Antibacterial Effect of Caprylic Acid and Monocaprylin on Major Bacterial Mastitis Pathogens

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ABSTRACT

Bovine mastitis is the most significant economic drain on the worldwide dairy industry. Concerns regarding poor cure rates, emergence of bacterial resistance, and residues in milk necessitate development of alternative therapeutic approaches to antibiotics for treatment of mastitis. A variety of free fatty acids and their monoglycerides have been reported to exert antimicrobial activity against a wide range of microorganisms. The objective of our study was to examine the efficacy of caprylic acid, a short-chain fatty acid, and its monoglyceride, monocaprylin, to inactivate common mastitis pathogens, including *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus*, and *Escherichia coli*. Milk samples containing 50 mM or 100 mM caprylic acid, and 25 mM or 50 mM monocaprylin were inoculated separately with a 3-isolate mixture of each of the 5 pathogens, and incubated at 39°C. Populations of surviving bacteria were determined at 0 min, 1 min, 6 h, 12 h, and 24 h of incubation. Both caprylic acid and monocaprylin reduced all 5 pathogens by >5.0 log cfu/mL after 6 h of incubation. Among the bacterial species tested, *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis* were most sensitive, and *E. coli* was most tolerant to caprylic acid and monocaprylin. Results of this study indicate that caprylic acid and monocaprylin should be evaluated as alternatives or adjuncts to antibiotics as intramammary infusion to treat bovine mastitis.

(Key words: caprylic acid, mastitis, monocaprylin)

Abbreviation key: DMSO = dimethyl sulfoxide, TSA = tryptic soy agar.

INTRODUCTION

Bovine mastitis is the single most important cause of economic loss to the dairy industry, and is character-

ized by inflammation of the mammary gland, usually caused by a microbial infection. Mastitis can lead to increased production costs due to culling, medication, discarded milk, delayed genetic progress, and reduced milk yield and milk quality, which combined lead to a huge economic drain on the industry (Natzke, 1981). Economic losses resulting from mastitis on a worldwide scale have been estimated to be approximately US\$35 billion (Wellenberg et al., 2002). The National Mastitis Council of the United States estimated the annual dollar loss to the dairy industry due to mastitis to be about US \$2 billion, which is equivalent to approximately \$180 per cow (Harmon, 1996). Based on the etiological agent involved, mastitis can be broadly classified into contagious mastitis (caused primarily by *Staphylococcus aureus* and *Streptococcus agalactiae*), and environmental mastitis (caused primarily by *Escherichia coli*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*; Riffon et al., 2001). Although contagious mastitis was responsible for majority of mastitis-related losses in the past, environmental pathogens have emerged as a major concern as well. Mastitis control programs against the latter have had limited success (Pyörälä, 2002; Erskine et al., 2003).

The most common treatment method for bovine mastitis is the intramammary infusion of antibiotics. However, the cure rates obtained with antibiotic therapy are generally poor against most mastitis pathogens. For example, the cure rates of *Staph. aureus* infections usually vary between 20 and 78% (Dingwell et al., 2003). Injudicious antibiotic therapy against bacterial diseases in cattle, including mastitis, has been recognized as a catalyst for emergence of antibiotic-resistant strains of bacteria (Berghash et al., 1983; Piddock, 1996; White, 1999). Moreover, use of antibiotics to treat bovine mastitis has been implicated as the most common source of harmful drug residues in milk (Erskine, 1996). Approximately 90% of the inhibitory residues detected in milk over a period of 5 yr in Michigan originated from antibacterial therapy for mastitis (Erskine et al., 2003). Because of the previous concerns, alternatives to antibiotic therapy are being explored in an attempt to evaluate their efficacy in treating intramam-

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mary infections. These include antibacterial teat seals (Woolford et al., 1998; Ryan et al., 1999), intramammary infusion of antimicrobials such as ozone (Ogata and Nagahata, 2000), and immune modulators like β 1,3-glucan (Persson et al., 2003).

Microbicidal activity of a variety of lipids, including free fatty acids and their esters, has been studied extensively in the past. Free fatty acids and their monoglycerides were found to be inhibitory towards an array of pathogenic microorganisms including enveloped viruses, gram-positive and gram-negative bacteria (Kabar, 1978; Isaacs et al., 1995; Petschow et al., 1996). Although a plethora of information is available on the antimicrobial properties of fatty acids, most research has focused on medium- and long-chain fatty acids (containing more than 10 carbons). Caprylic acid (octanoic acid) is an 8-carbon, short-chain fatty acid naturally present in breast milk, bovine milk, and coconut oil (Jensen et al., 1990; Sprong et al., 2001; Jensen, 2002). Caprylic acid and its monoglyceride, monocaprylin, were found to be effective in inactivating infant pathogens such as herpes simplex virus, respiratory syncytial virus, *Haemophilus influenzae*, and Group B streptococci (Isaacs et al., 1995).

