

Activity of organic acid salts in combination with lauric arginate against *Listeria monocytogenes* and *Salmonella* Rissen

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ABSTRACT: The objective of this study was to investigate the in vitro activities of sodium diacetate (SD), sodium citrate (SC), or sodium lactate (SL) in combination with lauric arginate (LAE) against *Listeria monocytogenes* and *Salmonella* Rissen. Bacteria isolated from a pig carcass were used to determine the minimum inhibitory and bactericidal concentrations (MIC and MBC), fractional bactericidal concentration index (FBCI), time-kill method, as well as to perform scanning and transmission electron microscopy characterizations. For *L. monocytogenes*, the MBC of SD, SC, LAE and SL were 62.5, 62.5, 0.032 mg/ml, and 8.4% (v/v), respectively. As for *S. Rissen*, the MBC of SD and LAE were 62.5 and 0.032 mg/ml, respectively. The effects of SD, SC, and SL in combination with LAE were synergistic against both bacteria, exhibiting FBCIs of 0.19, 0.50, and 0.50, respectively, for *L. monocytogenes* and 0.19, ≤ 0.50 and ≤ 0.50 , respectively, for *S. Rissen*. In time-kill studies, all salts of organic acids plus LAE combinations added at their MBC produced a bactericidal effect that was dependent on the type of bacteria and antimicrobial. This resulted in a loss and change of the cytoplasm and membrane in cells of both bacteria. Furthermore, SC and SL alone were not active in killing *S. Rissen*. The present investigation revealed that salts of organic acid in combination with LAE are potentially antilisterial and anti-salmonella agents, which can be applied to meat products.

KEYWORDS: sodium diacetate, sodium citrate, sodium lactate, lauroyl arginate ethyl, pathogenic foodborne bacteria

INTRODUCTION

Listeria monocytogenes and *Salmonella* spp. are the pathogenic foodborne bacteria^{1,2}. Salmonellae are most often associated with any raw food of animal origin which may be subject to faecal contamination, especially raw meat and poultry². *Salmonella* testing in the slaughter environment is important as intestinal pathogens are carried into the abattoir in the bowels and on the skin of the animals³. In pork carcasses, a prevalence of *Listeria* spp. and *Salmonella* spp. can be as high as 23% and 100%, respectively, from floor slaughtering process and 12% and 56%, respectively, from hanging slaughtering process in abattoirs of Southern Thailand. Furthermore, *L. monocytogenes* was the third most specie of *Listeria* isolate in carcasses from both slaughtering processes (28% and 25%, respectively). *Salmonella* Rissen was the most frequently found serotype of *Salmonella* isolate in carcasses from both slaughtering processes (32% and 37%, respectively)^{4,5}.

Safe preservation in the meat industry is complicated, as nowadays 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 by using chemical antimicrobials. Salts of organic acid are commonly used to control the growth of undesirable microorganisms. Several treatments using organic acids or their salts have been demonstrated to be effective at reducing populations of spoilage and pathogenic bacteria on, or in, meat or meat products. However, the study of the antimicrobial effects of organic acid salts on spoilage bacteria growth by incorporating them during the preparation of meat products is limited. Conversely, extensive research has examined the effect of lactate and diacetate on the viability of pathogens such as *Salmonella* spp. during refrigerated storage of vacuum-packaged meat products dipped or sprayed with chemicals⁶. Citric and lactic acid salts have been considered as preservatives in a number of meat systems⁷. 2% (w/w) sodium

lactate (SL) or 2% (w/w) sodium citrate (SC) or 1.5% (w/w) SC+1.5% (w/w) SL into fresh pork sausage has been found to be effective in reducing contamination by *Salmonella* Kentucky⁸.

Lauroyl arginate ethyl (LAE) is a new surfactant derived from lauric acid and arginine whose antimicrobial properties have been shortly described in literature. In addition to these advantages, the antimicrobial properties of LAE remain stable from pH 3 to pH 7, suggesting that this substance may be useful as antimicrobial agent for a wide range of food⁹. Toxicological studies have determined that LAE was rapidly metabolized by humans to the naturally occurring dietary components lauric acid and arginine¹⁰ and thus it is considered as a safe product. Besides, LAE has been Generally Recognized as Safe by the FDA in 2005. Hence LAE represents a potential non odorous alternative to essential oils for the development of new food preservation alternatives including antimicrobial active packaging⁹.

The individual effectiveness of salts of organic acid or LAE against foodborne pathogenic bacteria has been examined⁶⁻⁹. The aim of this study was to compare the antibacterial activity of sodium diacetate (SD), SC, SL, and LAE on the growth of *L. monocytogenes* and *S. Rissen* isolated from pig carcasses.

MATERIALS AND METHODS

Test strains

L. monocytogenes TSULM1 and *S. Rissen* TSUSR1 used in the present study were previously isolated from pig carcasses from southern Thailand abattoirs using the standard procedure^{11,12} and its identity confirmed by the 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 10⁸ CFU/ml (McFarland standard of 0.5). These suspensions were further diluted with saline as required. An initial concentration of approximately 5 × 10⁵ CFU/ml was used for the susceptibility, synergy and kill-time tests.

Antimicrobial agents

SD and SC were supplied by Chemipan Corporation Co. Ltd. (Bangkok). SL was provided by Purac Inc. (Bangkok). LAE was obtained from A&B Ingredients (Fairfield, NJ, USA). All antibacterials were food

grade. For the agar disk diffusion and broth dilution assays, the concentrations of SD, SC, and LAE were assessed as mg/ml, but for SL the concentrations were measured as % (v/v).

