Control of Pathogenic Bacteria in Raw Pork using Organic Acid Salts in Combination with Freezing and Thawing

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ABSTRACT

The effect of sodium lactate, sodium acetate and potassium sorbate on growth inhibition of seven bacterial species: *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Salmonella* Rissen, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Yersinia enterocolitica* was investigated by determining the minimum inhibitory concentration (MIC) values at pH 4.5-7.0. Sodium lactate and potassium sorbate provided overall greater inhibition to all tested bacterial strains in comparison with sodium acetate. At a low pH level (4.5-5.5), sodium lactate was more effective in inhibiting most tested bacteria than at a high pH level (6.0-7.0). The most sensitive bacteria to sodium lactate at pH 4.5 were *L. monocytogenes* and *E. coli*. Potassium sorbate had good inhibitory activity against *E. coli* and *P. fluorescens* at pH 4.5-5.0. The pH value had less effect on the antibacterial action of sodium acetate particularly at pH 5.0-6.5. *Y. enterocolitica* was the most sensitive strain to sodium acetate at pH 4.5-7.0. The effect of these organic acid salts in combination with freezing (-23±2°C for 72 h) and slow thawing on the survival of *Salmonella* Rissen and *S. aureus* in pork was investigated. Survival cells of *Salmonella* Rissen and *S. aureus* decreased as the freezing time increased. Of all the tested organic acid salts, potassium sorbate showed the greatest inhibitory effect on *Salmonella* Rissen, while sodium lactate inhibited *S. aureus* well. These organic acid salts provided a synergistic effect with freezing and thawing on lowering survival of both bacterial species.

Key words: raw pork, organic acid salts, freezing, *Salmonella* spp., *Staphylococcus aureus*

INTRODUCTION

Raw pork can be contaminated with pathogenic bacteria including *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Yersinia* spp. (Schaft et al., 1992; Quiñones et al., 2000; Nanasombat et al., 2002). Recently, Ganyarat (2007) also found *Salmonella* spp., *S. aureus* and coliform bacteria contaminated in 86.7, 43.3 and 96.7% of 30 raw pork samples sold in Bangkok, respectively. These bacteria can enter and spread through the pork supply chain in all stages of processing including primary stages on the farm, during transportation and in the slaughterhouse by cross-contamination from the environment and infecting animals (van der Gaag et al., 2004). If this contaminated pork is used as raw material to produce pork products, it can be hazardous to consumers as the pathogenic bacteria may survive during fermentation. Nanasombat et al. (2002) reported that five serotypes of *Salmonella* such as *S. Agona*, *S.
Anatum, S. Panama, S. Rissen and S. Senftenberg were found in nham, a Thai fermented pork sausage. Ganyarat (2007) identified that some pathogenic bacteria had survived in nham. *Salmonella* spp., *S. aureus*, *E. coli*, and *Clostridium perfringens* were detected in 36.67, 26.67, 53.33 and 6.67% of 30 nham samples, respectively. Therefore, the control of these bacteria is necessary.

Microorganisms in meat and meat products may be controlled by organic acids and their salts. Acetic, lactic, propionic and sorbic acids and their salts exert antimicrobial activity. They have been traditionally used as food preservatives (Ray, 1996). In the meat industry, these preservatives were used to decontaminate the products by dipping and spraying. Lactic acid, acetic acid, sodium acetate, sodium diacetate, potassium sorbate and potassium benzoate at a concentration of 2.5-5.0 g/100ml in the dipping solution provided extensive growth inhibition of *L. monocytogenes* in refrigerated bologna (Samelis et al., 2001). Moreover, Mbandi and Shelef (2001) reported that addition of sodium lactate into meat enhanced growth inhibition of *L. monocytogenes* and *Salmonella enteritidis* in meat. Similarly, sodium lactate was reported to cause a delay in the proliferation of *Enterobacteriaceae* in ground beef (Sallam and Samejima, 2004).

