2-dependent pathway, while IL-10 production depends on MSK 1/2 (Bachstetter and Van Eldik, 2010). Our results show that after infection with *S. aureus* tilmicosin activates p-ERK 1/2 but at the same time is a potent inhibitor of p-P38 in MAC-T. These results could explain the reduction of IL-1 β , TNF- α , and IL-6 production and increased viability of MAC-T infected with *S. aureus* after tilmicosin treatment.

Therefore, tilmicosin appears to differentially modulate MAPK phosphorylation in MAC-T stimulated with *S. aureus*, which may be related to the reported effect of p-ERK and P38 on cell viability and cytokine production, respectively (Shinkai

M et al., 2006; Bachstetter and Van Eldik, 2010). This differential modulation of MAPK has shown to be induced by other drugs such clarithromycin, which selectively activated p-ERK but not p-P38 in human bronchial epithelial cells, promoting cell viability by modulation of the inflammatory process (Shinkai M et al., 2006).

Staphylococcus aureus mastitis impacts the secretory cells differentiation and the alveolar structure with the consequent reduction of casein micelles and lipids droplets as a result of tissue damage caused by inflammation (Akers and Nickerson, 2011). After evaluating the effect of tilmicosin on MAPK phosphorylation and the synthesis of pro-inflammatory cytokines, we sought to determine the effects on the physiologic function of MAC-T cells by evaluating intracellular casein production. The pretreatment with tilmicosin was more efficient in preserving casein production than the posttreatment up to 24 h. However, as previously reported (Scorneaux and Shryock, 1999), we cannot rule out the possibility of further improvements in casein production with higher doses of tilmicosin. It is worth pointing out that this is the first report that shows a positive effect of an antibiotic treatment on the physiological function of the mammary gland; considering that caseins have important economic

Figure 7. Putative model of Tilmicosin on *Staphylococcus aureus* infection in immortalized bovine mammary cells (MAC-T). After *S. aureus* recognition by epithelial cells, the increase in ROS production elicits an increase in phosphorylation of MAPK (P38, ERK) favoring a micro-inflammatory environment leading to increased production of pro-inflammatory cytokines. Beyond a certain threshold, the high production of ROS becomes toxic for the cells, affecting specific physiological functions and increasing cell death. Tilmicosin treatment is able to inhibit ROS production, which decreases MAPK phosphorylation and pro-inflammatory cytokine production, resulting in a protective effect promoting cell survival and preserving the physiological functions of the mammary gland. Under these conditions, casein production is preserved.

value in dairy production, the effect of tilmicosin appears clinically relevant.

Staphylococcus aureus infection leads to an inflammatory response of MAC-T that includes ROS and pro-inflammatory cytokine production (IL-1 β , TNF- α , and IL-6) as a result of MAPK (P38, ERK) activation. Overproduction of ROS can lead to toxicity and affect cell viability and the physiological functions of mammary cells. Tilmicosin treatment appears to decrease the infection of MAC-T and modulate the inflammatory microenvironment by inhibiting ROS and pro-inflammatory cytokine production, in association with a selective phosphorylation of MAPK (P38 and ERK), thereby preserving the viability and function of MAC-T. In conclusion, tilmicosin exerts a protective effect of the mammary epithelium, restraining the inflammatory process induced by *S. aureus* infection and promoting cell survival and casein production by the mammary gland. This proposed model of tilmicosin action is provided in Figure 7.

Our data suggest that tilmicosin could be used as a prophylactic drug against *S. aureus* infection in dry dairy cow therapy, as it may reduce the parenchymal damage caused by infection and preserve the physiological function of the mammary gland, thereby diminishing the economic costs caused by mastitis.

