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Citrus-derived oil inhibits *Staphylococcus aureus* growth and alters its interactions with bovine mammary cells

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ABSTRACT

This experiment examined the effects of cold-pressed, terpeneless citrus-derived oil (CDO) on growth of *Staphylococcus aureus,* which a major cause of contagious bovine mastitis, and invasion of bovine mammary cells (MAC-T). To determine minimum inhibitory concentration, we used the broth dilution method, using CDO concentrations range from 0.0125 to 0.4% with 2-fold dilutions. Growth inhibition was examined by adding 0.00, 0.05, 0.025, 0.0125, and 0.00625% CDO to 10^5 cfu/mL *S. aureus* in nutrient broth and enumerating colonies after serial dilution. In a 96-well plate, *S. aureus* (10^7 cfu/mL) was allowed to form a biofilm, treated with 0, 0.025, 0.5, or 1% CDO, and then was measured using a spectrophotometer. Cytotoxic effect on immortalized MAC-T cells was also examined at various concentrations of CDO using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. We observed that the minimum inhibitory concentration of CDO to inhibit the growth of *S. aureus* in vitro was 0.025% CDO. A time kill curve for CDO action on *S. aureus* over 4 h was generated. The CDO completely eliminated *S. aureus* after 3 h of incubation at a concentration of 0.25%, or after 2 h of incubation at concentrations of 0.05%. It was also observed that CDO had no effect on preformed biofilms except at a concentration of 0.05%, in which a significant reduction in the measured absorbance was noted. In addition, the association and invasion of *S. aureus* to MAC-T cells were significantly inhibited after 1 h of treatment with CDO. Citrus-derived oil was also able to increase cellular proliferation of MAC-T cells at concentrations up 0.05% and had no effect at a concentration of 0.1% after 1 h. Our data suggests that CDO should be considered for further research as a preventive and therapeutic against bovine mastitis.

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INTRODUCTION

Mastitis, an inflammation of the mammary gland, is one of the most costly diseases affecting dairy farming, exceeding $2 billion annually, with economic losses resulting from premature culling, additional labor, management, and veterinary costs, and reduced milk production and quality (Seegers et al., 2003; Bar et al., 2008). This cost excludes irreversible damage to the mammary gland that can lead to future reduced milk production, milk composition changes, and reproductive inefficiency from cows with mastitis (Harmon, 1994).

Mastitis is characterized by an increase in milk SCC and may be accompanied by the presence of an intramammary pathogen (Paape et al., 2003). The most common bacteria associated with mastitis are *Staphylococcus* species, *Streptococcus* species, and coliforms, such as *Escherichia coli* and *Corynebacterium. Staphylococcus* species, specifically *Staphylococcus aureus*, are considered more virulent and the most damaging causative agent (Reyher et al., 2012). Mastitis can be clinical or subclinical; clinical mastitis is characterized by abnormal milk secretions with or without local or systemic signs of inflammation, whereas subclinical mastitis has a higher incidence rate and is most commonly diagnosed by the presence of a pathogen in milk (Barlow, 2011). Subclinical mastitis is the most common form of mastitis but is asymptomatic, and therefore may go undetected (Barlow, 2011). A recent surveillance study showed that *S. aureus* is also the main cause for subclinical mastitis (Botrel et al., 2010; Moser et al., 2013). Antibiotic treatment is widely used to treat clinical mastitis caused by *S. aureus*. However, antibiotic use is controversial not only due to economic relevance, but also growing consumer concerns (Moser et al., 2013). Treatment of the causative bacteria using antibiotics is coming under increasing public scrutiny due to the possible development of resistant pathogens

(Craven, 1987; Guterbock et al., 1993) and risk of residues appearing in the milk. One study, which included conventional dairy farms in New York, found *S. aureus* resistance to ampicillin, erythromycin, penicillin, and tetracycline to be common among isolates obtained from mastitic milk (Tikofsky et al., 2003). In addition, cure rates using antibiotics for *S. aureus* mastitis vary considerably, ranging from 4 to 92% (Barkema et al., 2006). A study on clinical mastitis cases in the Netherlands found a cure rate of only 52% (Sol et al., 2000). Thus, alternative strategies for control and treatment for *S. aureus* is essential for improving animal health and economic outcome for the farmer (Barkema et al., 2006).