The present study was carried out to evaluate the potential application of treating bovine intramammary infections by applying caprylic acid and monocaprylin to inactivate the common bacterial mastitis pathogens in milk.

MATERIALS AND METHODS

Maintenance and Preparation of Bacterial Cultures

Three isolates each of *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *Staph. aureus*, and *E. coli*, all isolated from clinical cases of bovine mastitis at the Diagnostic Testing Services Laboratory, University of Connecticut, were used in the study. All bacteriological media used in the study were purchased from Difco (Sparks, MD). Cultures were maintained on tryptic soy agar (TSA) slants at 4°C, and 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 for 24 h at 37°C. The cells were then sedimented by centrifugation (3600 $\times$ 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 population in the individual isolates and in the 3-isolate mixture of bacteria was determined by plating 0.1-mL portions of appropriate dilutions on 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.

Lipids

Caprylic acid and monocaprylin were purchased from Sigma (St. Louis, MO). The lipids were dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific Co., Pittsburgh, PA) before adding to milk to get a final concentration of 50 and 100 mM of caprylic acid, and 25 and 50 mM of monocaprylin. Concentrations of caprylic acid and monocaprylin were selected based on our previous research on the bactericidal concentration of these lipids on *E. coli* O157:H7 and *Listeria monocytogenes* in milk (Nair et al., 2004). The final concentration of DMSO in milk was always 2%, which did not affect bacterial viability (Isaacs et al., 1995).

Sample Preparation

Fresh, antibiotic residue-free, raw milk was collected from the bulk tank at the University of Connecticut dairy, and autoclaved at 121°C, 103.4 kPa of pressure for 15 min. Appropriate amounts of caprylic acid and monocaprylin dissolved in 200 μ L of DMSO were added to separate tubes containing 9.8 mL of whole milk to obtain the aforementioned final concentrations. All milk samples were inoculated separately with 100 μ L of the 3-isolate mixture of each pathogen to obtain an inoculation density of approximately 7.0 log cfu/mL. Control samples were prepared by inoculating 100 μ L of the bacterial culture into 9.8 mL of milk containing 200 μ L of DMSO (DMSO control) or no DMSO (milk control). All treatment and control milk samples were incubated at 39°C (normal body temperature of cattle).

Enumeration of Bacteria

Surviving populations of each bacterial pathogen were enumerated at 0 min, 1 min, 6 h, 12 h, and 24 h by plating 0.1-mL portions of the milk samples directly or after serial dilutions (1:10 in PBS) on duplicate TSA plates containing 0.6% yeast extract. When the pathogens under study were not detected by direct plating, the samples were tested for surviving bacteria by enriching 1 mL of milk sample in 100 mL of tryptic soy broth at 37°C for 24 h. When growth was observed in the broth, the culture was streaked on mannitol salt agar (*Staph. aureus*), sorbitol MacConkey agar (*E. coli*), or blood agar (streptococci) and observed for typical colonies of each pathogen. Duplicate samples were assayed for each treatment and control at each concentration at each specified sampling time, and the study was replicated 3 times.

Statistical Analyses

For each analysis, data from 3 independent replicate trials in duplicate were analyzed using the GLM procedure (SAS Inst, Inc., Cary, NC). For each pathogen (*Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *Staph. aureus*, and *E. coli*), the model included the 3 replicates, 5 treatments (control, caprylic acid at 50 and 100 mM, and monocaprylin at 25 and 50 mM), time (0, 1, 6, 12, and 24 h), and all the interactions of treatment and time. The model equation used was: model bacterial count = replicate, treatment, time, and treatment $\times$ time. The degrees of freedom associated with each source were as follows; replication = 2, treatment = 4, time = 4, time $\times$ treatment = 16, error = 123, and total = 149. Least significant difference test was used to determine significant differences ($P < 0.05$) with appropriate corrections for multiple comparisons due to replication, treatment, and time on bacterial counts for each pathogen.