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LITERATURE CITED

- Akers, R. M., and S. C. Nickerson. 2011. Mastitis and its impact on structure and function in the ruminant mammary gland. *J. Mammary Gland Biol. Neoplasia*. 16:275–289. doi:10.1007/s10911-011-9231-3
- Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell*. 124:783–801. doi:10.1016/j.cell.2006.02.015
- Alva-Murillo, N., I. Medina-Estrada, M. Báez-Magaña, A. Ochoa-Zarzosa, and J. E. López-Meza. 2015. The activation of the TLR2/p38 pathway by sodium butyrate in bovine mammary epithelial cells is involved in the reduction of staphylococcus aureus internalization. *Mol. Immunol.* 68(2 Pt B):445–455. doi:10.1016/j.molimm.2015.09.025
- Alva-Murillo, N., A. Ochoa-Zarzosa, and J. E. López-Meza. 2017. Sodium octanoate modulates the innate immune response of bovine mammary epithelial cells through the TLR2/P38/JNK/ERK1/2 Pathway: implications during staphylococcus aureus internalization. *Front. Cell. Infect. Microbiol.* 7:1–15. doi:10.3389/fcimb.2017.00078
- Asli, A., E. Brouillette, C. Ster, M. G. Ghinet, R. Brzezinski, P. Lacasse, M. Jacques, and F. Malouin. 2017. Antibiofilm and antibacterial effects of specific chitosan molecules on staphylococcus aureus isolates associated with bovine mastitis. *PLoS One*. 12:e0176988. doi:10.1371/journal.pone.0176988
- Bachstetter, A. D., and L. J. Van Eldik. 2010. The p38 MAP kinase family as regulators of proinflammatory cytokine production in degenerative diseases of the CNS. *Aging Dis.* 1:199–211.
- Bae, H., C. H. Jeong, W. N. Cheng, K. Hong, H. G. Seo, and S. G. Han. 2017. Oxidative stress-induced inflammatory responses and effects of N-acetylcysteine in bovine mammary alveolar cells. *J. Dairy Res.* 84:418–425. doi:10.1017/S002202991700067X
- Bergstrom, B., M. H. Aune, J. A. Awuh, J. F. Kojen, K. J. Blix, L. Ryan, T. H. Flo, T. E. Mollnes, T. Espevik, and J. Stenvik. 2015. TLR8 senses *staphylococcus aureus* RNA in human primary monocytes and macrophages and induces IFN- β production via a TAK1-ikkb-IRF5 signaling pathway. *J. Immunol.* 195:1100–1111. doi:10.4049/jimmunol.1403176
- Buret, A. G. 2010. Immuno-modulation and anti-inflammatory benefits of antibiotics: the example of tilmicosin. *Can. J. Vet. Res.* 74:1–10. PMC2801304.
- Campillo-Navarro, M., K. Leyva-Paredes, L. Donis-Maturano, M. González-Jiménez, Y. Paredes-Vivas, A. Cerbulo-Vázquez, J. Serafin-López, B. García-Pérez, S. E. Ullrich, L. Flores-Romo, et al. 2017. *Listeria monocytogenes* induces mast cell extracellular traps. *Immunobiology*. 222:432–439. doi:10.1016/j.imbio.2016.08.006
- Cao, X. Y., M. Dong, J. Z. Shen, B. B. Wu, C. M. Wu, X. D. Du, Z. Wang, Y. T. Qi, and B. Y. Li. 2006. Tilmicosin and tylosin have anti-inflammatory properties via modulation of COX-2 and iNOS gene expression and production of cytokines in LPS-induced macrophages and monocytes. *Int. J. Antimicrob. Agents*. 27:431–438. doi:10.1016/j.ijantimicag.2005.12.010
- Dego, O. K., J. E. van Dijk, and H. Nederbragt. 2002. Factors involved in the early pathogenesis of bovine staphylococcus aureus mastitis with emphasis on bacterial adhesion and invasion. A review. *Vet. Q.* 24:181–198. doi:10.1080/01652176.2002.9695135
- Dingwell, R. T., K. E. Leslie, T. F. Duffield, Y. H. Schukken, L. DesCoteaux, G. P. Keefe, D. F. Kelton, K. D. Lissemore, W. Shewfelt, P. Dick, et al. 2003. Efficacy of intramammary tilmicosin and risk factors for cure of staphylococcus aureus infection in the dry period. *J. Dairy Sci.* 86:159–168. doi:10.3168/jds.S0022-0302(03)73596-6
- Er, A., and E. Yazar. 2012. Effects of tylosin, tilmicosin and tulathromycin on inflammatory mediators in bronchoalveolar lavage fluid of lipopolysaccharide-induced lung injury. *Acta Vet. Hung.* 60:465–476. doi:10.1556/AVet.2012.041
- Ezzat Alnakip, M., M. Quintela-Balujá, K. Böhme, I. Fernández-No, S. Caamaño-Antelo, P. Calo-Mata, and J. Barros-Velázquez. 2014. The immunology of mammary gland of dairy ruminants between healthy