Susceptibility test methods

Susceptibility tests were performed by the disk diffusion method of Bauer et al¹³ with MHA. All the antimicrobials were dissolved in distilled water. Subsequent two-fold serial dilutions were performed in culture medium so that the final concentrations of the test samples on disks ranged from 0–62.5 mg/ml for SD and SC, 0–8.4% (v/v) for SL and 0–0.128 mg/ml for LAE. Zones of inhibition were measured after 18 h of incubation at 35 °C.

The minimal inhibitory concentration (MIC) was determined by a broth microdilution method¹⁴ for each bacterium. Serial two-fold dilutions of the test substances were mixed with MHB in microtitre plates. The final concentrations of the inhibitors in the broth were the same as those used for the disk diffusion method. A 20 µl aliquot of the inoculum suspension was added to each well. Then, the inoculated plates were incubated at 35 °C for 18 h. 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 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. The 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.

Synergistic effects

To determine whether lauric acid or monolaurin acted synergistically with lactic acid, the fractional inhibitory concentration index (FICI) and fractional bactericidal concentration index (FBCI) in MHB using chequerboard 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 > 0.5–1.0; 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 ^{15–18}.

Determination of kill-time

The effect of SD, SC, LAE, and SL alone (62.5, 62.5, 0.032 mg/ml, and 8.4% (v/v), respectively) and in combinations of SD (7.8 mg/ml) and LAE (0.002 mg/ml), SC (15.6 mg/ml) and LAE (0.008 mg/ml), SL (2.1% v/v) and LAE (0.008 mg/ml) on the cell viability of *L. monocytogenes* and *S. Rissen* over 12 h was evaluated by the viable cell count procedure. To do so, 8 ml of MHB was inoculated with 1 ml of the bacterial inoculum of *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1 and 1 ml of antimicrobial solution (final concentration as shown above) was combined and gently shaken for 30 s. The resulting suspension was incubated at 35 °C¹⁸. At different time intervals (0, 5, 10, 15, 30, 60, 120, 180, 360, and 720 min), the cells that were capable of growth on solid selective media were enumerated using spread plate count on MHA in order to determine the total culturable population. When the concentration of culturable cells was < 300 CFU/ml, a portion (0.1 ml) of each resuspension was plated onto MHA. When the culturable cell counts were lower than the detection limit, culturability was assessed by plating 1 ml on MHA. Two 0.3 ml aliquots or a 0.4 ml aliquot of each resuspension were added onto MHA¹⁹. The cell numbers (CFU) were determined following incubation at 35 °C for 48 h.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

SEM and TEM were performed using a modification of methods described^{19–21}. *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1 samples for SEM and TEM were centrifuged at 16 000g for 5 min and the supernatant was discarded. The pellets were washed 3 times with Sorensen's phosphate buffer (SPB), and subsequently fixed in 2.5% glutaraldehyde in SPB for 1–2 h at 4 °C, followed by 1% osmium in SPB for 1–2 h. The sample was washed 3 times with SPB between fixatives. The pellets were dehydrated by passage through a graded ethanol series (3 × 5 min each at 50, 70, 80, 90, and 95 and 2 × 10 min at 100% v/v) and then stored overnight. For SEM, the sample was dehydrated to the critical point drying using a Polaron CPD 7501 (VG Microtech, 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, USA) prior to examination with a Quanta

400 scanning electron microscope (FEI Ltd., 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 Ltd., USA) operated at 160 kV.

Statistical analysis

Data are 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.

RESULTS AND DISCUSSION

Susceptibility test

The results of the antimicrobial activity of salts of organic acid and LAE tested by the disk diffusion method against *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1 are given in Fig. 1 and Fig. 2. SD, and LAE exhibited a favourable activity against both bacteria tested. They were inhibited at > 3.91 mg/disc of SD for both bacteria and > 0.004 and > 0.008 mg/disc of LAE for *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1, respectively. SC and SL inhibited *L. monocytogenes* TSULM1 but did not inhibit *S. Rissen* TSUSR1. They were inhibited *L. monocytogenes* TSULM1 at > 7.8 mg/disc of SC and $> 2.1\%$ (v/v)/disc of SL. The findings of this study are in agreement with those of other researchers for the efficacy of salts of organic acid in inhibiting the growth of food-related pathogens^{6,22,23}. It has long been known that salts of organic acid have an inhibitory effect on *L. monocytogenes*^{24–28} and *Salmonella*^{25,29}. Salts of organic acid have also been suggested to have antimicrobial effects by causing hyper-acidification via proton donation at the plasma membrane interface of the microorganism and intracellular cytosolic acidification, an excess of which can disrupt the H⁺-ATPase enzyme required for ATP synthesis^{24,28}. Furthermore, the growth-delaying effect of lactate on *L. monocytogenes* in food products or microbiological growth media has also been reported by^{30–33}. Gram-positive bacteria are more sensitive towards lactate than Gram-negative bacteria³⁴. The antimicrobial activity of sodium citrate has been attributed to its strong chelating activity³⁵. LAE interacts with the lipids from the bacterial membranes, producing disturbance in membrane potential and structural changes in *S. Typhimurium* and