Many plant derivatives and essential oils derived from the citrus fruits and other plants contain secondary metabolites that can inhibit bacterial growth (Burt and Reinders, 2003; Burt, 2004). Essential oils, or fractions thereof, have been traditionally used as flavoring agents in foods, and it has frequently been noted that many possess antimicrobial properties (Smith-Palmer et al., 1998; Alzoreky and Nakahara, 2003; Ahn et al., 2014; Salaheen et al., 2014a; Yang et al., 2014; Budri et al., 2015). One past study examined clove and cinnamon oil for use in the treatment of mastitis-causing *S. aureus* biofilms and found them to be effective in reducing preformed biofilms (Budri et al., 2015). Citrus-derived oils (**CDO**) have recently been shown to inhibit growth of methicillin-resistant *S. aureus* (Muthaiyan et al., 2012b). Therefore, examining its use as an alternative to antibiotic therapy for mastitis is warranted. To the best of our knowledge, no studies have examined the toxicity of CDO on mammary epithelial cells for use as an alternative therapy for mastitis. Our objective was to determine the inhibitory effect of CDO on the growth of bovine pathogen *S. aureus*, its role in host cell, and invasion of bovine mammary cells (**MAC-T**) *S. aureus* interactions to explore the potential strategy to control bovine mastitis.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Staphylococcus aureus ATCC 29740 was used in this study. This strain has been isolated from bovine mastitic milk. Response time and infection rate of this *S. aureus* strain has been documented previously with consistent changes in onset and duration of the host response beginning as early as 56 h and continuing through 240 h. (Bannerman, 2009). Bacteria were maintained in nutrient broth or nutrient agar (Gibco, Thermo Fisher Scientific, Waltham, MA) and were grown for 18 to 24 h before use.

Preparation of CDO

Terpeneless, cold-pressed Valencia orange oil was provided by Firmenich Citrus Center (Safety Harbor, FL). A stock solution was prepared by dissolving CDO in dimethyl sulfoxide (**DMSO**; Sigma-Aldrich, St. Louis, MO) to a final concentration of 40% CDO. However, for the preformed biofilm experiment, a 10% CDO in DMSO stock solution was needed due to orange oil evaporation. The 10% stock solution was also used for detection of cytotoxicity.

Determination of MIC

The broth dilution method was used to determine the MIC of *S. aureus* (Muthaiyan et al., 2012b). Concentrations of CDO ranging from 0.0125 to 0.4% CDO with half-step dilutions were added to 24-well plates containing 10^5 cfu/mL of *S. aureus*. Plates were incubated overnight and MIC was determined as the lowest concentration of CDO inhibiting visual growth of bacteria in the wells of 24-well plate (Greiner Bio-One, Monroe, NC). This was confirmed by colony-forming unit assay.

Growth Inhibition Assay

Using a spectrophotometer, *S. aureus* was diluted 10^8 cfu/mL in nutrient broth (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ) by adjusting it to an optical density at 600 nm ($\mathbf{OD_{600}}$) of ~0.08 to 0.12. This was further diluted to 10^5 cfu/mL, and placed in cell culture tubes. *Staphylococcus aureus* was treated with CDO at concentrations of 0.00, 0.05, 0.025, 0.0125, and 0.00625%. The control used a concentration of 1% DMSO. For 4 h, the cell culture tubes were kept in a shaking incubator at 37°C. We took 100 μL of *S. aureus* every 15 min for 1 h, then every 30 min for 1 h, and then every 60 min for a total of 4 h of incubation. This was serially diluted and plated on to nutrient agar (Difco). Viability was determined by colonies count after overnight culture at 37°C.

Inhibition of Preformed Biofilms and Biofilm Formation

The ability of CDO to affect preformed *S. aureus* biofilms and biofilm formation was examined using methods adapted from Karaolis et al. (2005), O'Toole (2011), and Ma et al. (2012). Briefly, bacterial cells were inoculated into nutrient broth and incubated for 18 h. The OD_{600} of the bacterial suspension was adjusted to ~0.08 to 0.12 (10^8 cfu/mL) and then further diluted with nutrient broth to 10^7 cfu/mL. To measure CDO