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Research article

Antibacterial properties of lauric acid in combination with organic acids against major pathogens causing dairy mastitis

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Abstract

The objective of this study was to determine the antibacterial properties of lauric acid in combination with acetic acid and lactic acid against major dairy mastitis pathogens including *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Escherichia coli* and *Klebsiella* spp. The antibacterial effect of each acid and the acid mixtures was evaluated by their minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) using broth microdilution method. The differences in, MIC and MBC values of lauric acid and acid mixtures for each pathogen were calculated by applying the one-way analysis of variance (ANOVA) and Turkey's multiple-range tests were used for pairwise comparison. Results demonstrated that acetic acid had the highest inhibitory and bactericidal effect against all tested pathogens with the lowest MIC and MBC values of 0.125% and 0.25-1 %w/v, respectively. The mixture of lauric acid with acetic and lactic acid exhibited significant higher inhibitory and bactericidal effects by having the lower MIC and MBC values against all tested pathogens when compared with lauric acid alone ($P < 0.05$). In conclusion, acetic and lactic acid can enhance antibacterial properties of lauric acid against major mastitis pathogens.

Keywords: Antibacterial properties, Acetic acid, Lactic acid, Lauric acid, Mastitis pathogens

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INTRODUCTION

Mastitis is the most costly disease in dairy industries worldwide (Halasa et al., 2007). It is usually caused by intramammary bacterial infection resulting in inflammatory reaction in individual udders (Piepers et al., 2013; Busanello et al., 2017; Petzer et al., 2017). The main or major pathogens causing mastitis that are of most concern to dairy farmers are contagious or environmental bacteria such as *Staphylococcus. aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli* and *Klebsiella* spp. (Cheng et al., 2019; Rysanek et al., 2009). To prevent and control mastitis, pre and post milking teat dipping containing various antiseptics are very important to minimize bacterial penetration into the teat canal and prevent new intramammary infection (Gleeson et al., 2018). With the emergence of bacterial resistance and the risk of residual problems of some antiseptic in milk, alternative teat dips containing medium chain fatty acids such as lauric acid have been widely distributed in the market. However, lauric acid has limited properties against bovine mastitis pathogens especially for Gram-negative bacteria (Fischer et al., 2012). Fitzpatrick et al. (2019) reported that the spectrum of antimicrobial activity of fatty acids can be broadened when combined with other substances such as acidulants (Batovska et al., 2009; Fitzpatrick et al., 2019). Organic acids including acetic and lactic acid are natural compounds that present in various food stuffs. They are widely used by the food industry as an antimicrobial and preservative to improve the microbiological safety of food products (Mani-López et al., 2012; Van Ba et al., 2018). Several reports show their broad spectrum to inhibit and eliminate several species of bacterial pathogens especially Gram-negative bacteria (Kovanda et al., 2019).

Therefore, the combination of these organic acids with lauric acid may lead to broadening the antibacterial properties of teat dipping formulations. Although the antibacterial properties of lauric acid combined with some organic acids have been reported, there are few reports on the combination effect of these acids against all major mastitis pathogens. In an effort to further develop the teat dipping of acid mixtures, the objective of this study was to determine the antibacterial properties of lauric acid in combination with acetic acid and lactic acid against major mastitis pathogens in vitro.

MATERIALS and METHODS

Preparation of acid solutions

Lauric acid (Sigma-Aldrich, St. Louis, USA) and organic acid such as acetic (C-2) (Alfa Aesar, Lancashire, UK), and lactic acid (Alfa Aesar, Lancashire, UK) used in this study were analytical grade. A stock solution of each acid was prepared by dissolving 2 g of the specified acid with 8 mL of 1:1 v/v mixture of 50% ethanol and 99.8% dimethylsulfoxide (DMSO) to yield the final concentration at 20% w/v. The stock solutions were used within 24 h after preparation.

