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 log₁₀ 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*). Carvacrol 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 caryophyllis*; 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 ($3,600 \times g$ 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.

Time-Kill Assay

The bactericidal kinetics of TC were studied by inoculating sterile milk containing the MIC (0.1%), MBC (0.4%), or a concentration greater than the MBC (0.7%) of TC with each pathogen at $6.0 \log_{10}$ cfu/mL. The concentration of 0.7% TC was chosen based on preliminary experiments in which it was found to be the lowest concentration above the MBC that brought about rapid killing of all pathogens. Control samples containing inoculated milk with no added antimicrobial were also included. The samples were incubated at 39°C for 24 h to replicate the cow's body temperature. Surviving populations of each bacterial pathogen were enumerated at 0, 6, 12, and 24 h of incubation by plating 0.1-mL portions of the samples directly or after serial dilutions (1:10 in PBS) on duplicate TSA plates. Pathogens that were not detected by direct plating were tested for surviving bacteria by enriching 1 mL of the sample in 100 mL of TSB at 39°C for 24 h. When growth was observed in the broth, the culture was streaked on TSA plates and observed for typical colonies of each pathogen. Each treatment was done in duplicate, and the experiment was replicated 3 times.

Determination of Persistence of Antimicrobial Activity of TC in Milk

Milk samples containing 0.45% (MBC) or 0.7% of TC were inoculated with a 3-strain mixture of *Staph. aureus* at $6.0 \log_{10}$ cfu/mL and incubated at 39°C for 14 d. Inoculated milk samples containing no TC served as controls. The surviving bacteria were determined immediately after TC addition and at 24-h intervals until d 14. To mimic the bacterial invasion of mammary gland after TC infusion, approximately $6.0 \log_{10}$ cfu/mL of *Staph. aureus* was inoculated into the same milk samples every 48 h until d 6 (d 2, 4, and 6) and bacterial populations were determined on TSA after 24 h. Duplicate samples were inoculated and the experiment was replicated 3 times.

Determination of pH

Approximately 5 mL of milk was transferred into 20-mL tubes. This was followed by the addition of various antimicrobial molecules and the pH was immediately determined (Accumet pH meter, Fisher Scientific, Fair Lawn, NJ).

Statistical Analysis

Time-Kill Assay. Bacterial counts (log values) from each pathogen were analyzed separately using PROC

MIXED (SAS Institute, Cary, NC). The model statement partitioned variation due to compound concentration, sample time, experiment replicate and concentration $\times$ time interaction. The REPEATED statement was used to indicate that samples within replicate $\times$ concentration interaction were collected from the same tube. Data are expressed as least squares means.

Persistency Determination. Data from persistency determination experiments were analyzed similarly to the method used for time-kill assays. Data from 3 independent replicate experiments were analyzed using PROC MIXED (SAS Institute). The model statement partitioned variation due to TC concentration, sample time, experiment replicate, and concentration $\times$ time interaction. The REPEATED statement was used to indicate that samples within replicate $\times$ concentration interaction were collected from the same tube. Data are expressed as least squares means.

RESULTS

The average pH of milk and milk with the addition of antimicrobial molecules ranged from 6.60 to 6.67. The MIC and MBC of TC, eugenol, carvacrol, and thymol on the mastitis pathogens are provided in Table 1. There was no variation in the MIC and MBC values among replicate samples. Among the 4 molecules tested, TC was most effective in killing all the mastitis pathogens tested. 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 other 3 molecules ranged from 0.4 to 0.8% and from 0.8 to 1.5%, respectively.

The bactericidal kinetics of TC on the mastitis pathogens in milk are depicted in Figure 1, panels A to E. The average initial bacterial population in all the treatment and control samples for the 5 mastitis pathogens was approximately $6.0 \log_{10}$ cfu/mL. In the control samples, the bacterial population increased during the 24-h incubation period, reaching about $8.5 \log_{10}$ cfu/mL. However, in the treatment samples containing TC at MBC and >MBC, the bacterial counts of all the pathogens were reduced substantially ($P < 0.001$). The presence of TC at MBC reduced the bacterial population by 4.0 to 5.0 $\log_{10}$ cfu/mL and to undetectable levels in 6 and 24 h, respectively. However, the concentration of TC above the MBC completely inactivated all bacterial pathogens by 12 h of incubation, except *Strep. uberis*, in which an approximately $5.0 \log_{10}$ cfu/mL kill was observed. As expected, for all bacteria, TC at MIC concentration did not allow growth to occur.