- and inflammatory conditions. *J. Vet. Med.* 2014:659801. doi:10.1155/2014/659801
- Gutiérrez-Barroso, A., J. L. Anaya-López, L. Lara-Zarate, P. D. Loeza-Lara, J. E. López-Meza, and A. Ochoa-Zarzosa. 2008. Prolactin stimulates the internalization of *Staphylococcus aureus* and modulates the expression of inflammatory response genes in bovine mammary epithelial cells. *Vet. Immunol. Immunopathol.* 121:113–122. doi:10.1016/j.vetimm.2007.09.007
- Ianaro, A., A. Ialenti, P. Maffia, L. Sautebin, L. Rombolà, R. Carnuccio, T. Iuvone, F. D'Acquisto, and M. Di Rosa. 2000. Anti-inflammatory activity of macrolide antibiotics. *J. Pharmacol. Exp. Ther.* 292:156–163. PMID: 10604943.
- Klein, J. A., T. R. Powers, and L. A. Knodler. 2017. Measurement of salmonella enterica internalization and vacuole lysis in epithelial cells. *Methods Mol. Biol.* 1519:285–296. doi:10.1007/978-1-4939-6581-6_19
- Li, L., J. Chen, G. Xiong, D. K. St Clair, W. Xu, and R. Xu. 2017. Increased ROS production in non-polarized mammary epithelial cells induces monocyte infiltration in 3D culture. *J. Cell Sci.* 130:190–202. doi:10.1242/jcs.186031
- Ling, Z. 2016. Preparation, characterization, and pharmacokinetics of tilmicosin- and florfenicol-loaded hydrogenated castor oil-solid lipid nanoparticles. *J. Vet. Pharmacol. Ther.* 40:293–303. doi:10.1111/jvp.12356
- López-Boado, Y. S., and B. K. Rubin. 2008. Macrolides as immunomodulatory medications for the therapy of chronic lung diseases. *Curr. Opin. Pharmacol.* 8:286–291. doi:10.1016/j.coph.2008.01.010
- Lutzow, Y. C., L. Donaldson, C. P. Gray, T. Vuocolo, R. D. Pearson, A. Reverter, K. A. Byrne, P. A. Sheehy, R. Windon, and R. L. Tellam. 2008. Identification of immune genes and proteins involved in the response of bovine mammary tissue to *staphylococcus aureus* infection. *BMC Vet. Res.* 4:18. doi:10.1186/1746-6148-4-18
- Matsunaga, T., S. Kamata, N. Kakiichi, and K. Uchida. 1993. Characteristics of *staphylococcus aureus* isolated from peracute, acute and chronic bovine mastitis. *J. Vet. Med. Sci.* 55:297–300. doi:10.1292/jvms.55.297
- Medina-Estrada, I., J. E. López-Meza, and A. Ochoa-Zarzosa. 2016. Anti-inflammatory and antimicrobial effects of estradiol in bovine mammary epithelial cells during *staphylococcus aureus* internalization. *Mediators Inflamm.* 2016:6120509. doi:10.1155/2016/6120509
- Mendoza, J., I. Martínez-Cortés, R. López-Ordaz, L. Gutiérrez, and H. Sumano. 2016. Concentrations of tilmicosin in mammary gland secretions of dairy cows following subcutaneous administration of one or two doses of an experimental preparation of tilmicosin and its efficacy against intramammary infections caused by *staphylococcus aureus*. *Am. J. Vet. Res.* 77:922–930. doi:10.2460/ajvr.77.9.922
- Menzies, M., and A. Ingham. 2006. Identification and expression of toll-like receptors 1–10 in selected bovine and ovine tissues. *Vet. Immunol. Immunopathol.* 109:23–30. doi:10.1016/j.vetimm.2005.06.014
- Mohammadsadegh, M. 2018. Impact of intramammary tilmicosin infusion as a dry cow therapy. *J. Vet. Pharmacol. Ther.* 41:22–27. doi:10.1111/jvp.12427
- Moloney, J. N., and T. G. Cotter. 2018. ROS signalling in the biology of cancer. *Semin. Cell Dev. Biol.* 80:50–64. doi:10.1016/j.semcdb.2017.05.023
- Nickerson, S. C. 2009. Control of heifer mastitis: antimicrobial treatment-an overview. *Vet. Microbiol.* 134:128–135. doi:10.1016/j.vetmic.2008.09.019