Microorganisms

Mastitis pathogens used in this study included referenced strains of *S. aureus* ATCC25923 and *E. coli* ATCC25922 and five isolates of field strains of each pathogen of *S. aureus*, *S. agalactiae*, *S. uberis*, *E. coli* and *Klebsiella* spp. which belonged to the collection of the Faculty of Veterinary Medicine, Chiang Mai University. These pathogens were isolated from milk samples from bovine mastitis cases in Chiang Mai province. Their species were confirmed by MALDI-TOP Mass Spectrometry (Shimadzu, Tokyo, Japan). The selected strains were recovered from stock and maintained by subculture on Tryptone Soya agar (TSA; Himedia, Mumbai, India) and incubated at 37°C for 24 hrs. Bacterial suspension was prepared by suspending 3 to 4 fresh bacterial colonies in a NaCl solution (0.85% w/v) to achieve a turbidity between 0.5 on the McFarland scale (10^8 CFU/mL) before used for further antibacterial assay.

Antibacterial assay

Antibacterial properties of lauric acid in combination with acetic and lactic acid were determined by minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values using microdilution method following Clinical Laboratory and Standard Institute guidelines (CLSI, 2014). Each acid was dissolved in a solvent containing 50% ethanol 1:1 DMSO (80 mg/mL of the final concentration) and was then diluted with Muller-Hinton Broth medium (MHB; Himedia, Mumbai, India) to a concentration of 4% w/v. Further, two-serial dilutions were performed by the addition of culture broth to reach concentrations ranging from 0.004 to 4% w/v using 96-well plates. Each well was inoculated with 10 µl of bacterial suspension to yield a final inoculum size of 10^5 CFU/mL and then incubated at 37°C for 24 hrs. The MIC and MBC evaluations were performed in duplicate for each combination of bacteria and acids. The lowest concentration which inhibited visible growth of bacteria in broth was defined as MIC. For MBC, 10 µl of transparent mediums was smeared on TSA and incubated at 37°C for 24 hrs. The lowest acid concentration that inhibited the growth of bacteria on agar medium was defined as MBC.

Statistical analysis

The differences in, MIC and MBC values of lauric acid and acid mixtures for each pathogen were calculated by applying the one-way analysis of variance (ANOVA) and Turkey's multiple-range tests were used for pairwise comparison. The significant level was defined at $P < 0.05$.

RESULTS

According to the results obtained, lauric acid was found to be the most resistant to all bacterial strains especially for *S. aureus*, *E. coli* and *Klebsiella* spp. The MIC and MBC of lauric acid against all microorganisms were 0.5-4% and 2-4 %w/v, respectively (Table 1). Among all acids, acetic acid exhibited stronger inhibitory and bactericidal effects with the lowest MIC and MBC values of 0.125 %w/v, and 0.25 to 1%w/v, respectively. Considering the combination effect, the mixture of lauric with acetic acid and lauric with lactic acid exhibited higher inhibitory and bactericidal effects than lauric acid alone ($P < 0.05$) (Figure 1). In addition, different levels of bacterial sensitivity for the different pathogens were observed. It was found that Gram-negative bacteria especially *E. coli* and *Klebsiella* spp. were more resistant to the acid mixtures than other pathogens ($P < 0.05$) (Figure 2).

Table 1 The range of MIC and MBC values of lauric acid, acetic acid, and lactic acid against major mastitis pathogens

Major pathogens	N	MIC (%w/v)			MBC (%w/v)		
		Acetic acid	Lactic acid	Lauric acid	Acetic acid	Lactic acid	Lauric acid
S. aureus	6	0.125	0.25-0.50	4.00	0.50	0.50	4.00
S. agalactiae	5	0.125	0.25	0.50-1.00	0.25	0.25	4.00
S. uberis	5	0.125	0.50	1.00	0.25	0.50	2.00
E. coli	6	0.125	0.25-0.50	1.00	0.50	0.50	4.00
Klebsiella spp.	5	0.125	0.25-0.50	1.00-2.00	0.25-1.00	0.50-1.00	4.00

