Combined actions of virgin coconut oil, lauric acid and monolaurin with lactic acid on *Staphylococcus aureus*

Pussadee Tangwatcharin[1]* and Prapaporn Khopaibool[2]
[1] Faculty of Technology and Community Development, Thaksin University, Phatthulung Campus, Phatthalung, 93110, Thailand
[2] Department of Food Science and Technology, King Mongkut's Institute of Technology, Ladkrabang, Bangkok, 10520, Thailand
* Corresponding author (phone & fax: 1100-66-74-693996; e-mail: putang3009@hotmail.com)

***Abstract* - The objective of this study was to investigate the combined actions of virgin coconut oil, lauric acid and monolaurin with lactic acid or used either alone on two strains of *Staphylococcus aureus*, CH1 and CH2 strains, which were isolated from pig carcasses, by determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and fractional inhibitory concentration index (FICI). MIC of lauric acid, monolaurin and lactic acid were 1.6 and 0.1 mg/ml and 0.1% (v/v), respectively. MBC of antimicrobials were 3.2 and 0.1 mg/ml and 0.4% (v/v), respectively. The effects of lauric acid + lactic acid and monolaurin + lactic acid combinations were synergistic against both isolates which there were 0.3125 and 0.6250, respectively. In contrast, virgin coconut oil did not inhibit growth of both strains. No difference was found MIC, MBC and FICI for CH1 and CH2 strains.**

Index Terms* - Combination, lipid, antimicrobial agent, *Staphylococcus aureus

I. INTRODUCTION

Staphylococcus aureus, which is commonly found on the skin and mucous membranes of animals and humans, is involved in a wide variety of infections (Loir, Baron & Gautier, 2003). Several types of food have been implicated in food poisoning incidents attributed to *S. aureus*, most frequently meat and meat products (Smith, Buchanan & Palumbo, 1983). In fresh pork meat a prevalence of this bacterium as high as 62.5% has been reported (Atanassova, Meindl & Ring, 2001).

The problem of safe preservation in the meat industry has grown to be more complex as today's products require more safety and greater assurance of protection from pathogens. Of all the organic acids evaluated for their application as meat decontaminants, lactic acid is one of the most widely accepted (Huffman, 2002). There is, therefore, extensive information of the application of lactic acid to control both spoilage and pathogenic organisms in foods of animal origin. Virgin coconut oil is obtained from cold press processing the kernel from the fruit of the coconut tree. The most major fatty acid in virgin coconut oil is lauric acid (C_{12}, 51.05%). Certain fatty acids (medium chain saturates) and their derivatives have adverse effects on various microorganisms. The antimicrobial effect of fatty acids are additive and total concentration is critical for inactivating bacteria (Kabara, 1978). Different preparations of lauric acid protect the skin from bacterial infections (Kabara, 1990). Monolaurin is monoglycerol ester of lauric acid containing 12 carbon atoms, and are present in many animals and plants. It has been shown to possess wide-spectrum activity against bacteria (Anang, Rusul, Bakar & Ling, 2007). It blocks the production of various exoenzymes and virulence factors, including protein A, alpha-hemolysin, β-lactamase and toxic shock syndrome toxin 1 in *S. aureus* (Ruzin & Novick, 2000). Monolaurin is currently used as a GRAS (generally recognized as safe) food emulsifier, approved by the FDA, and is considered essentially a non-toxic compound even at relatively high dose levels (Kabara & Marshall, 2005).

The aim of this study was to compare antibacterial activity of virgin coconut oil, lauric acid, monolaurin and lactic acid used either alone or in combination on the growth of *S. aureus*, isolated from pig carcasses.

II. MATERIALS AND METHODS

Test strains. *S. aureus* CH1 and CH2, which were isolated from pig carcasses in the Southern Thailand abattoirs by the standard procedure (Bacteriological Analytical Manual, BAM online, 2001). The organisms were confirmed strain by Department of Medical Sciences, Ministry of Public Health of Thailand. These organisms were maintained on Mueller Hinton agar (MHA) (Merck, Germany). Overnight cultures were prepared by inoculating approximately 2 ml Mueller Hinton broth (MHB) (Merck, Germany) with 2-3 colonies taken from MHA. Broths were incubated overnight at 35°C. Inocula were prepared by diluting overnight culture in saline to 10^8 cfu/ml (McFarland standard of 0.5). These suspensions were further diluted with saline as required. The initial concentrations of approximately 5 x 10^5 cfu/ml were adopted for susceptibility test and synergy.