RESULTS

Antimicrobial activity of caprylic acid and monocaprylin against the mastitis pathogens was evaluated in autoclaved milk because of the lack of an effective selective medium for specifically enumerating the various streptococci. The average pH of milk before adding caprylic acid or monocaprylin was 6.6. The pH of milk containing 2% DMSO, 25 mM or 50 mM of monocaprylin did not vary from that of normal milk. Addition of 50 and 100 mM of caprylic acid reduced the pH of milk to 5.51 and 5.4, respectively.

Caprylic acid and monocaprylin reduced ($P < 0.05$) the counts of all the 5 pathogens compared with control samples. Effect of caprylic acid and monocaprylin on the 3 streptococci species in milk was similar (Figures 1, 2, and 3). The average initial population of the pathogens at 0 min in all samples was approximately 7.0 log cfu/mL. Caprylic acid at 100 mM and monocaprylin at 50 mM killed the pathogens rapidly, reducing *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis* by approximately 5.0 log cfu/mL following 1 min of incubation. After 6 h of incubation, the population of the 3 species of streptococci in all treatment samples was reduced to less than 1.0 log cfu/mL (detected by enrichment), without substantial, further reduction after 24 h. In control samples containing no caprylic acid or monocaprylin, *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis* grew slightly by 6 h, with no further increase in their population at the end of 24 h (Figures 1, 2, and 3).

The effect of caprylic acid and monocaprylin on the viability of *Staph. aureus* in milk is depicted in Figure 4. Among the 4 treatments, 100 mM caprylic acid and

50 mM monocaprylin were most effective in reducing *Staph. aureus*. After 1 min of incubation, these 2 treatments reduced the population of *Staph. aureus* by >2 log cfu/mL. After 6 h, the population of *Staph. aureus* decreased by more than 5.0 log cfu/mL in all treatment samples, without further reduction after 24 h. In contrast, in the samples containing 100 mM caprylic acid or 50 mM monocaprylin, *Staph. aureus* was reduced to less than 1.0 log cfu/mL by 24 h (Figure 4). In the control samples devoid of caprylic acid or monocaprylin, the population of *Staph. aureus* increased to approximately 8.0 log cfu/mL after 24 h of incubation.

Similar to the results observed with streptococci and *Staph. aureus*, both caprylic acid and monocaprylin were effective in killing *E. coli* in milk (Figure 5). Among the 4 treatments tested, 100 mM caprylic acid and 50 mM monocaprylin were most effective, with the former reducing *E. coli* populations by approximately 5.0 log cfu/mL after 1 min of incubation (Figure 5). Both of these treatments decreased *E. coli* counts to approximately 1.0 log cfu/mL after 24 h of incubation, whereas in the milk samples containing less caprylic acid or monocaprylin, the pathogen tended to recover and grow back, resulting in a final count of approximately 2.0 log cfu/mL (Figure 5). The population of *E. coli* in the control samples increased by approximately 1.0 log cfu/mL after 24 h incubation. Throughout the study, the population of inoculated pathogens at any of the sampling periods did not differ between controls with and without 2% DMSO, indicating that DMSO had no inhibitory effect on bacterial cells at the concentration used. Because adding 100 mM caprylic acid reduced the pH of milk to 5.40, we determined the viability of the pathogens in milk at pH 5.40 to rule out any potential inhibitory effect of the low pH on the bacteria. Results revealed that none of the pathogens was inactivated in the milk samples at pH 5.4 after 24 h of incubation at 39°C (data not shown).

DISCUSSION

Although fatty acids have been exploited for centuries as antimicrobials in soaps, the antimicrobial properties of individual fatty acids received little attention before the 1930s (Sun et al., 2002). A substantial body of literature exists on the antimicrobial properties of free fatty acids and their esters in various synthetic laboratory media (Hogan et al., 1987; Wang and Johnson, 1992; Petschow et al., 1996), but their efficacy in killing bacteria in milk or in a menstruum with a high organic content is limited. For example, Wang and Johnson (1992) investigated the antimicrobial effect of a variety of fatty acids ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$), and monoglycerides of lauric acid (monolaurin),

Figure 1. Effect of caprylic acid and monocaprylin on *Streptococcus agalactiae* in milk containing (◆) 50 mM caprylic acid, (■) 100 mM caprylic acid, (▲) 25 mM monocaprylin, (●) 50 mM monocaprylin, (◇) no treatment (milk control), and (□) 2% dimethyl sulfoxide (DMSO control). Surviving bacterial populations (expressed in log cfu/mL) in milk samples stored at 39°C for 24 h were enumerated at specified time points on tryptic soy agar plates containing 0.6% yeast extract.