MIC: minimum inhibitory concentration

MBC: minimum bactericidal concentration

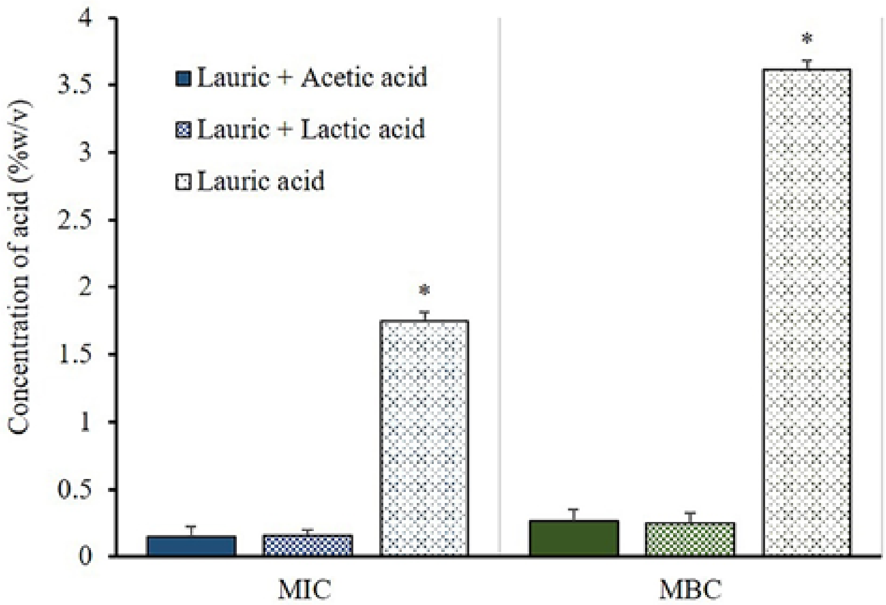

Figure 1 The antibacterial properties represent by MIC and MBC values of lauric acid and acid mixture (lauric+lactic acid, lauric+acetic acid) against all tested mastitis pathogens. * The means difference of MIC and MBC value of each acid types is significant at P<0.05.

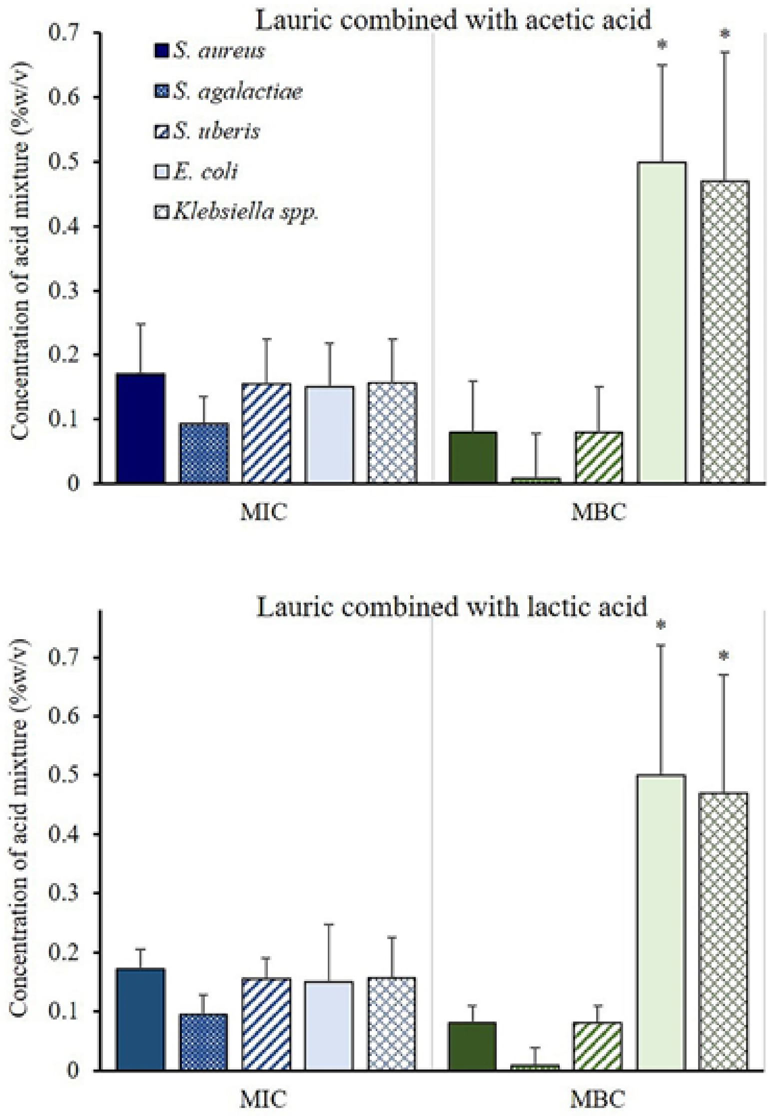

Figure 2 The antibacterial properties represent by MIC and MBC values of acid mixture (lauric+lactic acid, lauric+acetic acid) against five strains of mastitis pathogens. * The means difference among each bacterial strain is significant at P<0.05.