- Ochoa-Zarzosa, A., E. Villarreal-Fernández, H. Cano-Camacho, and J. E. López-Meza. 2009. Sodium butyrate inhibits *staphylococcus aureus* internalization in bovine mammary epithelial cells and induces the expression of antimicrobial peptide genes. *Microb. Pathog.* 47:1–7. doi:10.1016/j.micpath.2009.04.006
- Ou, X. M., Y. L. Feng, F. Q. Wen, K. Wang, J. Yang, Z. P. Deng, D. S. Liu, and Y. P. Li. 2008. Macrolides attenuate mucus hypersecretion in rat airways through inactivation of NF- κ B. *Respirology.* 13:63–72. doi:10.1111/j.1440-1843.2007.01213.x
- Paape, M. J., A. V. Capuco, A. J. Guidry, and C. Burvenicht. 1995. Morphology, function, and adaptation of mammary cells in normal and disease states. *J. Anim. Sci.* 73:1–17.
- Reshi, A. A., I. Husain, S. A. Bhat, M. U. Rehman, R. Razak, S. Bilal, and M. R. Mir. 2015. Bovine mastitis as an evolving disease and its impact on the dairy industry. *Int. J. Curr. Res. Rev.* 7:48–55.
- Rubin, B. K. 2004. Immunomodulatory properties of macrolides: overview and historical perspective. *Am. J. Med.* 117(Suppl. 9A):2S–4S. doi:10.1016/j.amjmed.2004.07.021
- Scali, F., C. Camussone, L. F. Calvino, M. Cipolla, and A. Zeconi. 2015. Which are important targets in development of *S. aureus* mastitis vaccine? *Res. Vet. Sci.* 100:88–99. doi:10.1016/j.rvsc.2015.03.019
- Scorneaux, B., and T. R. Shryock. 1999. The determination of the cellular volume of avian, porcine and bovine phagocytes and bovine mammary epithelial cells and its relationship to uptake of tilmicosin. *J. Vet. Pharmacol. Ther.* 22:6–12. doi:10.1046/j.1365-2885.1999.00185.x
- Seegers, H., C. Fourichon, and F. Beaudeau. 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Vet. Res.* 34:475–491. doi:10.1051/vetres:2003027
- Sharma, N., and D. K. Jeong. 2013. Stem cell research: a novel boulevard towards improved bovine mastitis management. *Int. J. Biol. Sci.* 9:818–829. doi:10.7150/ijbs.6901
- Shinkai, M., G. H. Foster, and B. K. Rubin. 2006. Macrolide antibiotics modulate ERK phosphorylation and IL-8 and GM-CSF production by human bronchial epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 290:L75–L85. doi:10.1152/ajplung.00093.2005
- Shinkai, M., Y. S. López-Boado, and B. K. Rubin. 2007. Clarithromycin has an immunomodulatory effect on ERK-mediated inflammation induced by *pseudomonas aeruginosa* flagellin. *J. Antimicrob. Chemother.* 59:1096–1101. doi:10.1093/jac/dkm084
- Shompole, S., K. T. Henon, L. E. Liou, K. Dziewanowska, G. A. Bohach, and K. W. Bayles. 2003. Biphasic intracellular expression of *staphylococcus aureus* virulence factors and evidence for agr-mediated diffusion sensing. *Mol. Microbiol.* 49:919–927. doi:10.1046/j.1365-2958.2003.03618.x
- Simone Reuter, B. B. A. 2011. Oxidative stress, inflammation, and cancer: how are they linked?. *Free Radic Biol Med.* 49:1603–1616. doi:10.1016/j.freeradbiomed.2010.09.006
- Singh, K., R. A. Erdman, K. M. Swanson, A. J. Molenaar, N. J. Maqbool, T. T. Wheeler, J. A. Arias, E. C. Quinn-Walsh, and K. Stelwagen. 2010. Epigenetic regulation of milk production in dairy cows. *J. Mammary Gland Biol. Neoplasia.* 15:101–112. doi:10.1007/s10911-010-9164-2
- Son, Y., S. Kim, H. T. Chung, and H. O. Pae. 2013. Reactive oxygen species in the activation of MAP

