ACTIVITY OF VIRGIN COCONUT OIL, LAURIC ACID OR MONOLAURIN IN COMBINATION WITH LACTIC ACID AGAINST *STAPHYLOCOCCUS AUREUS*

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Abstract. The objective of this study was to investigate the *in vitro* activities of virgin coconut oil, lauric acid and monolaurin in combination with lactic acid against two strains of *Staphylococcus aureus*, ATCC 25923 and an isolate from a pig carcass, by determination of Fractional Bactericidal Concentration Index (FBCI), time-kill method, as well as scanning and transmission electron microscopy. Minimum bactericidal concentrations (MBC) of lauric acid, monolaurin and lactic acid were 3.2 mg/ml, 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 strains, exhibiting FBCIs of 0.25 and 0.63, respectively. In time-kill studies, lauric acid and monolaurin + lactic acid combinations added at their minimum inhibitory concentrations produced a bactericidal effect. The induction of stress in non-stressed cells was dependent on the type and concentration of antimicrobial. This resulted in a loss and change of the cytoplasm and membrane in cells of the bacterium. In contrast, virgin coconut oil (10%) was not active against *S. aureus*. The bacterial counts found in pork loin treated with lauric acid and monolaurin alone were significantly higher (p <0.05) than those treated with both lipids in combination with lactic acid at sub-inhibitory concentrations. The color, odor and overall acceptability of the pork loins were adversely affected by treatment with the three lipids and lactic acid alone but when combinations of the agents were used the sensory quality was acceptable.

Keywords: *Staphylococcus aureus*, virgin coconut oil, lauric acid, monolaurin, lactic acid, lipid, antimicrobial agent

INTRODUCTION

Staphylococcus aureus, commonly found on the skin and mucous membranes of animals and humans, is involved in a wide variety of infections (Loir *et al*, 2003). Several foods have been implicated in food poisoning incidents attributed to *S. aureus*, with meat and meat products being the most frequent vehicles of intoxication (Smith *et al*, 1983). In fresh pork meat a prevalence of this bacterium as high as 62.5% has been reported (Atanassova *et al*, 2001).

The problem of safe preservation in

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the meat industry has grown to be more complex as today's products require more safety and greater assurance of protection from pathogens. Many attempts have been made to control the growth of pathogens on the surface of meat and meat products with the use of chemical antimicrobials. Considering all the organic acids that have been evaluated for their application as meat decontaminants, lactic acid is among the most widely accepted (Huffman, 2002). There is, therefore, extensive information on the application of lactic acid to control both spoilage and pathogenic organisms in foods of animal origin. For example, significant inhibition of *S. aureus* growth was obtained by dipping or spraying meat with lactic acid solutions. However, it is difficult to stabilize preservatives on the surface of food due to evaporation, neutralization and diffusion into the matrix (Smulder and Greer, 1998).

Virgin coconut oil is obtained by cold press processing of the kernel from the fruit of the coconut tree. The major fatty acid in virgin coconut oil is lauric acid (C_{12}, 54.61%) (Pumedin Natural Products, 2010). Certain fatty acids (medium chain saturated fatty acids) and their derivatives have adverse effects on various microorganisms. The antimicrobial effects of fatty acids are additive and their total concentration is critical for inhibition of bacterial growth. The medium chain fats in lauric oils are comparable to fats in human milk and have similar nutraceutical effects. Preparations of lauric acid can protect the skin from bacterial infections (Kabara, 1978, 1990). Monolaurin is the monoglycerol ester of lauric acid and is present in many animals and plants. It has been shown to possess wide-spectrum activity against bacteria, fungi and viruses (Homung *et al*, 1994; Anang *et al*, 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* (Projan *et al*, 1994; Ruzin and Novick, 2000). Monolaurin is currently used as a GRAS (generally recognized as safe) food emulsifier, approved by the US Food and Drug Administration, and is considered essentially a non-toxic compound even at relatively high dose levels. It is however, insoluble in water and therefore must be dissolved in appropriate medium before its application. It has been investigated for its effect against both pathogenic and spoilage microorganisms in some foods and food processing surfaces. Furthermore, the inhibition produced by monolaurin is greatest at low pH (Bautista *et al*, 1993; Razavi-Rohani and Griffiths, 1994; Oh and Marshall, 1996; Kitahara *et al*, 2004; Kabara and Marshall, 2005; Kamdem *et al*, 2008; Zhang *et al*, 2009).

The individual effectiveness of lauric acid, monolaurin or lactic acid against foodborne pathogenic bacteria, has been examined (Skřivanová *et al*, 2006). The aim of this study was to compare the 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.

MATERIALS AND METHODS

Test strain

Staphylococcus aureus ATCC 25923 and an isolate from a pig carcass, CH1, were used in the present study. *S. aureus* ATCC 25923 was provided by the Department of Medical Sciences, Ministry of Public Health of Thailand. *S. aureus* CH1 (MSSA) was previously isolated from pig carcasses in southern Thailand abattoirs by the standard procedure (FDA, 2001)

and its identity was confirmed by the Department of Medical Sciences, Ministry of Public Health of Thailand. These organisms were maintained on Mueller Hinton agar (MHA) (Merck, Darmstadt, Germany). The overnight cultures were prepared by inoculating approximately 2 ml Mueller Hinton broth (MHB) (Merck, Darmstadt, 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. An initial concentration of approximately 5 x 10^5 CFU/ml was used for the susceptibility, synergy and kill-time tests.

Antimicrobial agents

Virgin coconut oil (100%) was provided by Grand 4C (Bangkok, Thailand). Lauric acid and monolaurin were supplied by Sigma Adrich (Lyon, France). Lactic acid 80% (v/v), food grade was obtained from Vichhi Enterprise (Bangkok, Thailand). For the agar disk diffusion and broth dilution assays, the concentrations of virgin coconut oil and lactic acid were assessed as % (v/v) but for lauric acid and monolaurin concentrations were measured as mg/ml.