The results from the experiment determining the persistence of antimicrobial activity of TC in milk are

Table 1. Minimum inhibitory concentration and minimum bactericidal concentration (MBC) of *trans*-cinnamaldehyde (TC), eugenol, carvacrol, and thymol against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* (expressed in percentage)

Pathogen	TC		Eugenol		Carvacrol		Thymol	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Staph. aureus</i>	0.1	0.45	0.6	1.4	0.5	1.2	0.6	1.2
<i>E. coli</i>	0.1	0.45	0.8	1.4	0.8	1.2	0.8	1.5
<i>Strep. agalactiae</i>	0.05	0.4	0.4	0.8	0.4	0.8	0.4	0.9
<i>Strep. dysgalactiae</i>	0.05	0.4	0.4	0.4	0.4	0.8	0.4	0.9
<i>Strep. uberis</i>	0.1	0.45	0.5	0.4	0.8	1.2	0.6	1.4

depicted in Figure 2. *Staphylococcus aureus* ($6.0 \log_{10}$ cfu/mL) inoculated in the milk samples on d 0 were completely inactivated by 0.7% TC after 24 h. On d 2, $6.0 \log_{10}$ cfu/mL of *Staph. aureus* reinoculated into the milk samples was reduced to $<1.0 \log_{10}$ cfu/mL on d 3. On d 4, a similar reduction in *Staph. aureus* counts ($>5.0 \log_{10}$ cfu/mL) was observed after 24 h. This trend in bacterial reduction was observed on the subsequent days, where $6.0 \log_{10}$ cfu/mL of *Staph. aureus* inoculated on d 6 was decreased to undetectable levels on d 12 of the experiment. A similar trend in *Staph. aureus* inactivation was also observed in milk samples containing 0.45% TC, but the magnitude of killing was slightly less than that observed with 0.70% TC. In control samples, the bacterial population increased to approximately $8.0 \log_{10}$ cfu/mL by 24 h, and remained at that level for the duration of the experiment.

DISCUSSION

Mastitis continues to be the single most expensive health-related problem confronting the dairy industry worldwide (Hortet and Seegers, 1998). Based on previous studies and the recommendations of the National Mastitis Council, DCT is considered to be one of the most effective methods for preventing mastitis during the dry period (Eberhart, 1986; Schukken et al., 1993). Given the various arguments that discourage widespread antibiotic use, development of alternative strategies using compounds not subject to limitations associated with antibiotics is needed. Herein, we present data indicating the efficacy of several plant-derived compounds for killing mastitis pathogens in vitro.

Antibacterial activity of several plant-derived molecules, including carvacrol, thymol, eugenol, and TC have been demonstrated against both gram-positive and gram-negative pathogens (Burt, 2004). For example, the antibacterial activity of TC has been reported against *Clostridium botulinum* (Bowles and Miller, 1993), *Staph. aureus* (Bowles et al., 1995), *E. coli* O157:H7, and *Salmonella* Typhimurium (Helander et al., 1998) in synthetic laboratory media. Similarly, the inhibi-

tory effect of carvacrol and thymol against a variety of pathogens such as *Salmonella* Typhimurium (Si et al., 2006), *E. coli* O157:H7 (Burt et al., 2005), *Campylobacter jejuni*, and *Listeria monocytogenes* (Friedman et al., 2002) has been demonstrated. Eugenol is another plant-derived molecule reported to possess a wide spectrum of antimicrobial activity against gram-positive and gram-negative pathogens (Gill and Holley, 2004; Gaysinsky et al., 2007). Although considerable information is available on the antimicrobial properties of essential oils, most studies determining the antimicrobial activity of essential oils have been carried out in model broth systems (Knight and McKellar, 2007). Several studies have reported a decreased antimicrobial effect of plant extracts when used in foods or complex systems (Shelef et al., 1984; Stecchini et al., 1993; Pandit and Shelef, 1994; Gaysinsky et al., 2007). The concentrations of plant-derived molecules needed to achieve antimicrobial activity in complex foods such as meat, fish, dairy products, and vegetables are significantly greater than that needed in laboratory media (Burt, 2004). Similarly, Gaysinsky et al. (2007), while evaluating the antimicrobial effect of eugenol in milk, reported that milk composition, especially fat level, reduces the efficacy of the molecule. In light of these findings, we determined the MIC of MBC of the various molecules against the mastitis pathogens directly in milk rather than in any synthetic laboratory medium.