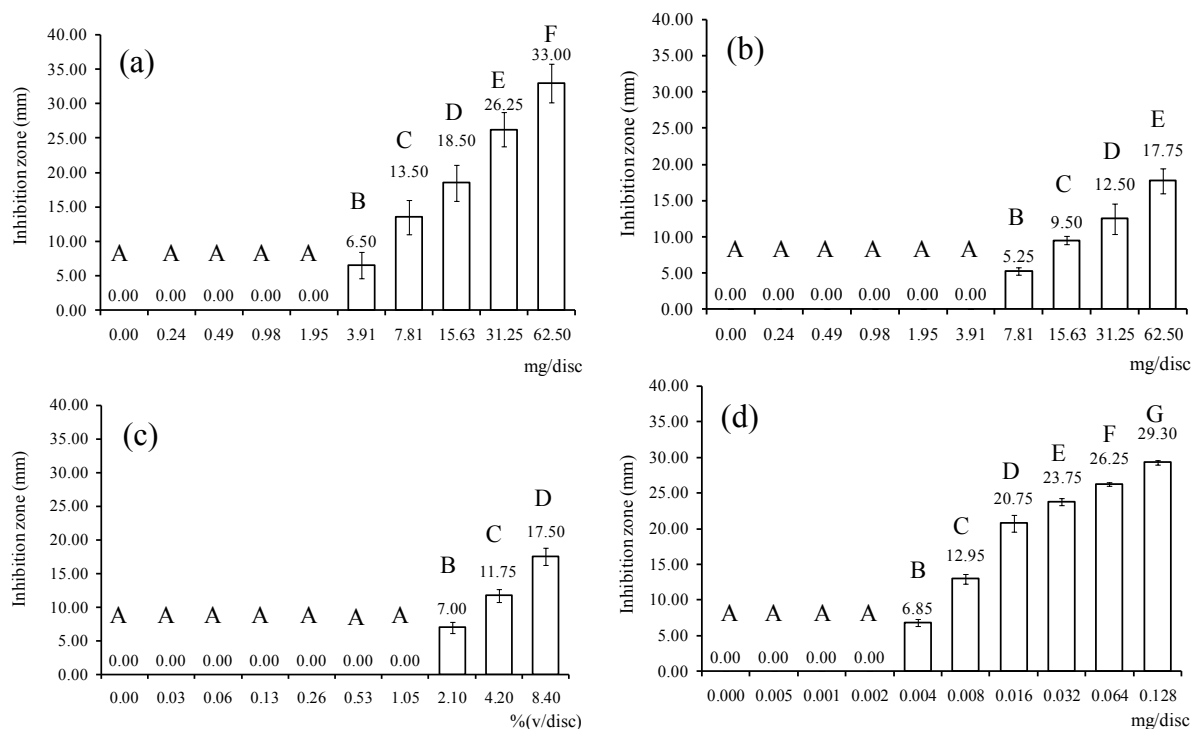


Fig. 1 Antimicrobial activity (zone of inhibition) of (a) SD, (b) SC, (c) SL, and (d) LAE against *L. monocytogenes* TSULM1. Different letters (A–G) indicate that values are significantly different ($p < 0.05$).

*Staphylococcus aureus*⁹.

In addition, the MICs of antibacterial against *L. monocytogenes* TSULM1 were determined to be 31.3 mg/ml for SD, 15.6 mg/ml for SC, 2.1% (v/v) for SL and 0.008 mg/ml for LAE. The MICs of SD and LAE against *S. Rissen* TSUSR1 were determined to be 31.3 and 0.016 mg/ml, respectively. However, the MBCs of antibacterial against both bacteria were two-fold for SD and four-fold for SC and SL, respectively, higher than the corresponding MIC. The MBCs of LAE against *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1 were both 0.032 mg/ml, or four- and two-fold, respectively, higher than the corresponding MIC (Table 1). Earlier studies found MIC of SD, SC, SL, and LAE against *L. monocytogenes* in the range of 4.00–5.00 mg/ml^{36,37}, 70 mg/ml³⁸, 2.5–4.8% (v/v)^{38–40}, and 0.0125–0.025 mg/ml^{9,41}, respectively.

Synergistic effects

The FICI for the combined application of organic acid salts with LAE on *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1 is shown in Table 2. FICI and FBCI indicate that application of organic acid salts in combination with LAE resulted in enhanced inhibition of both pathogens. The enhancing effect of the combination was also evidenced by bactericidal

Table 1 The MIC and MBC values of salts of organic acid and LAE against *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1.

Antimicrobials	<i>L. monocytogenes</i> TSULM1		<i>S. Rissen</i> TSUSR1	
	MIC	MBC	MIC	MBC
SD (mg/ml)	31.3	62.5	31.3	62.5
SC (mg/ml)	15.6	62.5	NI*	NI
SL (mg/ml)	2.1	8.4	NI	NI
LAE (% v/v)	0.008	0.032	0.016	0.032

SD, sodium diacetate; SC, sodium citrate; SL, sodium lactate; LAE, lauric arginate.

* NI, no inhibition.

responses produced at sub-MBC levels for each bacterium. FICIs of the combined action of SD + LAE, SC + LAE, and SL + LAE were 0.25 (3.91 mg/ml + 0.001 mg/ml), 0.50 (3.91 mg/ml + 0.002 mg/ml), and 0.38 (0.26% (v/v) + 0.002 mg/ml), respectively, for *L. monocytogenes* TSULM1 and 0.38 (1.95 mg/ml + 0.002 mg/ml), < 0.38 (3.91 mg/ml+0.002 mg/ml) and < 0.50 (1.05% (v/v) + 0.002 mg/ml), respectively, for *S. Rissen* TSUSR1 suggesting synergy of the assayed antimicrobials. Similarly, FBCIs of the combined

Table 2 FICI and FBCI of the combined action of lipid with lactic acid to *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1.

Strain	Combination [*]	FICI [†]		FBCI [†]	
		Concentration [‡]	Value	Concentration [‡]	Value
<i>L. monocytogenes</i> TSULM1	SD + LAE	3.91 + 0.001	0.25	7.8 + 0.002	0.19
	SC + LAE	3.91 + 0.002	0.50	15.6 + 0.008	0.50
	SL + LAE	0.26 + 0.002	0.38	2.1 + 0.008	0.50
<i>S. Rissen</i> TSUSR1	SD + LAE	1.95 + 0.002	0.19	7.8 + 0.002	0.19
	SC + LAE	3.91 + 0.002	< 0.16	15.6 + 0.008	< 0.50
	SL + LAE	1.05 + 0.002	< 0.25	2.1 + 0.008	< 0.50

* SD, sodium diacetate; SC, sodium citrate; SL, sodium lactate; LAE, lauric arginate.