Susceptibility test methods

Susceptibility tests were performed by the disk diffusion method of Bauer *et al* (1966) with MHA. All the antimicrobials were dissolved in sterile 20% (v/v) dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) in water, except for lactic acid, which was dissolved in distilled water. Subsequent two-fold serial dilutions were performed in culture medium that final concentration of DMSO was 2% (v/v). Both 20% (v/v) DMSO and distilled water were used as negative controls. Final concentrations of the test samples on disks ranged from 0 - 10%, 0 - 6.4 mg/ml, 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 hours of incubation at 35°C.

The minimal inhibitory concentration (MIC) was determined by a broth microdilution method (CLSI, 2002) for each isolate. Serial two-fold dilutions of the test substances were mixed with MHB in microtiter plates. The final concentrations of the inhibitors in the broth were the same as those used for the disk diffusion method. An aliquot (20 µl) of the inoculum suspension was added to each well. The inoculated plates were incubated at 35°C for 18 hours. The 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, 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 hours. The MBC was then recorded as the lowest concentration that killed at least 99.9% of the initial number of bacteria. All MIC and MBC experiments were repeated three times.

Synergistic effects

To determine whether lauric acid or monolaurin acted synergistically with lactic acid, the Fractional Inhibitory Con-

centration Index (FICI) and Fractional Bactericidal Concentration Index (FBCI) in MHB using checkerboard titration was estimated. The experiments were repeated three times and the mean MIC, MBC, FICI and FBCI were obtained. Synergy was indicated by an FICI and FBCI ≤ 0.5; partial synergy/additive effect was apparent when the FICI and FBCI ranged from > 0.5 to 1.0; an FICI and FBCI of > 1 to < 2 suggested that there was no interaction, and antagonism was exhibited when the FICI and FBCI was ≥ 2 (Bharadwaj *et al*, 2003; Doores, 2005; Gutierrez *et al*, 2008; Vasconcelos de Oliveira *et al*, 2010).

Determination of kill-time

The effect of the antimicrobial solutions alone (MBC) and in combinations of lauric acid and lactic acid (¼ MBC + 1/16 MBC; ⅛ MBC + ⅛ MBC; 1/16 MBC + ¼ MBC), and monolaurin and lactic acid (½ MBC + ⅛ MBC; ½ MBC + ¼ MBC; ⅛ MBC + ½ MBC) on the cell viability of *S. aureus* over 8 hours was evaluated by the viable cell count procedure. For this, 8 ml of MHB was inoculated with 1 ml of the bacterial inoculum of *S. aureus* CH1 and 1 ml of antimicrobial solution (final concentration shown above) were combined and gently shaken for 30 seconds. The resulting suspension was incubated at 35ºC (Vasconcelos de Oliveira *et al*, 2010). At different time intervals (0, 5, 10, 15, 30, 60, 120, 240 and 480 minutes), the cells that were capable to grow on solid selective media were enumerated using spread plate count on Baird-Parker agar (BP) containing 50 ml/l of egg-yolk tellurite emulsion and 0.05 g/l of sulphamethazine (Merck, Darmstadt, Germany) and MHA in order to determine the culturable and total culturable population, respectively. When the concentration of culturable cells was < 300 CFU/ml, a portion (0.1 ml) of each resuspension was plated onto BP and MHA. When the culturable cell counts were lower than the detection limit, culturability was assessed by plating 1 ml on BP and MHA. Two 0.3 ml aliquots or a 0.4 ml aliquot of each resuspension were added onto BP and MHA (Tangwatcharin *et al*, 2007). The cell numbers (CFU) were determined following incubation at 35ºC for 48 hours. The culturable and total culturable populations were determined by plating on BP and MHA, respectively. The densities of stressed and non-stressed cell sub-populations were determined using the assumptions and calculations described in these below equations (Thomas *et al*, 2002).

$$\text{Stressed cells} = \text{Total culturable cells} - \text{Culturable cells}$$

$$\text{Non-stressed cells} = \text{Culturable cells}$$

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

SEM and TEM were performed using a modification of the methods described by Chaveerach *et al* (2002), Tangwatcharin *et al* (2007) and Zakaria *et al* (2007). *S. aureus* CH1 samples for SEM and TEM were centrifuged at 16,000*g* for 5 minutes and the supernatant was discarded. The pellets were washed 3 times with Sorensen's phosphate buffer (SPB). The pellets were subsequently fixed in 2.5% glutaraldehyde in SPB for 1-2 hours and kept at 4ºC, followed by 1% osmium in SPB for 1-2 hours. The sample was washed 3 times with SPB between fixatives. The pellets were dehydrated by passage through a graded ethanol series [3 x 5 minutes each at 50, 70, 80, 90 and 95 and 2 x 10 minutes at 100% (v/v)] and then stored overnight. For SEM, the sample was dehydrated for critical point drying using a Polaron CPD 7501 (VG Microtech, E Sussex, UK). The

dried specimens were mounted onto a stub with double-sided carbon tape. The specimens were coated with a thin layer of gold by a Sputter Coater (SPI suppliers, PA, USA) prior to examination with a Quanta 400 scanning electron microscope (FEI, Brno, Czech Republic). For TEM, ethanol was replaced with propylene oxide, which was gradually replaced with Spurr's resin (Polysciences, Warrington, PA). Following polymerization, specimen blocks were thin sectioned (70 - 90 nm). Sections were stained with 5% uranyl acetate and 10% lead citrate for examination with a JEM-2010 transmission electron microscope (JEOL, MA) operated at 160 kV.

