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Antibacterial effect of plant-derived antimicrobials on major bacterial mastitis pathogens in vitro

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ABSTRACT

The objective of this study was to investigate the antimicrobial effect of plant-derived antimicrobials including *trans*-cinnamaldehyde (TC), eugenol, carvacrol, and thymol on major bacterial mastitis pathogens in milk. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the aforementioned compounds on *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus*, and *Escherichia coli* were determined. In addition, the bactericidal kinetics of TC on the aforementioned pathogens and the persistence of the antimicrobial activity of TC in milk over a period of 2 wk were investigated. All 4 plant-derived molecules exhibited antimicrobial activity against the 5 mastitis pathogens tested, but TC was most effective in killing the bacteria. The MIC and MBC of TC on *Staph. aureus*, *E. coli*, and *Strep. uberis* were 0.1 and 0.45%, respectively, whereas that on *Strep. agalactiae* and *Strep. dysgalactiae* were 0.05 and 0.4%, respectively. The MIC and MBC of the other 3 molecules ranged from 0.4 to 0.8% and 0.8 to 1.5%, respectively. In time-kill assays, TC at the MBC reduced the bacterial pathogens in milk by 4.0 to 5.0 $\log_{10}$ cfu/mL and to undetectable levels within 12 and 24 h, respectively. The antimicrobial effect of TC persisted for the duration of the experiment (14 d) without any loss of activity. Results of this study suggest that TC has the potential to be evaluated as an alternative or adjunct to antibiotics as intramammary infusion to treat bovine mastitis.
Key words: mastitis, *trans*-cinnamaldehyde, antimicrobial

INTRODUCTION

Bovine mastitis is an inflammatory condition of mammary gland most often caused by bacterial intramammary infection, resulting in significant economic losses to the dairy industry. The increased production costs associated with mastitis can be attributed to culling, medication, discarded milk, and reduced milk quality (Natzke, 1981). The economic losses due to mastitis in the United States and worldwide have been estimated at US $2 billion (Ott, 1999) and $35 billion (Wellenberg et al., 2002), respectively. Based on the bacteriological etiologic agent, mastitis can be classified into contagious and environmental mastitis. An infected quarter is the source of contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*, whereas environmental pathogens such as *Escherichia coli*, *Streptococcus dysgalactiae,* and *Streptococcus uberis* originate from a variety of sources including bedding, manure, pastures, and pond water. Bacteria gain access to a healthy gland most frequently during and after the milking process, when vacuum fluctuations, liner slips, and relaxed teat canal sphincter muscle tone afford the greatest opportunity for invasion.

Intramammary infusion of antibiotics is the most common treatment method available for treating mastitis. However, the cure rates obtained with antibiotics are generally poor and vary for different mastitis pathogens. For example, the cure rates of mastitis caused by *Staph. aureus* range from 20 to 75% (Eberhart et al., 1987; Dingwell et al., 2003). Use of antibiotics against bacterial diseases in cattle, including mastitis, may potentially lead to the emergence of antibiotic resistant strains of bacteria (Berghash et al., 1983; White, 1999). Moreover, the use of antibiotics to treat bovine mastitis has been implicated as a common source of drug residues in milk (Erskine, 1996). Approximately 90% of the residues detected in milk over a period of 5 yr in Michigan originated from antibacterial therapy for mastitis (Erskine et al., 2003). In light of the aforementioned problems and concerns, there is a need for alternative approaches for controlling mastitis in dairy cows.

Plant-derived essential oils represent a group of natural antimicrobials that have been traditionally used to preserve foods as well as enhance food flavor. The antimicrobial properties of several plant-derived essential oils have been demonstrated (Bilgrami et al., 1992; Burt, 2004; Holley and Patel, 2005), and a variety of

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active components of these oils have been identified. *Trans*-cinnamaldehyde (**TC**) is an aromatic aldehyde present as a major component of bark extract of cinnamon (*Cinnamomum verum*). Carvacol and thymol are antimicrobial ingredients in oregano oil obtained from *Origanum glandulosum* (Bendahou et al., 2008). Similarly, eugenol is an active ingredient in the oil from cloves (*Eugenia caryophillis*; Ali et al., 2005). All the aforementioned substances are classified as GRAS (generally regarded as safe) by the United States Food and Drug Administration. Moreover, plant-derived antimicrobials have been reported not to induce resistance in gram-positive and gram-negative bacteria after prolonged exposure (Ohno et al., 2003; Domadia et al., 2007).