† FICI, fractional inhibitory concentration index; FBCI, fractional bactericidal concentration index.

‡ The units of antimicrobial concentration are mg/ml for SD, SC, and LAE and % (v/v) for SL.

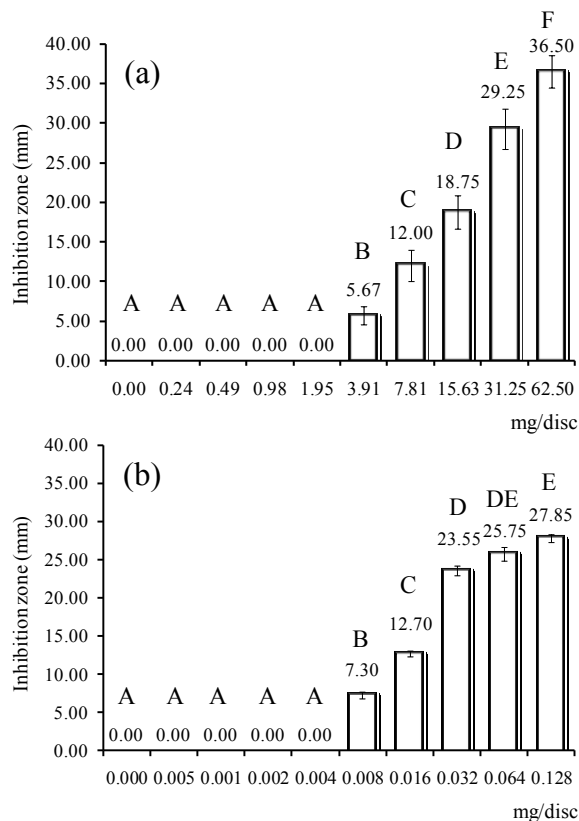


Fig. 2 Antimicrobial activity (zone of inhibition) of (a) SD and (b) LAE against *S. Rissen* TSUSR1. Different letters (A–F) indicate that values are significantly different ($p < 0.05$).

action of SD + LAE, SC + LAE, and SL + LAE were 0.19 (7.8 mg/ml + 0.002 mg/ml), 0.50 (15.6 mg/ml + 0.008 mg/ml), and 0.50 (2.1% (v/v) + 0.008 mg/ml), respectively, for *L. monocytogenes* TSULM1 and 0.19 (7.8 mg/ml + 0.002 mg/ml), < 0.50 (15.6 mg/ml +

0.008 mg/ml), and < 0.50 (2.1% (v/v) + 0.008 mg/ml), respectively, for *S. Rissen* TSUSR1 again suggesting synergy. The test strain was able to grow at sub-bactericidal concentrations ($\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC) of all antimicrobials when applied alone (data not shown). The subsequent calculation and analysis of FICIs and FBCIs (presented in Table 2) indicate that application of organic acid salt with LAE resulted in synergistic inhibition of the pathogen, potentially resulting from salts of organic acid, SD, SC, and SL, a weak organic acid salt was effective in inhibiting most tested bacteria. In addition, the synergistic effect is mainly due to the dissociation effect of LAE caused by the presence of organic acids. The pKa of lactic acid is 3.5, whereas that of citric acid is 3.15, and that of diacetic acid 3.58 or 3.77 depending on the source^{42–44}. Undissociated acid molecules are able to penetrate rapidly through the bacterial cell membrane, dissociating and acidifying the cell interior. When the internal pH of cells decreases below a certain threshold value, cellular functions are inhibited⁴⁵. Sodium lactate dissociates into uncharged acid molecules, anions and cations⁴⁶. Furthermore, the inhibitory effect of sodium lactate and sodium citrate were probably due to their chelating properties⁴². Lactate and citrate are able to chelate a large portion of the metallic nutrient ions, depleting the cell of its essential nutrients⁴⁷. In addition, the LAE interacts with the lipids from the bacterial membranes, producing disturbance in membrane potential and structural changes in Gram positive and Gram negative bacteria and sensitize the cell to undissociated salts of organic acid⁴⁸.

Time-kill

To determine the rates at which bacteria were killed, *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1 were exposed to SD, SC, SL, and LAE alone and in

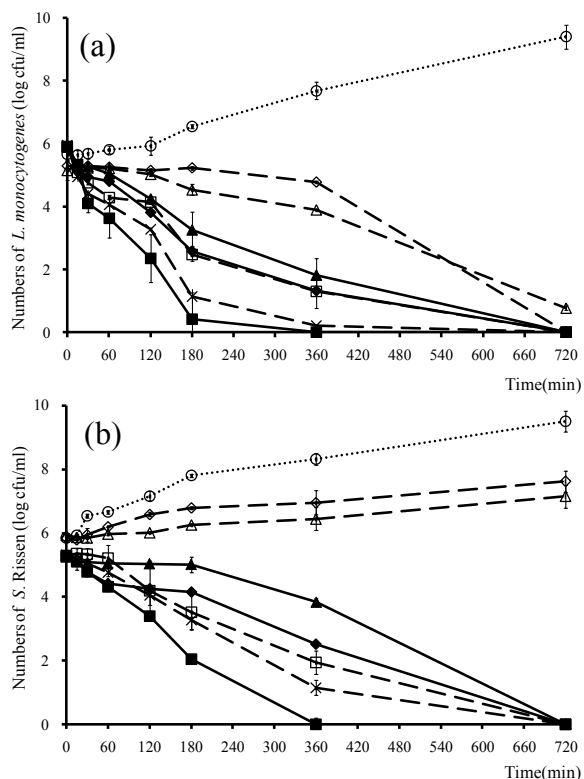


Fig. 3 Survivors curves of (a) *L. monocytogenes* TSULM1 and (b) *S. Rissen* in MHB at 35 °C as a function of organic acid salts and LAE alone (dashed line) and in combinations (solid line); control (circles), SD (squares), SC (triangles), SL (diamonds) and LAE (crosses).