myristic acid, and palmitic acid against *Listeria monocytogenes* in brain-heart infusion broth and milk. These researchers found that although C_{12:0}, C_{14:0}, and monolaurin were bactericidal in brain-heart infusion broth,

none of the fatty acids or monolaurin was inhibitory on *L. monocytogenes* in milk. Reduced antimicrobial activity of fatty acids and monoglycerides observed in milk has been attributed to its complex composition

Figure 2. Effect of caprylic acid and monocaprylin on *Streptococcus dysgalactiae* in milk containing (◆) 50 mM caprylic acid, (■) 100 mM caprylic acid, (▲) 25 mM monocaprylin, (●) 50 mM monocaprylin, (◇) no treatment (milk control), and (□) 2% dimethyl sulfoxide (DMSO control). Surviving bacterial populations (expressed in log cfu/mL) in milk samples stored at 39°C for 24 h were enumerated at specified time points on tryptic soy agar plates containing 0.6% yeast extract.

Figure 3. Effect of caprylic acid and monocaprylin on *Streptococcus uberis* in milk containing (◆) 50 mM caprylic acid, (■) 100 mM caprylic acid, (▲) 25 mM monocaprylin, (●) 50 mM monocaprylin, (◇) no treatment (milk control), and (□) 2% dimethyl sulfoxide (DMSO control). Surviving bacterial populations (expressed in log cfu/mL) in milk samples stored at 39°C for 24 h were enumerated at specified time points on tryptic soy agar plates containing 0.6% yeast extract.

(Wang and Johnson, 1992). Proteins, especially lipophilic proteins such as albumin, and other nutrients, including fat and starch, interact with fatty acids and monoglycerides, thereby reducing their bioavailability

(Kabara, 1978). Glassman (1949) reported that proteins reduce the bactericidal activity of surface-active agents like monoglycerides due to the formation of lipid-protein complexes. However, results of this study indicate

Figure 4. Effect of caprylic acid and monocaprylin on *Staphylococcus aureus* in milk containing (◆) 50 mM caprylic acid, (■) 100 mM caprylic acid, (▲) 25 mM monocaprylin, (●) 50 mM monocaprylin, (◇) no treatment (milk control), and (□) 2% dimethyl sulfoxide (DMSO control). Surviving bacterial populations (expressed in log cfu/mL) in milk samples stored at 39°C for 24 h were enumerated at specified time points on tryptic soy agar plates containing 0.6% yeast extract.

Figure 5. Effect of caprylic acid and monocaprylin on *Escherichia coli* in milk containing (◆) 50 mM caprylic acid, (■) 100 mM caprylic acid, (▲) 25 mM monocaprylin, (●) 50 mM monocaprylin, (◇) no treatment (milk control), and (□) 2% dimethyl sulfoxide (DMSO control). Surviving bacterial populations (expressed in log cfu/mL) in milk samples stored at 39°C for 24 h were enumerated at specified time points on tryptic soy agar plates containing 0.6% yeast extract.

that both caprylic acid and monocaprylin were effective in killing the 5 mastitis pathogens in milk. Moreover, preliminary experiments conducted in our laboratory revealed that the antibacterial effect of caprylic acid and monocaprylin in raw and autoclaved milk did not differ significantly (data not shown). We have used DMSO as a solvent for monocaprylin in this study and have previously observed that in addition to DMSO, ethanol (2%) could also be used as an effective solvent for dissolving monocaprylin in milk (Nair et al., 2004). The pronounced antimicrobial effect of caprylic acid and monocaprylin on bacteria observed in the present study could be partly attributed to its short chain length. The antimicrobial property of fatty acids decreases with increasing chain length, with medium-chain fatty acids exhibiting stronger activity than longer-chain fatty acids (Wang and Johnson, 1992). Similarly, Isaacs et al. (1995) reported that fatty acids and monoglycerides containing 8 to 12 carbons were more antiviral and antibacterial than their longer-chain counterparts. Galbraith et al. (1971) noted that fatty acids must be in solution and remain sufficiently lipophilic to enable adsorption to bacterial cell surfaces for antibacterial activity. Caprylic acid being an 8-carbon, short-chain fatty acid, is less hydrophobic and potentially more soluble than medium-chain and long-chain fatty acids, and more effective in killing bacteria.