Antimicrobial agents. Virgin coconut oil (100%) was provided by Grand 4C Co., Ltd. (Bangkok, Thailand). Lauric acid and monolaurin were supplied by Sigma Adrich (Sigma, France). Lactic acid (80% (v/v), food grade) was obtained from Vichhi Enterprise Co., Ltd. (Bangkok, Thailand). Virgin coconut oil and lactic acid were diluted v/v in both agar disc diffusion and broth dilution methods but lauric acid and monolaurin were diluted mg/ml.

Susceptibility test methods. Susceptibility tests were performed by the disc diffusion method of Bauer, Kirby, Sherris & Turck (1966) with MHA. All agents were dissolved in sterile solution of 20% (v/v) dimethyl sulfoxide (DMSO, Merck, Germany) in water, except lactic acid was dissolved in distilled water, and subsequent two-fold serial

dilutions were performed in the culture medium. DMSO and distilled water was used as a negative control. Final concentrations of the test samples on disc ranged from 0-10% (v/v), 0-6.4, 0-1.6 mg/ml and 0-1.6% (v/v) for virgin coconut oil, lauric acid, monolaurin and lactic acid, respectively. Zones of inhibition were measured after 18 h of incubation at 35^{o}C.

The minimal inhibitory concentration (MIC) was determined by a broth microdilution method (CLSI M7-A4, 2002) for each strain. Serial two-fold dilution of the test substances were mixed with MHB in microtiter plates. Final concentrations of the test samples in broth ranged similarly in disc diffusion method. Add 20 µl of inoculum suspension in each well. The inoculated plates were incubated at 35^{o}C for 18 h. MIC was recorded as the lowest concentration that limited the turbidity of the broth to < 0.05 at absorbance of 600 nm by UVM 340 Microplate Reader (Biochrom Ltd., Cambridge, UK). Solvent controls were also included, though no significant effect on bacterial growth was observed at the highest concentration employed.

The minimal bactericidal concentration (MBC) was determined by comparing the number of remaining viable bacteria with the initial number of bacteria. All wells from the MIC experiments that showed no visible turbidity were serially diluted and spread onto MHA plates for viable cell counting. The plates were incubated for 24-48 h. MBC was then recorded as the lowest concentration that killed at least 99.99% of the initial number of bacteria. All MIC and MBC experiments were repeated three times.

Synergy methods. The methods of synergy of lauric acid and monolaurin with lactic acid were carried out by determining Fractional inhibitory concentration index (FICI) in MHB using checkerboard titration. Organisms were tested three times and the mean MIC and FICI was obtained to report the synergism. Synergy was FICI $\leq$ 0.5; partial synergy/addition was FICI > 0.5 to 1.0; indifference was FICI > 1.0 to <2.0; and antagonism was FICI $\geq$ 2.0 (Bharadwaj, Vidya, Dewan & Pal, 2003).

Statistical analysis. Data were presented as means and standard deviations. All statistical computations were performed to determine significant differences ($P < 0.05$) by ANOVA followed by Duncan's new multiple range test (SAS, 1998).