combination at MBC concentration in MHB (Fig. 3). Addition of SD (62.5 mg/ml) and LAE (0.032 mg/ml) to the culture media caused a sharp drop in both bacterial counts after 360 min, and values under two log cycle were maintained for the remainder of the time studied. LAE proved to be more effective against both bacteria in MHB than the three salts of organic acid ($p < 0.05$). Moreover, SC (62.5 mg/ml) and SL (8.4% v/v) alone was not active in killing *S. Rissen*. SC and SL had little effect on *S. Rissen* counts until 120 and 60 min, respectively, and subsequently, bacterial counts increased by more than one log cycle after a further 600 and 660 min, respectively. After an incubation period of 180 min, the combination of SD with LAE at sub-bactericidal concentrations reduced *L. monocytogenes* and *S. Rissen* counts by greater than five and four log cycles, respectively, compared with the initial bacterial load. The bacterial counts found in MHB containing salts of organic acid alone were significantly higher ($p < 0.05$) than the counts obtained for the broth to which had been added the

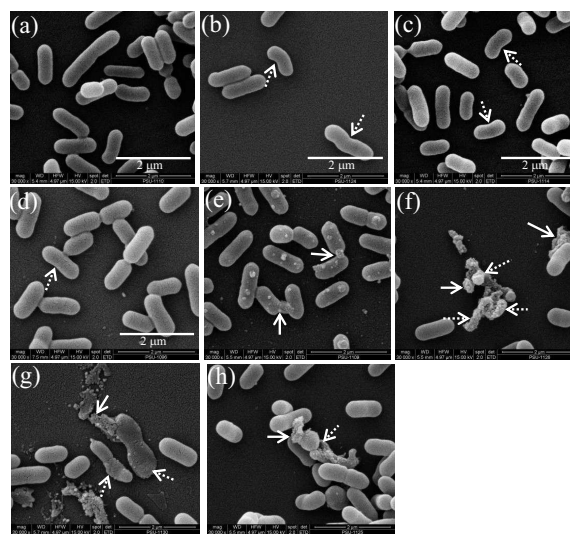


Fig. 4 Scanning electron micrographs of *L. monocytogenes* TSULM1 in MHB containing antimicrobials: (a) control, (b) 62.5 mg/ml of SD, (c) 62.5 mg/ml of SC, (d) 8.4% (v/v) of SL, (e) 0.032 mg/ml of LAE, (f) 7.8 mg/ml of SD + 0.002 mg/ml of LAE, (g) 15.6 mg/ml of SC and 0.008 mg/ml of LAE, and (h) 2.1% (v/v) of SL + 0.008 mg/ml of LAE at 35 °C for 720 min, except (e) and (f) for 360 min. Membrane cells were disturbed and leaked (solid arrow) and subsided (hatched arrow). Bars = 2 μm.

mixture of 7.8 mg/ml SD and 0.002 mg/ml LAE, the mixture of 15.6 mg/ml SC and 0.008 mg/ml LAE, and the mixture of 2.1% (v/v) SL and 0.008 mg/ml LAE.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Cells of *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1 treated with SD, SC, SL, and LAE alone and in combination underwent considerable morphological alterations in comparison with the control when studied by SEM ((Figs. 4 and 6) and TEM (Figs. 5 and 7). Untreated cells of *L. monocytogenes* TSULM1 (control) appear as a smooth bacilli (Figs. 4a and 5a). Treated cells lost and changed the cytoplasm following exposure to SD, SC, and SL (Figs. 5b, 5c, and 5d). For LAE, some membrane leakage was observed (Figs. 4e and 5e). The images demonstrated that LAE led to dramatic changes in cell envelope, indicating that membranes are the main target of this substance. This hypothesis agrees with the results showing that LAE interacts with the lipids from the bacterial membranes disturbing the membrane potential and causing structural changes in *S. Typhimurium* and *S. aureus*⁴⁵. Images obtained in the present study demonstrated that LAE also disrupts the membrane

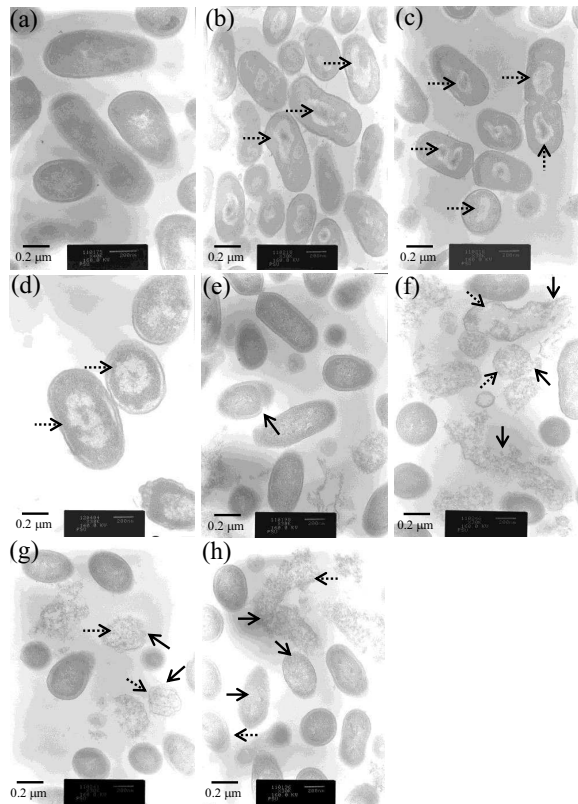


Fig. 5 Transmission electron micrographs of *L. monocytogenes* TSULM1 in MHB containing antimicrobials: (a) control, (b) 62.5 mg/ml of SD, (c) 62.5 mg/ml of SC, (d) 8.4% (v/v) of SL, (e) 0.032 mg/ml of LAE, (f) 7.8 mg/ml of SD + 0.002 mg/ml of LAE, (g) 15.6 mg/ml of SC and 0.008 mg/ml of LAE, and (h) 2.1% (v/v) of SL + 0.008 mg/ml of LAE at 35 °C for 720 min, except (e) and (f) for 360 min. Membrane cells were disturbed and leaked (solid arrow) and subsided (hatched arrow). Bars = 0.2 μm.