Among the 5 bacterial species tested in this study, *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis*

were most sensitive to caprylic acid and monocaprylin in milk. In general, caprylic acid at 100 mM and monocaprylin at 50 mM levels resulted in a rapid decline (4 to 6 log cfu/mL) in the populations of streptococci after 1 min of treatment. At the same time point, the effect of monocaprylin on *E. coli* and both caprylic acid and monocaprylin on *Staph. aureus* was less pronounced. Differences in the instantaneous antibacterial effect of these lipids on various pathogens could be attributed to the difference in their cell surface properties. Most mastitis-causing strains of *Staph. aureus* are surrounded by a layer of exopolysaccharide or pseudocapsule slime (Sutra et al., 1990), which could interfere with the lipids coming in direct contact with the bacterial cell membranes and exert their antimicrobial activity. The 3 *Staph. aureus* isolates used in the study were found to produce slime when grown on Congo red agar plates (data not shown). Similarly, Butcher et al. (1976) reported that long-chain fatty acids were generally more inhibitory to hemolytic streptococci than *Staph. aureus*. In comparison with the other 4 bacterial species, *E. coli* is a gram-negative mastitis pathogen, and differences in the cell wall structure could have contributed to the comparatively lesser effect of the lipids on *E. coli*. Other investigators also have reported that gram-negative bacteria are less sensitive to the antimicrobial effect of fatty acids and monoglycerides than gram-positive bacteria (Kabara, 1978; Monk et al., 1996).

Although the exact mechanism of action of caprylic acid and monocaprylin on bacteria is not known, numerous hypotheses have been suggested to explain the general mode of antimicrobial activity of free fatty acids and their monoglycerides. One theory maintains that monoglycerides as nonionic surfactants penetrate and are incorporated into bacterial plasma membrane, thereby altering membrane permeability. Electron microscopic studies with bacterial cells exposed to monoglycerides have shown plasma membrane disintegration leaving the peptidoglycan cell wall intact (Bergsson et al., 1998). Another school of thought hypothesizes that short- and medium-chain fatty acids diffuse into bacterial cells in their undissociated form and dissociate within the protoplasm, thereby leading to intracellular acidification (Sun et al., 1998). A lower intracellular pH can lead to inactivation of intracellular enzymes (Viegas and Sa-Correia, 1991), and inhibition of amino acid transport (Freese et al., 1973). At sublethal concentrations, monoglycerides such as monolaurin can interfere with bacterial signal transduction, and inhibit the expression of virulence factors and antibiotic resistance genes (Projan et al., 1994; Ruzin and Novick, 1998).

Emergence of antimicrobial resistant strains of animal pathogens and their potential health risk to humans through foodborne transmission have engaged the attention of the public and scientific community. Because of the extensive use of antibiotics, many gram-negative pathogens such as *E. coli* and *Salmonella* spp. were found to acquire multiple drug-resistance genes that restrict treatment options (White, 1999). The pressure to limit antibiotic use in food-producing animals is one of the biggest challenges for the dairy industry, even though antibiotics remain the most effective tool to combat mastitis (Bradley, 2002). Therefore, a need exists for an effective, alternative strategy for treating bovine mastitis that would reduce the use of antibiotics. Because fatty acids and their monoglycerides are believed to kill bacteria by multiple mechanisms, the potential for developing bacterial resistance to these molecules is relatively negligible. Bergsson et al. (1999) suggested that free fatty acids and monoglycerides exert their antimicrobial effect on bacterial biological membranes; thus, the chances of emergence of bacterial resistance are very unlikely. Petschow et al. (1996) observed relatively lower frequency of resistance development in *Helicobacter pylori* against C-10 and C-12 monoglycerides and C-12 fatty acids compared with other common antimicrobials tested. Fatty acids and their monoglycerides, being present in natural secretions such as milk are assumed, at least in smaller concentrations, to be nontoxic to mucosa (Bergsson et al., 1998). Caprylic acid is a food-grade chemical approved by the Food and Drug Administration as Gener-

ally Regarded as Safe (GRAS 184.1025). Toxicity studies have shown that caprylic acid is nontoxic even at large concentrations, with an oral LD₅₀ of 10.1 g of caprylic acid per kg of BW in rats (Jenner et al., 1964).

In conclusion, caprylic acid and monocaprylin at the concentrations tested were bactericidal against the major bovine mastitis pathogens *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *Staph. aureus*, and *E. coli* in milk. These results provide justification for evaluation of caprylic acid and monocaprylin as alternatives or adjuncts to antibiotics for treatment of bovine mastitis. Future experiments are needed, however, to ascertain the in vivo efficacy of these lipid molecules as an intramammary treatment of bovine mastitis, and their potential effects on the mammary gland tissue.

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