III. RESULTS AND DISCUSSION

Susceptibility test methods. The results of antimicrobial activity of the lipid and lactic acid tested by disc diffusion method against *S. aureus* CH1 and CH2 are given in Table 1. The lauric acid, monolaurin and lactic acid exhibited a favorable activity against the bacteria tested. The blind control (20% (v/v) DMSO or stilled water) and virgin coconut oil did not inhibit both isolates. They were inhibited at $\geq$ 1.6 mg/ml of lauric acid, $\geq$ 0.1 mg/ml of monolaurin and $\geq$ 0.1% (v/v) lactic acid which were MIC of antimicrobials. However, MBC of lauric acid and lactic acid against both strains were two and four-fold, respectively higher than the corresponding MIC which there were 3.2 mg/ml and 0.4% (v/v) (Table 2). In contrast, virgin coconut oil also did not inhibit growth of *S. aureus*. Finding of this study supports the observations of the other researchers about the efficacy of lauric acid, monolaurin and lactic acid in inhibiting the growth of food-related pathogens (Anang et al., 2007; Skřivanová, & Marounek, 2007). Kabara, Swieezkowski, Conley & Truant (1972) examined several specific straight-chain saturated fatty acids and found lauric acid to be one of the most potent bacteriostatic fatty acid when tested on gram-positive organisms. They also investigated the effect of esterification and found that monolaurin was the only monoacylglycerol more active than the free fatty acid form, similar to our results (Table 1) that monolaurin had lower MIC and MBC than lauric acid against both *S. aureus* strains. The efficacy of lauric acid was ca. 32 fold that of monolaurin. This could be due to the higher is hydrophobicity and accumulation of lauric acid into the membrane bilayer. This causes a change in the hydrogen bonding and the dipole-dipole interaction between acyl chains and, at high concentrations, cell inactivation by the disruption of the glycerophospholipids organization with the membrane (Bergsson, Arnfinnsson, Steingrìmsson & Thormar, 2001). Monolaurin is known to produce highly ordered membranes, which is thought to disrupt membrane function by affecting signal transduction due to blockage of promoters, uncoupling of energy systems, altered respiration, and altered amino acid uptake (Kabara & Marshall, 2005). For lactic acid, undissociated forms of organic acid penetrate the lipid membrane of bacterial cell and dissociate within the cell. As bacteria maintain a neutral pH of the cytoplasm, the export of excess protons consumes cellular ATP and results in depletion of energy, intracellular pH drop and cell death; causing a loss and change of the cytoplasm and a subsided of membrane (Doores, 2005). Eariler studies found MIC of lauric acid, monolaurin and lactic acid against *S. aureus* in a range of 0.050-0.498, 0.025-0.064 mg/ml and 0.000025-0.01% (v/v), respectively (Kabara et al., 1972; Vasconcelos de Oliveira, Stamford, Neto & Leite de Souza, 2010). These MIC of antimicrobials were lower than what we observed (1.6 mg/ml). This may be due to differences in the sensitivity of *S. aureus* strains to certain lipids (Kelsey, Bayles, Shafii & McGuire, 2006).

Synergy methods. FICI for the combined application of lauric acid and monolaurin with lactic acid on *S. aureus* strains are shown in Table 3. FICIs of the combined action of lauric acid and monolaurin with lactic acid were 0.3125 and 0.6250 for both strains suggesting a synergic and partial synergic, respectively, interaction of the assayed antimicrobial. Test strains presented capability to grow at sub-inhibitory concentrations (½MIC and ¼MIC) of all antimicrobials when applied alone (data not showed). Antimicrobial compounds, used as preservative in foods, often impart some flavor to products. Therefore, researchers have searched for optimized combinations of substances to reach antimicrobial efficacy at sufficient low concentration so as not to adversely affect the organoleptic acceptability of foods (Vasconcelos de Oliveira et al., 2010). At theory, synergy is found when the effect of the combined compounds is

greater than the sum of the individual effects (Gutierrez, Barry-Ryan & Bourke, 2008). For combinations between lauric acid and monolaurin with lactic acid, there was more antimicrobial activity when compared with antimicrobial alone. This could be due to lactic acid improved the uptake of lauric acid into the membrane, which probably affects membrane function and furthermore, leads to measurable synergism of the combined antimicrobial treatment (Oh & Marshall, 1994). Moreover, the antimicrobial synergy between monolaurin and lactic acid might be related to changes in both membrane function and fluidity (Tokarskyy & Marshall, 2008).