Meat model

Fresh pork loin was purchased from the local slaughter house of Phatthalung Province, Thailand. Meat pieces with a thickness of 1.5 cm, width of 5 cm, length of 7 cm and weight 50-70 g were prepared. After that, meat pieces were divided into 3 groups. Two groups were used to determine the effects of antimicrobials on total plate count (TPC) and sensory evaluation, for which the pieces were not inoculated with the bacterial suspension. The other group was used to determine the effect of antimicrobials on *S. aureus*, for which the pieces were inoculated with *S. aureus* CH1 suspension as follows: the pieces were individually submerged in 50 ml of the bacterial inoculum [*S. aureus* CH1 containing approximately 10^7 CFU/ml, prepared in sterile 0.85% (w/v) saline solution] for 10 minutes, air dried for 20 minutes in a bio-safety cabinet before washing with the antimicrobials. The initial count of *S. aureus* on each piece was approximately 10^5 CFU/g. The pieces were randomly divided into eight groups and immersed for 10 minutes as follows: (1) control – non treated; (2) dipped in sterile distilled water; (3) dipped in 10% virgin coconut oil; (4) dipped in lauric acid MBC solution; (5) dipped in lactic acid MBC, (6) dipped in lauric acid solution ($\frac{1}{16}$ MBC) + lactic acid ($\frac{1}{4}$ MBC); (7) dipped in monolaurin MBC solution; (8) dipped in monolaurin ($\frac{1}{2}$ MBC) + lactic acid ($\frac{1}{4}$ MBC). The sample meats were submitted to count for *S. aureus* (FDA, 2001) and TPC (Anang *et al*, 2010) according to standard procedures. The results were transformed to log CFU per gram of meat (log CFU/g). For sensory color, odor and overall acceptability scores, the meat samples were prepared approximately 1 hour before sensory evaluation. Forty students who have knowledge of food science and sensory quality of food were requested to score the color, odor and overall (general appearance color, and wetness or dryness, and odor) acceptability on the basis of a nine point hedonic rating scale. The scale was as follows: 1=extremely unacceptable, 2=very much unacceptable, 3=moderately unacceptable, 4=slightly unacceptable, 5=between acceptable and unacceptable, 6= lightly acceptable, 7=moderately acceptable, 8=very much acceptable and 9=extremely acceptable (Ranganna, 1994; Shestha and Min, 2006). All the samples were served in Petri dishes and were returned for further chemical analysis.

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 (Statistical Analysis System Institute, Version 6.12; Cary, NC).

RESULTS

Susceptibility test methods

The results of the antimicrobial activity of the lipid and lactic acid tested by the

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

Concentrations of antimicrobial[1]	Zone of inhibition (mm)[2, 3, 4]					
	S. aureus ATCC 25923			*S. aureus* CH1		
	Lauric acid	Monolaurin	Lactic acid	Lauric acid	Monolaurin	Lactic acid
0.1	0.0 ± 0.00[a]	8.0 ± 0.71[a]	7.0 ± 0.00[a]	0.0 ± 0.00[a]	7.5 ± 0.71[a]	6.5 ± 0.00[a]
0.2	0.0 ± 0.00[a]	13.5 ± 0.00[b]	8.0 ± 1.41[a]	0.0 ± 0.00[a]	13.5 ± 0.71[b]	8.0 ± 0.00[a]
0.4	0.0 ± 0.00[a]	14.5 ± 1.41[b]	21.0 ± 0.71[a]	0.0 ± 0.00[a]	14.0 ± 2.83[b]	20.5 ± 1.41[b]
0.8	0.0 ± 0.00[a]	19.0 ± 0.71[c]	27.5 ± 0.71[c]	0.0 ± 0.00[a]	18.0 ± 1.41[c]	27.0 ± 1.41[c]
1.6	6.0 ± 0.71[a]	19.0 ± 2.83[c]	33.0 ± 0.00[d]	6.0 ± 1.41[b]	18.5 ± 2.12[c]	32.5 ± 0.71[d]
3.2	10.0 ± 0.71[c]	ND[5]	ND	10.0 ± 0.00[c]	ND	ND
6.4	11.0 ± 1.41[c]	ND	ND	10.5 ± 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 different concentration of antimicrobials.
[3] [a-d]Different letters within each column indicate that values are significantly different ($p < 0.05$).
[4] The diameters of inhibition zone at different concentration of virgin coconut oil against both *S. aureus* strains were 0.00 mm.
[5] ND, no detection.

Table 2
The MIC and MBC values of lipid and lactic acid against *S. aureus*.

Antimicrobials [a]	*S. aureus* ATCC 25923		*S. aureus* CH1	
	MIC	MBC	MIC	MBC
Virgin coconut oil	NI	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

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; NI, no inhibition.
[a]The units of antimicrobial agents are mg/ml for lauric acid and monolaurin and %(v/v) for virgin coconut oil and lactic acid.

disk diffusion method against *S. aureus* ATCC 25923 and CH1 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 distilled water] and virgin coconut oil did not inhibit the isolate. The MICs were determined to be 1.6 mg/ml for lauric acid, 0.1 mg/ml for monolaurin and 0.1% (v/v) for lactic acid. However, the MBC of lauric acid and lactic acid against both strains were two- (3.2 mg/ml) and four-fold [0.4% (v/v)], respectively, higher than the corresponding MIC (Table 2).