of *E. coli*, producing leakage of cytosolic components and subsequent cell death. The results obtained in the survival study agree with this hypothesis, since LAE showed fast bactericidal activity that is in consistent with a fast mechanism of action such as membrane disruption. In all cells exposed to the combinations of antimicrobials, the cytoplasm was disorganized and the integrity of the membrane was compromised (Figs. 4f, 4g, 4h, 5f, 5g and 5h) and the antimicrobial activity was higher when compared with that of the antimicrobials alone. This could be due to the presence of LAE improving the uptake of undissociated organic acid salts into the membrane, which probably affects membrane function and furthermore leads to measurable synergism of the combined antimicrobial treatment⁴⁵. For *S. Rissen* TSUSR1, cells treated

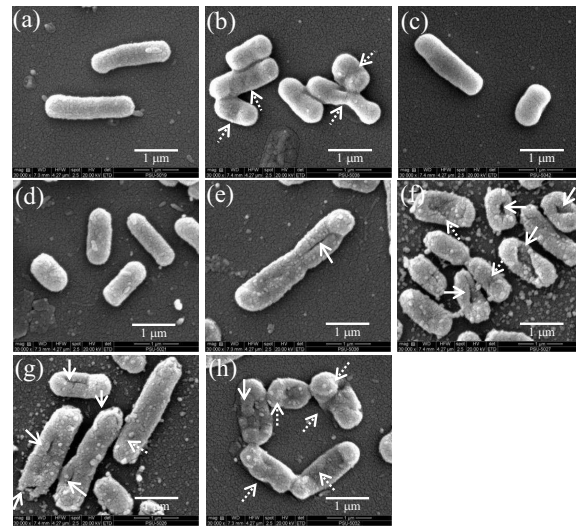


Fig. 6 Scanning electron micrographs of *S. Rissen* TSUSR1 in MHB containing antimicrobials: (a) control, (b) 62.5 mg/ml of SD, (c) 62.5 mg/ml of SC, (d) 8.4% (v/v) of SL, (e) 0.032 mg/ml of LAE, (f) 7.8 mg/ml of SD + 0.002 mg/ml of LAE, (g) 15.6 mg/ml of SC and 0.008 mg/ml of LAE, and (h) 2.1% (v/v) of SL + 0.008 mg/ml of LAE at 35 °C for 720 min, except (e) and (f) for 360 min. Membrane cells were disturbed and leaked (solid arrow) and subsided (hatched arrow). Bars = 1 μm.

with salts of organic acid and LAE alone and in combination appeared similar to cells of *L. monocytogenes* TSULM1, except for SC alone and SL alone (Figs. 6c, 6d, 7c, and 7d), for which the membrane and cytoplasm of cells were not different from untreated cells.

In summary, this study confirms that salts of organic acid and LAE alone and in combination exhibit *in vitro* antimicrobial effects against *L. monocytogenes* TSULM1 and *S. Rissen* TSUSR1 *S. aureus*, isolated from pig carcasses. There was a synergistic effect of LAE in the presence of SD, SC, and SL probably due to increased uptake of the fatty acids into the membrane, resulting in membrane disruption. However, whether they can be used for food or meat preservation, issues of *in vivo* antimicrobial activity and sensory effects during storage would need to be addressed.

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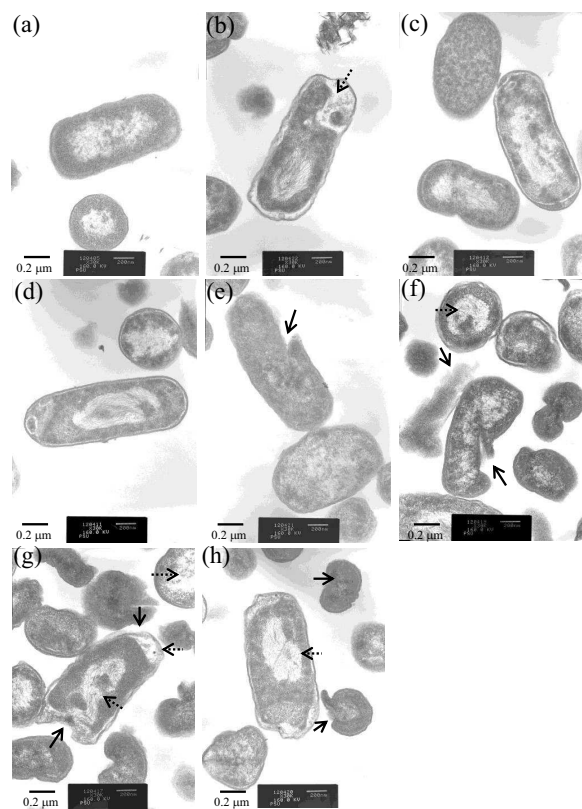


Fig. 7 Transmission electron micrographs of *S. Rissen* TSUSR1 in MHB containing antimicrobials: (a) control, (b) 62.5 mg/ml of SD, (c) 62.5 mg/ml of SC, (d) 8.4% (v/v) of SL, (e) 0.032 mg/ml of LAE, (f) 7.8 mg/ml of SD + 0.002 mg/ml of LAE, (g) 15.6 mg/ml of SC and 0.008 mg/ml of LAE, and (h) 2.1% (v/v) of SL + 0.008 mg/ml of LAE at 35 °C for 720 min, except (e) and (f) for 360 min. Membrane cells were disturbed and leaked (solid arrow) and subsided (hatched arrow). Bars = 0.2 μm.