Table 1. Antimicrobial activity (zone of inhibition) of lipids and lactic acid against *S. aureus* strains

Concentrations of antimicrobial[1]	Zone of inhibition (mm)[2, 3, 4]					
	S. aureus CH1			*S. aureus* CH2		
	lauric acid	monolaurin	lactic acid	lauric acid	monolaurin	lactic acid
0.1	0.0 ± 0.00^{a}	7.5 ± 0.71^{a}	6.5 ± 0.00^{a}	0.0 ± 0.00^{a}	8.0 ± 1.41^{a}	7.0 ± 0.71^{a}
0.2	0.0 ± 0.00^{a}	13.5 ± 0.71^{b}	8.0 ± 0.00^{a}	0.0 ± 0.00^{a}	13.5 ± 0.00^{b}	8.0 ± 0.71^{a}
0.4	0.0 ± 0.00^{a}	14.0 ± 2.83^{b}	20.5 ± 1.41^{b}	0.0 ± 0.00^{a}	14.5 ± 0.71^{b}	21.0 ± 1.41^{b}
0.8	0.0 ± 0.00^{a}	18.0 ± 1.41^{c}	27.0 ± 1.41^{c}	0.0 ± 0.00^{a}	19.0 ± 1.41^{c}	27.0 ± 0.71^{c}
1.6	6.0 ± 1.41^{b}	18.5 ± 2.12^{c}	32.5 ± 0.71^{d}	6.0 ± 0.00^{b}	19.5 ± 2.83^{c}	33.0 ± 0.00^{d}
3.2	10.0 ± 0.00^{c}	ND[5]	ND	10.0 ± 0.00^{c}	ND	ND
6.4	10.5 ± 0.71^{c}	ND	ND	11.0 ± 0.71^{c}	ND	ND

[1] The units of antimicrobial are mg/ml for lauric acid and monolaurin and %(v/v) for lactic acid.

[2] The values (average of triplicate ± standard deviation) are diameter of inhibition zone at difference concentration of antimicrobials.

[3] [a-d] Different letters within each column indicate that values are significantly different ($P \leq 0.05$).

[4] The diameters of inhibition zone at difference concentration of virgin coconut oil against both *S. aureus* strains were 0.00 mm.

[5] ND, not detection.

Table 2. The MIC and MBC values[1] of oil and lactic acid against *S. aureus* strains

Antimicrobials[2]	*S. aureus* CH1		*S. aureus* CH2	
	MIC	MBC	MIC	MBC
virgin coconut oil	NI[3]	NI	NI	NI
lauric acid	1.6	3.2	1.6	3.2
monolaurin	0.1	0.1	0.1	0.1
lactic acid	0.1	0.4	0.1	0.4

[1] MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

[2] The units of antimicrobial are mg/ml for lauric acid and monolaurin and %(v/v) for virgin coconut oil and lactic acid.

[3] NI, not inhibition.

Table 3. FICI of the combined action of oil with lactic acid to *S. aureus* strains

Strains	Combinations of oil and lactic acid [1]	FICI	Interpretation
S. aureus CH1	lauric acid + lactic acid	0.3125	Synergy
	monolaurin + lactic acid	0.6250	Partial synergy
S. aureus CH2	lauric acid + lactic acid	0.3125	Synergy
	monolaurin + lactic acid	0.6250	Partial synergy

[1] The units of antimicrobial are mg/ml for lauric acid and monolaurin and %(v/v) for lactic acid.

IV. CONCLUSION

This study confirms that laurin acid, monolaurin, lactic acid and in combinations posses *in vitro* against *S. aureus*, isolated from pig carcasses. In combinations was more antibacterial activity than antimicrobial alone. However, if they are to be used for food or meat preservation purposes, issues of killing time, *in vivo* antimicrobial activity and sensory will need to be addressed.

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REFERENCES

Anang, D.M., Rusul, G., Bakar, J. & Ling, F.H. (2007). Effects of lactic acid and lauricidin on the survival of *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* O157:H7 in chicken breast stored at 4°C. *Food Control*, 18, 961-969.

Atanassova, V., Meindl, A. & Ring, C. (2001). Prevalence of *Staphylococcus aureus* and staphylococcal enterotoxin in raw pork and uncooked ham, a comparison of classical culturing detection and RFLP-PCR. *International Journal of Food Microbiology*, 68, 105-113.