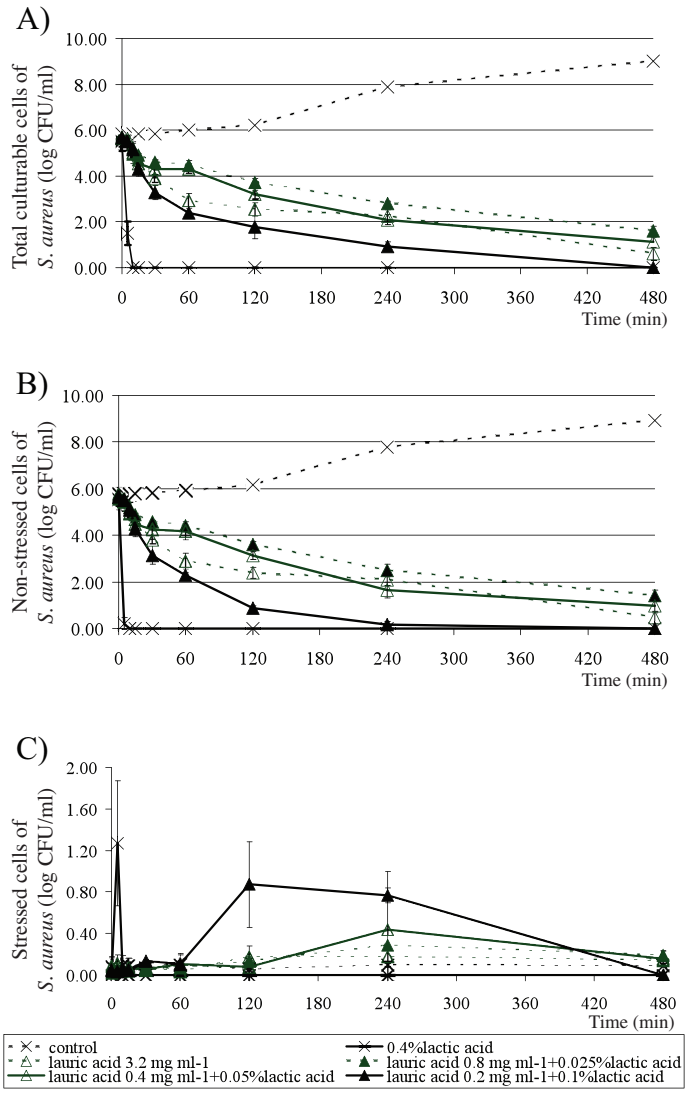

Fig 1–Survival curves for (A) total culturable, (B) non-stressed and (C) stressed cells of *S. aureus* CH1 in MHB at 35ºC as a function of lauric acid alone and in combinations.

The FICI for the combined application of lauric acid and monolaurin with lactic acid on *S. aureus* is shown in Table 3. FICIs of the combined action of lauric acid and monolaurin with lactic acid were 0.31 and 0.63 for both strains suggesting synergy and partial synergy, respectively, of the assayed antimicrobials. Similarly, FBCIs of the combined action of lauric acid and monolaurin with lactic acid were 0.25 and 0.63 for both strains again suggesting synergy and partial synergy, respectively. The test strain was able to grow at sub-bactericidal concentrations (½ MIC and ¼ MIC) of all antimicrobials when applied alone (data not shown).

Time-kill

To determine the rates at which bacteria were killed, *S. aureus* CH1 was exposed to lauric acid, monolaurin and lactic acid alone and in combination in MHB (Figs 1 and 2, respectively). Addition of lauric acid (3.2 mg/ml) and monolaurin (0.1 mg/ml) to broth caused a sharp drop in the total and non-stressed bacterial counts after 120 minutes, and values under two log cycles were maintained for the remainder of the time studied. Non-stressed cells were induced to mostly stressed cells ($p < 0.05$) during the incubation period. Lactic acid proved to be more effective against *S. aureus* in MHB than the three lipids ($p < 0.05$). Lactic acid caused a linear decrease of total and non-stressed bacterial counts until 10 minutes, after which the bacteria could not be cultured. Moreover, stressed cells became the largest proportion of the population after 5 minutes ($p < 0.05$). Virgin coconut oil had little effect on total and non-stressed bac-