DISCUSSION

Lauric acid and its derivatives are commonly included in teat disinfectants for preventing intramammary infection and reducing the incidence of clinical mastitis caused by major pathogens (Boddie and Nickerson, 1992). This acid exhibits antimicrobial properties primarily against Gram-positive bacteria including *S. agalactiae* and *S. aureus* but is less effective against Gram-negative microorganisms (Boddie and Nickerson, 1992; Hovorková et al., 2018). However, our study found lauric acid gaining more resistance to all major mastitis pathogen especially for *S. aureus*, *E. coli* and *Klebsiella* spp. based on the highest MIC and MBC values. This finding is similar to previous studies which found that *S. aureus* and Gram-negative strains appeared to be generally less susceptible to fatty acids than streptococcal strains (Heczko et al., 1979; Piotr B. Heczko, 1979). The emergence of antimicrobial tolerance might be related to the routine exposure to sub-lethal concentrations of teat germicide and the ability of pH adaptation in each bacteria such as exopolysaccharides capsule or slime production of *S. aureus* (Baselga et al., 1994). In addition, our study found that acetic acid was the most active substance by having low values of MIC and MBC on all tested microorganisms both Gram-positive and Gram-negative pathogens followed by lactic acid. This can be explained by the low pKa properties of acetic and lactic acids producing more hydrogen ion when compared with lauric acid, resulting in more accumulation of toxic anion in bacterial cells (Alakomi et al., 2000; Kim and Rhee, 2013)

Several routine use of teat dips containing one mainly active ingredient as germicides tended to be more resistant against several bacteria species. Therefore, the combination of various substances for broadening or enhancing the antibacterial properties is widely applied in teat dip formulations. Several studies reported that the spectrum of antimicrobial properties of lauric acid can be broadened when combined with other substances such as organic acids (Boddie and Nickerson, 1992; Tangwatcharin and Khopaibool, 2012). Boddie and Nickerson (1992) also reported that the effects of lauric acid in combination with lactic acid were synergistic against *S. aureus* (Boddie and Nickerson, 1992). Similar to our study, lower MIC and MBC values showed a marked bacteriostatic and bactericidal synergistic effect with the combination of lauric acid with acetic and lactic acid, compared with lauric acid alone ($P<0.05$). This can be explained by lauric acid mainly disrupting the integrity of bacterial cells by damaging the cell membrane (Kim and Rhee, 2013). The membrane disruption accelerates the entry of acetic and lactic with direct and rapid influx into the intracellular resulting in altering the intracellular pH while enhancing the bacteriostatic and bactericidal properties (Desbois and Smith, 2010). Considering the sensitivity of each pathogen, it was found that *E. coli* and *Klebsiella* spp. were more resistant to the acid mixtures than others. The differences in susceptibility of *E. coli* and *Klebsiella* spp. are generally attributed to the complex structure of their cell wall disrupting the main mechanism of lauric acid to the damaged cell membrane, resulting in low cell permeability and a reduction in hydrogen ions passing into the cell, causing more resistance (Nair et al., 2005; Nobmann et al., 2010). Although Gram-negative bacteria has more resistance to acid mixtures, their antimicrobial activity was still greater than with lauric acid alone.

From all these results, it can be concluded that using acetic acid or lactic acid as a part of teat disinfectant combined with lauric acid can be beneficial by broadening antimicrobial properties for preventing and controlling mastitis in dairy cows. Further experiments are needed to optimize the formulation and concentration of acid mixture for teat-dipping agent, study their stability, toxicity, tolerance of the product on teat skin as well as evaluate the effectiveness of this formulation for prevention of new-intramammary infections in vivo.

CONCLUSION

In conclusion, acetic and lactic acid led to bacteriostatic and bactericidal synergistic effect with lauric acid against the major bovine mastitis pathogens including *S. aureus*, *S. agalactia*e, *S. uberis*, *E. coli* and *Klebsiella* spp. There can be alternative compositions in teat dipping formulations for preventing intramammary infection.

CONFLICT of INTEREST

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The authors declare that there is no conflict of interest.

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