Because plant-derived molecules contain several different chemical groups in their structure, their antimicrobial activity is not attributable to one specific mechanism (Skandamis et al., 2001; Carson et al., 2002). A critical property of essential oils or their components is their hydrophobicity, which helps them to target the lipid-containing bacterial cell membrane and mitochondria (Knobloch et al., 1986; Sikkema et al., 1994). This makes these membranes more permeable, leading to leakage of ions and other cell contents (Cox et al., 2000; Carson et al., 2002; Ultee et al., 2002). In addition to the effect on cell membranes, TC is also believed to kill bacteria by inhibiting energy generation and glucose uptake (Gill and Holley, 2004). Yet another

Figure 1. Inactivation of A) *Staphylococcus aureus* (SEM = 0.052), B) *Escherichia coli* (SEM = 0.039); C) *Streptococcus agalactiae* (SEM = 0.038); D) *Streptococcus dysgalactiae* (SEM = 0.083); and E) *Streptococcus uberis* (SEM = 0.091) in milk containing 0% (control, ◆), 0.1% (■), 0.45% (▲), and 0.7% (×) trans-cinnamaldehyde.

mechanism by which cinnamon oil and its components kill microorganisms is by their inhibitory effect on enzymes such as amino acid decarboxylases (Wendakoon and Sakaguchi, 1995).

Although all the 4 plant-derived molecules exhibited antimicrobial activity against the 5 mastitis pathogens tested, TC was found to be most effective in killing the bacteria. As is evident in Table 1, the MIC and MBC

of TC were the lowest for all the mastitis pathogens compared with the other 3 molecules. Therefore, TC was selected for further studies. To be an effective antimicrobial as a DCT under field conditions, TC should maintain its antimicrobial activity over an extended period. However, the MIC and MBC experiments and time-kill assay determined the antimicrobial activity of TC in milk only up to 24 h. Moreover, TC could be

Figure 2. Persistence of antimicrobial activity of *trans*-cinnamaldehyde on *Staphylococcus aureus* in milk containing (♦) 0.45% and (■) 0.7% *trans*-cinnamaldehyde.

degraded or inactivated in milk over a period of time. Therefore, we determined the persistence of antimicrobial activity of TC on *Staph. aureus* in milk for 14 d to confirm if TC could maintain antimicrobial activity over this period. *Staphylococcus aureus* was chosen for this experiment because *Staph. aureus* (along with *E. coli* and *S. uberis*) required the highest MIC and MBC of TC compared with the other pathogens. Moreover, *Staph. aureus* is more commonly associated with contagious mastitis than the other species (Makovec and Ruegg, 2003). The period of 14 d was chosen because the endogenous protection mechanism of keratin plug formation in teat canal takes about 10 to 14 d during drying off of cows (Bitman et al., 1991; Williamson et al., 1995). Moreover, the invasion of pathogens into the mammary gland could occur multiple times after the intramammary infusion of antimicrobials.

Therefore, to investigate whether TC would maintain its effectiveness over a period of time in a milk environment, *Staph. aureus* was reinoculated into the milk samples on d 2, 4, and 6 of the experiment after the addition of TC on d 0. As observed in Figure 2, the antimicrobial effect of TC persisted for the duration of the experiment without any loss of activity. It was also found that TC added to milk on d 0 was effective in killing large populations of *Staph. aureus* inoculated multiple times on subsequent days.

Results from this study suggest that TC may be useful as an alternative or adjunct to antibiotics for use in DCT to control mastitis. In fact, given the antimicrobial activity documented herein, TC may also be useful in controlling mastitis during lactation. However, future experiments are needed to determine the pharmacoki-

netics of TC in bovine mammary gland studies, and to ascertain the in vivo efficacy of this molecule for treating bovine mastitis as an intramammary treatment, in addition to its potential effect on the mammary gland tissue.

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