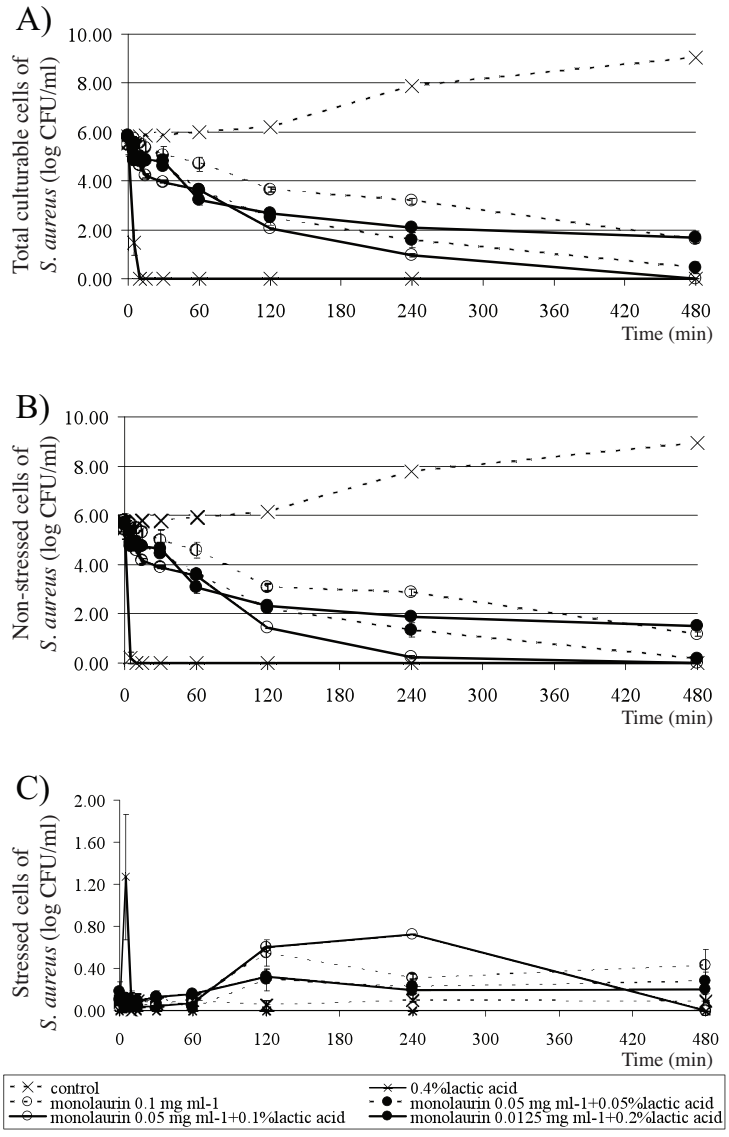

Fig 2–Survival curves for (A) total culturable, (B) non-stressed and (C) stressed cells of *S. aureus* CH1 in MHB at 35°C as a function of monolaurin alone and in combinations.

terial counts until 240 minutes, and subsequently, bacterial counts increased by one log cycle after a further 240 minutes. After an incubation period of 480 minutes the combination of lauric acid and monolaurin with lactic acid at sub-bactericidal concentrations reduced counts by greater than two log cycles compared with the initial total and non-stressed bacterial load. The bacterial counts found in MHB containing lauric acid and monolaurin alone were significantly higher ($p < 0.05$) than the counts obtained for the broth to which had been added the mixture of $\frac{1}{16}$ MBC lauric acid (0.2 mg/ml) and $\frac{1}{4}$ MBC lactic acid [0.1% (v/v)] and the mixture of $\frac{1}{2}$ MBC monolaurin (0.05 mg/ml) and $\frac{1}{4}$ MBC lactic acid [0.05% (v/v)].

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

Cells treated with virgin coconut oil, lauric acid, monolaurin and lactic acid alone and in combination underwent considerable morphologic alterations in comparison with the control when studied by SEM and TEM (Figs 3 and 4, respectively). Untreated cells (control) appear as coccoid and smooth (Figs 3A and 4A). For treated cells, there was some loss and change of the cytoplasm in cells of the bacterium following exposure to lactic acid (Figs 3C and 4C, respectively). For lauric acid and monolaurin, some membrane leakage was observed (Figs 3E, 3G, 4E and 4G). For virgin coconut oil (Figs 3B and 4B), the membrane and cytoplasm of cells were not different from untreated