Bacteriological Analytical Manual (BAM) Online. (2001). *Staphylococcus aureus*. U.S. Food and Drug Administration. Available at: http://www.cfsan.fda.gov/~ebam /bam-12.html. 9 Dec. 2006.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 45, 493-496.

Bergsson, G., Arnfinnsson, J.H., Steingrìmsson, O. & Thormar, H. (2001). Killing of Gram-positive cocci by fatty acids and monoglycerides. Acta Pathological Microbiological et Immunological Scandinavica, 109, 670-678.

Bharadwaj, R., Vidya, A., Dewan, B. & Pal, A. (2003). An *in vitro* study to evaluate the synergistic activity of norfloxacid and metronidazole. *Indian Journal of Pharmacology*, 35, 220-226.

CLSI. (2002). Reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. Clinical Laboratory Standards Institute, Wayne, Pa.

Doores, S. (2005). Organic acid. In P.M. Davidson, J.N. Sofos & J.I. Brannen, Antimicrobials in foods, 3rd ed (pp.91-142). Boca Raton, FL: CRC Press.

Gutierrez, J., Barry-Ryan, C. & Bourke, P. (2008). The antimicrobial efficacy of plat essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, 124, 91-97.

Huffman, R.D. (2002). Current and future technologies for the decontamination of carcass and fresh meat. *Meat Science*, 62, 285-294.

Kabara, J.J. (1978). Health oils from the tree of life. In J.J. Kabara, Pharmacological effect of lipids (pp. 624-629). London: AOCS Press.

Kabara, J.J. (1990). Lauric oils and health benefits. In J.J. Kabara, Pharmacological effect of lipids, 3rd ed (pp. 120-130). London: AOCS Press.

Kabara, J.J. & Marshall, D.L. (2005). Medium-chain fatty acids and esters. In P.M. Davison, J.N. Sofos & J.L. Brannen, Antimicrobials in food, 3rd ed (pp. 327-360). Boca Raton, FL: CRD Press.

Kabara, J.J., Swieezkowski, D.M., Conley, A.J. & Truant, J.P. (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrobiological Agents Chemotherapy*, 2, 23-28.

Kelsey, J.A., Bayles, K.W., Shafii, B. & McGuire, M.A. (2006). Fatty acid and monoacylglycerols inhibit growth of *Staphylococcus aureus*. *Lipids*, 41, 951-961.

Loir, Y.L., Baron, F. & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genetics Molecular Research*, 2, 63-76.

Oh, D.H. & Marshall, D.L. (1994). Enhanced inhibition of *Listeria monocytogenes* by glycerol monolaurate with organic acids. *Journal of Food Science*, 59, 1258-1261.

Ruzin, A. & Novick, R.P. (2000). Equivalence of lauric acid and glycerol monolaurate as inhibitors of signal transduction in *Staphylococcus aureus*. *Journal of Bacteriology*, 182, 2668-2671.

SAS. (1998). SAS/STAT User's Guide. Version 6.12. USA: Statistical Analysis Systems Institute Inc.

Skřirivanová, E. & Marounek, M. (2007). Influence of pH on antimicrobial activity of organic acids against rabbit enteropathogenic strain of *Escherichia coli*. *Folia Microbiology*, 52, 70-72.

Smith, J.L., Buchanan, R.L. & Palumbo, S.A. (1983). Effect of food environment on staphylococcal enterotoxin synthesis: A review. *Journal of Food Protection*, 46, 545-555.

Tokarskyy, O. & Marshall, D.L. (2008). Mechanism of synergistic inhibition of *Listeria moncytogenes* growth by lactic acid, monolaurin and Nisin. *Applied and Environmental Microbiology*, 74, 7126-7129.

Vasconcelos de Oliveira, C.E., Stamford, T.L.M., Neto, N.J.G. & Leite de Souza, E. (2010). Inhibition of *Staphylococcus aureus* in broth and meat broth using synergies of phenolics and organic acid. *International Journal of Food Microbiology*, 137, 312-316.