Because dry cow therapy (**DCT**) is a common strategy for controlling mastitis, we investigated the antimicrobial properties of TC, eugenol, carvacrol, and thymol in milk for future application as a DCT in cows. Upon entry into the mammary gland through the teat canal, pathogens come in contact with milk, where they need to adapt, survive, and replicate before establishing an infection (Lammers et al., 2000). Moreover, milk is a complex medium in which lipophilic proteins such as albumin, and other nutrients, including fat and starch, can potentially interact with the antimicrobial molecules, thereby reducing their bioavailability. Therefore, milk was chosen as the in vitro model for studying the antimicrobial potential of TC, eugenol, carvacrol, and thymol for controlling mastitis.

The objective of this study was to determine the efficacy of TC, eugenol, carvacrol, and thymol for killing the major bacterial mastitis pathogens in milk. Specifically, the antimicrobial effect of the aforementioned plant-derived antimicrobials was investigated on *Staph. aureus*, *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, and *E. coli*.

MATERIALS AND METHODS

Maintenance and Preparation of Bacterial Cultures

Three isolates each of *Staph. aureus* (DTSL-35, 17, and 38), *Strep. agalactiae* (DTSL-45, 41, and 7), *Strep. dysgalactiae* (DTSL-34, 20, and 28), *Strep. uberis* (DTSL-31, 27, and 19), and *E. coli* (DTSL-2, 39, and 40) isolated from clinical bovine mastitis cases were obtained from the University of Connecticut Diagnostic Testing Services Laboratory. All bacteriological media used in the study were purchased from Difco, Becton Dickinson (Sparks, MD). The purity of each culture was ensured by characteristic morphology on mannitol salt agar (*Staph. aureus*), sorbitol MacConkey agar (*E. coli*), or blood agar (streptococci). For preparation of inocula, each isolate of the pathogen was grown separately in 10 mL of tryptic soy broth (**TSB**) for 24 h at 37°C. The cells were then sedimented by centrifugation (3,600 × *g* for 15 min at 4°C), washed twice with sterile PBS (pH 7.2), and resuspended in PBS. Equal portions from each of the 3 isolates were combined to make a 3-isolate mixture of each species of the pathogen. The bacterial concentrations (cfu/mL) in the individual and 3-isolate mixtures were determined by plating 0.1-mL portions of appropriate dilutions on tryptic soy agar (**TSA**) plates, and incubating the plates at 37°C for 24 h. Appropriate dilutions of the 3-isolate mixture in PBS were used to obtain the desired level of inoculum.

Sample Preparation

Fresh, raw milk free from antibiotic residues was collected from the bulk tank at the University of Connecticut dairy farm and autoclaved at 121°C and 103.4 kPa of pressure for 15 min.

Antimicrobials

Trans-cinnamaldehyde (# 239968), eugenol (# 46100), carvacrol (# 282197), and thymol (# T0501) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO).

Determination of MIC and Minimum Bactericidal Concentration

The MIC and minimum bactericidal concentration (**MBC**) of TC, eugenol, carvacrol, and thymol against each bacterial pathogen were determined by the broth dilution assay described by Andrews (2001). Milk tubes containing TC, eugenol, carvacrol, or thymol in the range of 0 to 1.5% (vol/vol) in increments of 0.05% were inoculated separately with each bacterial pathogen at 6.0 $\log_{10}$ cfu/mL and incubated at 39°C for 24 h. Control samples included milk inoculated with each pathogen. Following incubation, the samples were serially diluted (1:10) in PBS and appropriate dilutions were plated on TSA plates. The plates were incubated at 37°C for 24 h. The lowest concentration of the antimicrobial treatment that inhibited visible growth of the pathogen after incubation was taken as the MIC of the treatment. The lowest concentration of the treatment that prevented growth of the organism after subculture on TSA following serial dilution and plating was taken as the MBC. Triplicate samples were included for each treatment, and the experiment was replicated 3 times.