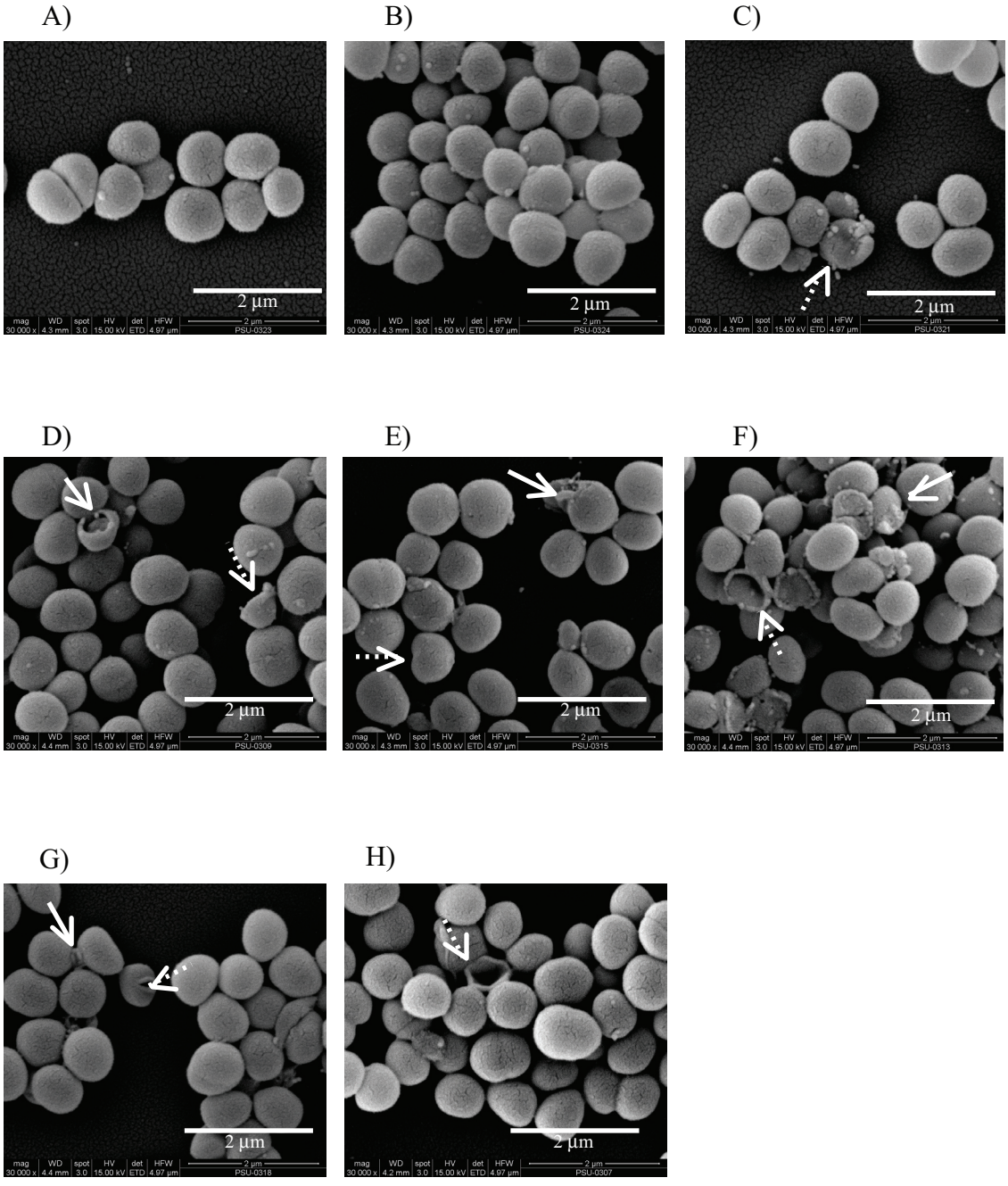

Fig 3–Scanning electronmicrographs of *S. aureus* CH1 in MHB containing antimicrobials: (A) control, (B) 10% virgin coconut oil, (C) 0.4% lactic, (D) 10% virgin coconut oil + 0.4% lactic acid, (E) 3.2 mg/ml of lauric acid, (F) 0.2 mg/ml of lauric acid + 0.1% of lactic acid, (G) 0.1 mg/ml of monolaurin and H) 0.05 mg/ml of monolaurin + 0.1% lactic acid at 35ºC for 120 minutes, except (C) and (D) for 10 minutes. Cell membranes were disturbed and leaked (solid arrow) and subsided (hatched arrow). Bars = 2 µm.

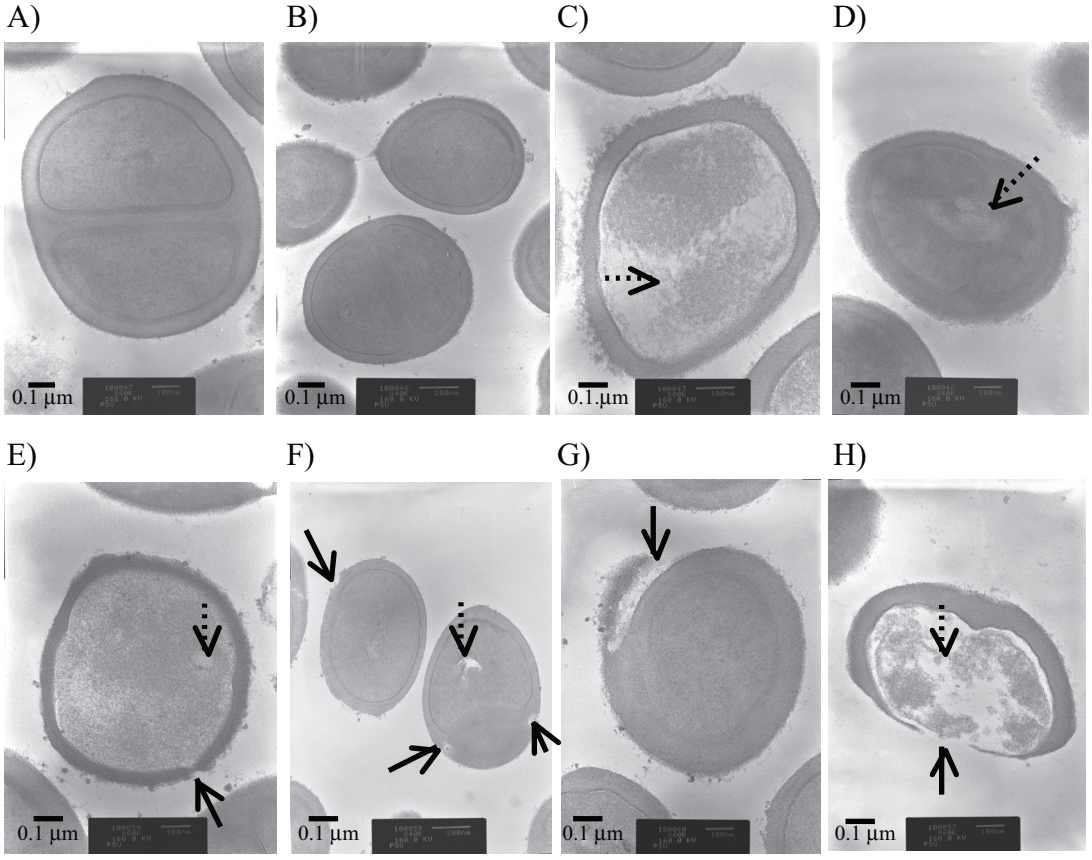

Fig 4–Transmission electronmicrographs of *S. aureus* CH1 in MHB containing antimicrobials: (A) control, (B) 10% virgin coconut oil, (C) 0.4% lactic, (D) 10% virgin coconut oil + 0.4% lactic acid, E) 3.2 mg/ml of lauric acid, F) 0.2 mg/ml of lauric acid + 0.1% of lactic acid, (G) 0.1 mg/ml of monolaurin and (H) 0.05 mg/ml of monolaurin + 0.1% lactic acid at 35ºC for 120 minutes, except (C) and (D) for 10 minutes. Cell membranes were disturbed and leaked (solid arrow) and subsided (hatched arrow). Bars = 0.1 mm.

cells. In all cells exposed to the combinations of antimicrobials, the cytoplasm was disorganized and the integrity of the membrane was compromised (Figs 3D, 3F, 3H, 4D, 4F and 4H).