REFERENCES

- McLauchlin J, Mitchell RT, Smerdon WJ, Jewell K (2004) *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. *Int J Food Microbiol* **92**, 15–33.
- Anonymous (2003) Zoonoses: Commissioner David Byrne welcomes new legislation to combat food-borne diseases such as *Salmonella* (IP/03/1306). European Commission.
- Grimont PAD, Grimont F, Bouvet P (2000) Taxonomy of the Genus *Salmonella*. In: Wray C, Wray A (eds) *Salmonella in Domestic Animals*, 1st edn, CABI Publishing, Oxon, UK, pp 1–17.
- Tangwacharin P, Wattanachant C (2008) Contamination of pathogens in hygienic and unhygienic slaughtering and cutting process in municipality of Hat Yai City, Songkhla province. Research Report of Thai Health Promotion Foundation, Thailand.
- Tangwacharin P, Wattanachant C (2009) Bacterial contaminations in floor and hanging pig slaughtering processes in municipality of Hat Yai City, Songkhla province. *King Mongkuts Agr J* **27**, 122–31.
- Mbandi E, Shelef LA (2001) Enhanced inhibition of *Listeria monocytogenes* and *Salmonella enteritidis* in meat by combinations of sodium lactate and diacetate. *J Food Protect* **64**, 640–4.
- Miller AJ, Call JE, Whiting RC (1993) Comparison of organic acid salts for *Clostridium botulinum* control in uncured turkey. *J Food Protect* **56**, 958–62.
- Scannell AGM, Hill C, Buckley DJ, Arendt EK (1997) Determination of the influence of organic acids and nisin on shelf-life and microbiological safety aspects of fresh pork. *J Appl Microbiol* **83**, 407–12.
- Becerril R, Manso S, Nerin C, Gómez-Lus R (2013) Antimicrobial activity of Lauroyl Arginate Ethyl (LAE), against selected food-borne bacteria. *Food Contr* **32**, 404–8.
- Hawkins DR, Rocabayera X, Ruckman S, Segret R, Shaw D (2009) Metabolism and pharmacokinetics of ethyl N^α-lauroyl-L-arginate hydrochloride in human volunteers. *Food Chem Toxicol* **47**, 2711–5.
- Hitchins AD, Jinneman K (2013) Detection and enumeration of *Listeria monocytogenes* in foods. *Bacteriological Analytical Manual (BAM) Online*, Ch. 10. U.S. Food and Drug Administration. URL: <http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm>.
- Andrews WH, Jacobson A, Hammack T (2011) *Salmonella*. *Bacteriological Analytical Manual (BAM) Online*, Ch. 5. U.S. Food and Drug Administration. URL: <http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm>.
- Bauer AW, Kirby WMM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**, 493–6.
- CLSI (2002) Reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. Wayne, Pa: Clinical Laboratory Standards Institute.
- Bharadwaj R, Vidya A, Dewan B, Pal A (2003) An in vitro study to evaluate the synergistic activity of norfloxacin and metronidazole. *Indian J Pharmacol* **35**, 220–6.
- Doores S (2005) Organic acids. In: Davidson PM, Sofos JN, Branen AL (eds) *Antimicrobials in Food*, 3rd edn, CRC Press, Boca Raton, FL, pp 91–142.
- Gutierrez J, Barry-Ryan C, Bourke P (2008) The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *Int J Food Microbiol* **124**, 91–7.
- de Oliveira CEV, Stamford TLM, Gomes Neto NJ, de Souza EL (2010) Inhibition of *Staphylococcus aureus* in broth and meat broth using synergies of phenolics