- kinases. *Methods Enzymol.* 528:27–48. doi:10.1016/B978-0-12-405881-1.00002-1.
- Stuart, A. D., T. D. K. Brown, and J. B. Tasker. 2007. Intracellular accumulation and trans-epithelial transport of Aivlosin, Tylosin and Tilmicosin. *Pig J.* 60:26–35.
- Van Bambeke, F., C. Gerbaux, J. M. Michot, M. B. d'Yvoire, J. P. Montenez, and P. M. Tulkens. 1998. Lysosomal alterations induced in cultured rat fibroblasts by long-term exposure to low concentrations of azithromycin. *J. Antimicrob. Chemother.* 42:761–767. doi:10.1093/jac/42.6.761
- Viguier, C., S. Arora, N. Gilmartin, K. Welbeck, and R. O'Kennedy. 2009. Mastitis detection: current trends and future perspectives. *Trends Biotechnol.* 27:486–493. doi:10.1016/j.tibtech.2009.05.004
- Wei, Z., C. Xiao, C. Guo, X. Zhang, Y. Wang, J. Wang, Z. Yang, and Y. Fu. 2017. Sodium acetate inhibits staphylococcus aureus internalization into bovine mammary epithelial cells by inhibiting NF- κ B activation. *Microb. Pathog.* 107:116–121. doi:10.1016/j.micpath.2017.03.030
- Whelehan, C. J., K. G. Meade, P. D. Eckersall, F. J. Young, and C. O'Farrelly. 2011. Experimental staphylococcus aureus infection of the mammary gland induces region-specific changes in innate immune gene expression. *Vet. Immunol. Immunopathol.* 140:181–189. doi:10.1016/j.vetimm.2010.11.013
- Yang, W., H. Zerbe, W. Petzl, R. M. Brunner, J. Günther, C. Draing, S. von Aulock, H. J. Schuberth, and H. M. Seyfert. 2008. Bovine TLR2 and TLR4 properly transduce signals from staphylococcus aureus and *E. coli*, but *S. aureus* fails to both activate NF- κ B in mammary epithelial cells and to quickly induce TNF α and interleukin-8 (CXCL8) expression in the udder. *Mol. Immunol.* 45:1385–1397. doi:10.1016/j.molimm.2007.09.004
- Zhao, X., and P. Lacasse. 2008. Mammary tissue damage during bovine mastitis: causes and control. *J. Anim. Sci.* 86(13 Suppl.):57–65. doi:10.2527/jas.2007-0302
- Zheng, L., Y. Xu, J. Lu, M. Liu, Bin Dai, J. Miao, and Y. Yin. 2016. Variant innate immune responses of mammary epithelial cells to challenge by staphylococcus aureus, *Escherichia coli* and the regulating effect of taurine on these bioprocesses. *Free Radic. Biol. Med.* 96:166–180. doi:10.1016/j.freeradbiomed.2016.04.022