Meat model

Table 4 presents the microbial loading and sensory attributes of fresh pork loin treated with the antimicrobials. The results show that there is no significant difference ($p > 0.05$) in *S. aureus* counts among meat pieces that were untreated (control), treated with water and 10% virgin coconut oil; counts ranged between 5.33 and 5.43 log CFU/g. Treatments with lauric acid, monolaurin and lactic acid alone and in combinations were more effective in limiting the growth of *S. aureus* compared to control ($p < 0.05$). Dipping meat pieces in the mixture of $^1/_{16}$ MBC lauric acid (0.2 mg/ml) and $^1/_4$ MBC lactic acid [0.1% (v/v)] caused the greatest reduction in *S. aureus*; resulting in a maxi-

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

Strains	Combinations of oil and lactic acid	FICI	Interpretation
S. aureus ATCC 25923	Lauric acid + lactic acid	0.3125	Synergy
	Monolaurin + lactic acid	0.6250	Partial synergy
S. aureus CH1	Lauric acid + lactic acid	0.3125	Synergy
	Monolaurin + lactic acid	0.6250	Partial synergy

Table 4
Effect of the lipid and lactic acid on microbial loading and sensory attributes of fresh pork loin.

Antimicrobials	Microbial loading (log CFU/g)[1,2]		Sensory attributes[1,2]		
	S. aureus	TPC	Color	Odor	Overall
Control	5.43±0.17^{a}	3.91±0.11^{a}	7.87±0.15^{a}	7.97±0.15	7.97±0.12^{a}
Water	5.36±0.13^{a}	3.78±0.10^{b}	7.83±0.15^{a}	7.93±0.25	7.90±0.10^{ab}
10%VCO	5.33±0.16^{a}	3.69±0.10^{b}	7.80±0.10^{ab}	7.90±0.10	7.80±0.10^{b}
0.4%LA	3.91±0.17^{d}	2.87±0.08^{f}	7.38±0.28^{d}	7.90±0.10	7.50±0.17^{c}
Lau 3.2 mg/ml	4.59±0.33^{b}	3.25±0.11^{cd}	7.77±0.15^{ab}	7.87±0.06	7.83±0.12^{ab}
Lau 0.2 mg/ml +0.1%LA	4.40±0.28^{c}	3.07±0.16^{e}	7.53±0.12^{c}	7.83±0.06	7.77±0.15^{b}
ML 0.1 mg/ml	4.67±0.28^{b}	3.32±0.11^{c}	7.80±0.10^{ab}	7.90±0.17	7.83±0.12^{ab}
ML 0.05 mg/ml +0.1%LA	4.42±0.29^{c}	3.14±0.11^{de}	7.67±0.12^{b}	7.87±0.21	7.80±0.17^{b}

VCO, virgin coconut oil; Lau, lauric acid; ML, monolaurin; LA, lactic acid
[1] Values correspond to mean ± standard deviation.
[2] $^{a-f}$ With different letters in columns are significantly different ($p < 0.05$).

mum reduction in count of *S. aureus* of 1 log CFU/g. Similarly, treating meat pieces with the mixture of lauric acid and lactic acid caused a significant reduction in TPC. In addition, dipping meat pieces in lipid alone resulted in a lower reduction in TPC than that observed for combinations of lipid and lactic acid ($p < 0.05$). Sensory analysis of fresh pork, including color, odor and overall acceptability scores, revealed that the use of lactic acid alone and in combinations reduced the color and overall acceptability ($p < 0.05$). However, the color, odor and overall acceptability of all samples were satisfactory; exhibiting scores in a range of 7.38-7.87, 7.83-7.97 and 7.50-7.97, respectively (Table 4).

DISCUSSION

The findings of this study are in accordance with those of other researchers for the efficacy of lauric acid, monolaurin and lactic acid in inhibiting the growth of food-related pathogens (Anang *et al*, 2007; Skřivanová and Marounek, 2007; Kamdem *et al*, 2008). It has long been known that lipids have an inhibitory effect on bacteria (Bayliss, 1936; Kodicek, 1949). Kabara *et al* (1972) examined several

straight-chain saturated fatty acids and found lauric acid to be one of the most potent bacteriostatic fatty acids 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 results were noted in the present study (Tables 1 and 2), in which monolaurin had lower MIC and MBC than lauric acid against *S. aureus*. The efficacy of lauric acid was approximately 32 fold that of monolaurin. Earlier studies found MIC of lauric acid, monolaurin and lactic acid against *S. aureus* in the ranges of 0.050 - 0.498 mg/ml, 0.025 - 0.064 mg/ml and 0.000025 - 0.01% (v/v), respectively (Kabara *et al*, 1972; Kelsey *et al*, 2006; Kitahara *et al*, 2006; Chaikulsareewath and Leekokseng, 2010; Vasconcelos de Oliveira *et al*, 2010). These MICs were lower than those observed in our study [1.6 mg/ml, 0.1 mg/ml and 0.1% (v/v), respectively]. This may be due to differences in the sensitivity of *S. aureus* strains to certain lipids (Kelsey *et al*, 2006). However, the MIC of monolaurin against *S. aureus* ATCC 25923 in our study (0.1 mg/ml) was similar to that observed by Smith *et al* (2008).

In addition, we have demonstrated that virgin coconut oil cannot inhibit growth of *S. aureus* (Tables 1 and 2) and the morphology of *S. aureus* cells exposed to the oil (Figs 3B and 4B) is not different from that of untreated cells (Figs 3A and 4A). Virgin coconut oil contained lauric acid at a concentration of 0.47 mg/ml (data not shown), which is well below the MIC of lauric acid (1.6 mg/ml) (Table 1). However, 10% (v/v) virgin coconut oil may extend the lag phase of *S. aureus* CH1 to about 240 minutes (data not shown). Therefore, the efficacy of virgin coconut oil as an antimicrobial should be investigated more in the future.