- and organic acid. *Int J Food Microbiol* **137**, 312–6.
19. Tangwacharin P, Chanthachum S, Khopaibool P, Griffiths MW (2007) Morphological and physiological responses of *Campylobacter jejuni* to stress. *J Food Protect* **69**, 2747–53.
 20. Chaveerach P, Keuzenkamp DA, Urlings HA, Lipman LJ, van Knipen F (2002) In vitro study on the effect of organic acids on *Campylobacter jejuni/coli* populations in mixtures of water and feed. *Poultry Sci* **81**, 621–8.
 21. Zakaria Z, Sreenivasan S, Mohamad M (2007) Antimicrobial activity of *Piper ribesoides* root extract against *Staphylococcus aureus*. *J Appl Biol Sci* **1**, 87–90.
 22. Serdengecti N, Yildirimi I, Gokoglu N (2006) Effects of sodium lactate, sodium acetate and sodium diacetate on microbiological quality of vacuum-packed beef during refrigerated storage. *J Food Saf* **26**, 62–71.
 23. Ukuku DO, Bari ML, Kawamoto S, Isshiki K (2005) Use of hydrogen peroxide in combination with nisin, sodium lactate and citric acid for reducing transfer of bacterial pathogens from whole melon surfaces to fresh-cut pieces. *Int J Food Microbiol* **104**, 225–33.
 24. Apostolidis E, Kwon Y-I, Shetty K (2008) Inhibition of *Listeria monocytogenes* by oregano, cranberry and sodium lactate combination in broth and cooked ground beef systems and likely mode of action through proline metabolism. *Int J Food Microbiol* **128**, 317–24.
 25. Hwang C-A, Sheen S, Juneja V (2011) Effects of sodium lactate on the survival of *Listeria monocytogenes*, *Escherichia coli* O157: H7, and *Salmonella* spp. in cooked ham at refrigerated and abuse temperatures. *Food Nutr Sci* **2**, 464–70.
 26. McMahon CMM, Doherty AM, Sheridan JJ, Blair IS, McDowell DA, Hegarty T (1999) Synergistic effect of heat and sodium lactate on the thermal resistance of *Yersinia enterocolitica* and *Listeria monocytogenes* in minced beef. *Lett Appl Microbiol* **28**, 340–4.
 27. Shelef LA, Yang Q (1991) Growth suppression of *Listeria monocytogenes* by lactates in broth, chicken, and beef. *J Food Protect* **54**, 283–7.
 28. Silva ENG, Almeida RCC, Figueiredo ACL, Miranda FA (2012) Effects of salts of organic acids on the survival of *Listeria monocytogenes* in soft cheeses. *Int J Food Sci Tech* **47**, 1819–25.
 29. Ibrahim SA, Yang H, Seo CW (2008) Antimicrobial activity of lactic acid and copper on growth of *Salmonella* and *Escherichia coli* O157:H7 in laboratory medium and carrot juice. *Food Chem* **109**, 137–43.
 30. Glass KA, Granberg DA, Smith AL, McNamara AM, Hardin M, Mattias J, Ladwig K, Johnson EA (2002) Inhibition of *Listeria Monocytogenes* by sodium diacetate and sodium lactate on wieners and cooked bratwurst. *J Food Protect* **65**, 116–23.
 31. Stekelenburg FK (2003) Enhanced inhibition of *Listeria monocytogenes* in frankfurter sausage by the addition of potassium lactate and sodium diacetate mixtures. *Food Microbiol* **20**, 133–7.
 32. Legan JD, Seman DL, Milkowski AL, Hirschey JA, Vandeven MH (2004) Modeling the growth boundary of *Listeria monocytogenes* in ready-to-eat cooked meat products as a function of the product salt, moisture, potassium lactate, and sodium diacetate concentrations. *J Food Protect* **67**, 2195–204.
 33. Abou-Zeid KA, Yoon KS, Oscar TP, Schwarz JG, Hashem FM, Whiting RC (2007) Survival and growth of *Listeria monocytogenes* in broth as a function of temperature, pH, and potassium lactate and sodium diacetate concentrations. *J Food Protect* **70**, 2620–5.
 34. Houtsma PC, de Wit JC, Rombouts FM (1993) Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. *Int J Food Microbiol* **20**, 247–57.
 35. Watanabe R, Hojo K, Nagaoka S, Kimura K, Ohshima T, Maeda N (2011) Antibacterial activity of sodium citrate against oral bacteria isolated from human tongue dorsum. *J Oral Biosci* **53**, 87–92.
 36. Patel JR, Sanglay GC, Solomon MB (2009) Control of *Listeria monocytogenes* on frankfurters with antimicrobials and hydrodynamic pressure processing. *J Muscle Foods* **20**, 227–41.
 37. Uhart M, Ravishankar S, Maks N (2004) Control of *Listeria monocytogenes* with combined antimicrobials on beef franks stored at 4 °C. *J Food Protect* **67**, 2296–301.
 38. DeVegt B (1999) Lactate controls *Listeria monocytogenes*. PURAC, Lincolnshire, IL, America.
 39. Houtsma PC, de Wit JC, Rombouts FM (1996) Minimum inhibitory concentration (MIC) of sodium lactate and sodium chloride for spoilage organisms and pathogens at different pH values and temperatures. *J Food Protect* **59**, 1300–4.
 40. Nanasombat S, Chooprang K (2009) Control of pathogenic bacteria in raw pork using organic acid salts in combination with freezing and thawing. *Kasetsart J (Nat Sci)* **43**, 576–83.
 41. Brandt AL, Castillo A, Harris KB, Keeton JT, Hardin MD, Taylor TM (2010) Inhibition of *Listeria monocytogenes* by food antimicrobials applied singly and in combination. *J Food Sci* **75**, M557–63.
 42. Manso S, Nerín C, Gómez-Lus R (2011) Antifungal activity of the essential oil of cinnamon (*Cinnamomum zeylanicum*), oregano (*Origanum vulgare*) and lauramide argine ethyl ester (Lae) against the mold *Aspergillus flavus* Cect 2949. *Ital J Food Sci* **SLIM-2010**, 151–7.
 43. Pezo D, Navascués B, Salafranca J, Nerín C (2012) Analytical procedure for the determination of Ethyl Lauroyl Arginate (LAE) to assess the kinetics and specific migration from a new antimicrobial active food packaging. *Anal Chim Acta* **745**, 92–8.
 44. Aznar M, Gómez-Estaca J, Vélez D, Devesa V, Nerín C (2013) Migrants determination and bioaccessibility study of ethyl lauroyl arginate (LAE) from a LAE based antimicrobial food packaging material. *Food Chem Toxicol* **56**, 363–70.

45. Shelef LA (1994) Antimicrobial effects of lactates: A review. *J Food Protect* **57**, 445–50.
46. Russell JB (1992) A review: Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *J Appl Bacteriol* **73**, 363–70.
47. Stratford M (2000) Traditional preservatives organic acids. In: Robinson RK, Batt CA, Patel PD (eds) *Encyclopedia of Food Microbiology* volume 1, 1st edn, Academic Press, New York. pp 1729–37.
48. Rodríguez E, Seguer J, Rocabayera X, Manresa A (2004) Cellular effects of monohydrochloride of L-arginine, N^ε-lauroyl ethylester (LAE) on exposure to *Salmonella typhimurium* and *Staphylococcus aureus*. *J Appl Microbiol* **96**, 903–12.