Lauric acid and monolaurin were reported to have higher antimicrobial activities against gram-positive bacteria than gram-negative bacteria, although most organisms are resistant to low concentrations of saturated fatty acids (Kitahara *et al*, 2004). Our study suggests that lauric acid and monolaurin inhibit the growth of *S. aureus* (Figs 1 and 2, respectively). This could be due to the high hydrophobicity that lauric acid presents and its accumulation 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 is achieved due to the disruption of the glycerophospholipid organization within the membrane (Bergsson *et al*, 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 and Marshall, 2005). A previous study demonstrated that monolaurin caused a constant increase in leakage of *S. aureus* CMCC(B) 26003 membrane to 91.6% over a period of 60 minutes (Zhang *et al*, 2009). Leakage of cell membranes was observed in this study (Figs 3E, 3G, 4E and 4G). For lactic acid, the bactericidal effect at MBC [0.4% (v/v)] required an exposure time of at least 10 minutes (Fig 1). This could be due to undissociated forms of organic acid, which penetrate the lipid membrane of the bacterial cell and dissociate within the cell. As the bacterial cytoplasm needs to be maintained at neutral pH, the excess export of protons results in consumption of cellular ATP and subsequent depletion of energy, with the intracellular pH

becoming more acidic. This results in loss and change of the cytoplasm, a loss of membrane integrity and concomitant cell injury and death (Ricke, 2003; Doores, 2005). Changes in cell activity and morphology associated with these events were observed in our study (Figs 1, 3C and 4C). For combinations between lauric acid and monolaurin with lactic acid (Figs 1, 2, 3H, 3F, 4H and 4F), the antimicrobial activity was higher when compared with that of the antimicrobials alone. This could be due to the presence of lactic acid improving 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 and Marshall, 1994). Moreover, the antimicrobial synergy (Gutierez *et al*, 2008) between monolaurin and lactic acid might be related to changes in both membrane function and fluidity (Tokarckyy and Marshall, 2008). For these reasons, the stressed bacterial counts found for MHB were higher when the lauric acid and monolaurin were added with lactic acid in the broth, than when they were added alone (Figs 1C and 2C; Table 3).

Previous studies have shown that the transition from non-stressed to stressed cells depends on many factors (such as pH, preservative, medium, etc). *S. aureus* is killed at pH 2, but is protected from killing by pre-exposure to pH 4 via a *sigβ*-dependent mechanism. These changes result in the adaptation of stressed cells (Netten *et al*, 1998; Gustafson, 2005), as was observed in this study (Fig 1C). The pH values ranged between 5.45 and 6.98 in the presence of lauric acid in combination, and between 5.41 and 7.00 for monolaurin in combination. The pH of the medium was 3.70, 6.80, 7.04 and 7.59 when lactic acid, virgin coconut oil, lauric acid and monolaurin, respectively, was present alone.

The inhibitory effect of lauric acid, and monolaurin against *S. aureus* in food has been reported previously (Razavi-Rohani and Griffiths, 1994) and other reports have demonstrated antilisterial effects of these lipids in food (Wang and Jonhson, 1992; Stecchini *et al*, 1996; Mbandi *et al*, 2004; Anang *et al*, 2007). Antimicrobial activity was demonstrated in skimmed and chocolate milk, but not in whole milk. This effect was attributed to the sequestration of monolaurin by fat globules or lipophilic proteins (Wang and Jonhson, 1992). The activities of monolaurin and lauric acid are also reduced or affected by the presence of carbohydrate and protein materials (Kato and Shibasaki, 1975). This may partly explain the reduced effect of monolaurin and lauric acid in pork loin, which is a high protein food. As reported by Anang (2007), the composition of meat can affect the inhibitory activity of monolaurin.

The most widely used chemical decontaminants in the meat industry are organic acids (Belk, 2001). 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 on the application of lactic acid to control *S. aureus* in meat. In the present study, treating pork loin with 0.4% (v/v) lactic acid (25 °C) for 10 minutes lead to a 1 log reduction in *S. aureus* counts. Netten *et al* (1998) similarly obtained a 1 log reduction of *S. aureus* when a pork belly cut was treated with 2% (v/v) lactic acid (37°C) for 120 seconds.

Results of the present study show that a concentration equal to the MBC of lactic acid [0.4% (v/v)] was the most effective against *S. aureus* and TPC compared to

other antimicrobials. However, the bacterial counts found in pork loin treated with lauric acid and monolaurin alone were significantly higher ($p < 0.05$) than the counts obtained for pork loin treated with lauric acid or monolaurin in combination with lactic acid at sub-inhibitory concentrations (Table 4).

Antimicrobial compounds, used as preservative in foods, often impart some flavor to products. Therefore, it is advisable to determine antimicrobial efficacy at sufficiently low concentrations so as not to adversely affect the organoleptic acceptability of foods (Bautista *et al*, 1993). Bautista *et al* (1993) observed that trained panelists were unable to distinguish difference in organoleptic traits of cottage cheese stored at 6ºC with different concentrations of monolaurin (0, 0.25 and 0.50 mg/g) over a storage period of 11 days. It is likely that the concentrations of antimicrobials found to be effective in our study would produce noticeable changes in the sensory properties of fresh pork (Table 4). However, the color and overall acceptability scores of the lactic acid treated samples were lower than those of other treated samples but scores for all treated samples were more than 7 (moderately acceptable). At low lactic acid concentrations, the discoloration effect was small (Smulders, 1987) and when 2% (v/v) lactic acid was sprayed on deboned meat there was no noticeable bleaching effect (Smulder and Woolthus, 1985).

In summary, this study confirms that lauric acid, monolaurin, lactic acid alone and in combination exhibit *in vitro* antimicrobial effects against *S. aureus*, isolated from pig carcasses. There was a synergistic effect of lactic acid in the presence of lauric acid and monolaurin, and this is thought to be due to increased uptake of the fatty acids into the membrane, resulting in membrane disruption. When the antimicrobial treatment was applied to fresh pork there was no loss of color or adverse odor and the overall acceptability scores remained satisfactory. However, if they can be used for food or meat preservation, issues of *in vivo* antimicrobial activity and sensory effects during storage will need to be addressed.

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