The antimicrobial activity of organic acid salts can be enhanced by combining them with physical treatments such as freezing and thawing. Some researchers reported that freezing can damage pathogens in meat (Barrell, 1988; Zhao et al., 2003). Using organic acid salts in combination with freezing and thawing effectively eliminated *L. monocytogenes* in smoked salmon (Yoon et al., 2004). Therefore, the objective of this study was to investigate whether organic acid salts in combination with freezing and thawing could effectively control the pathogenic bacteria in raw pork.

### MATERIALS AND METHODS

#### Determination of minimum inhibitory concentration (MIC) of sodium lactate, sodium acetate and potassium sorbate

**Microorganisms and inoculum preparation**

Seven bacterial strains were used in this study. *Staphylococcus aureus* SAP 17971/05 and *Salmonella* Rissen SAP 08946/02 were isolated from nham. *Salmonella Typhimurium* SAP08957/02 was isolated from raw pork. *Pseudomonas fluorescens* DMST 20076, *Listeria monocytogenes* DMST 11256, *Escherichia coli* DMST 4212 and *Yersinia enterocolitica* DMST 9380 were obtained from the culture collection at the Department of Medical Sciences, Ministry of Public Health, Thailand. These bacteria were transferred to Tryptic Soy Broth (TSB, pH 7.3 ± 0.2, Difco) and incubated at 37°C for 24 h. After incubation, bacterial cells were collected by centrifugation at 3000 rpm for 20 min, washed twice and resuspended in 0.1% peptone water. Turbidity was adjusted to match that of 2 McFarland standard to obtain a final concentration of 10^7 cells/ml.

**MIC determination**

The MICs of sodium lactate (60% wt/wt, S-LAC FG60, Vicchi Enterprise Co., Ltd), sodium acetate (Merck, Germany) and potassium sorbate (Fluka, Germany) against *S. aureus*, *Salmonella Rissen*, *Salmonella Typhimurium*, *P. fluorescens*, *L. monocytogenes*, *E. coli* and *Y. enterocolitica* were determined in Mueller Hinton Broth (MHB, pH 7.3 ± 0.1, Difco) adjusted to pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 with 0.1 N HCl or 0.1 N NaOH. MHB (100 µl) was dispensed into wells on a microtiter plate. Then, 100 µl of each organic acid salt solution (459 mg/ml sodium lactate, 504 mg/ml sodium acetate and 615.3 mg/ml potassium sorbate) were added, and subsequently two-fold serially diluted with MHB. The cell suspension (20 µl) of each bacterial strain
was inoculated in each well. The final concentrations of organic acid salts were 191.25-0.09 mg/ml for sodium lactate, 210.0-0.10 mg/ml for sodium acetate and 256.34-0.12 mg/ml for potassium sorbate. The microtiter plates were incubated at 37°C for 48 h. The turbidity was measured at 620 nm using a microplate reader (iEMS Reader MF, Labsystems). The lowest salt concentration at each pH level that inhibited visible growth of the tested bacteria was recorded as the MIC value.

**Effect of sodium lactate, sodium acetate and potassium sorbate in combination with freezing and thawing on survival of Staphylococcus aureus and Salmonella Rissen in raw pork**

**Raw pork preparation**

Raw pork (pH 5.65) was purchased at local markets in Bangkok, immediately transported to the laboratory, washed and decontaminated by dipping into hot water at 75°C for 15 sec. The pork was subsequently air dried for 30 min.

**Bacterial inoculation and treatments of inoculated samples**

The inoculum suspension (10^7 cells/ml) of *S. aureus* SAP 17971/05 and *Salmonella* Rissen SAP 08946/02 were prepared and inoculated into raw pork using the procedure described by Jung and Beuchat (1999). Each portion (400 g) of inoculated pork was divided into four batches for the addition of three organic acid salts: sodium lactate, sodium acetate and potassium sorbate, with the fourth batch as a control (no organic acid salts added). Concentrations of organic acid salts were selected based on the previous results of the MIC at pH 5.5. The treated pork samples were then stored at -23 ± 2°C for 72 h to apply freezing stress to *S. aureus* and *Salmonella* Rissen cells. The pork samples were slowly thawed at 5 ± 2°C for 24 h after freezing for 0, 12, 24, 48 and 72 h. Viable cells were enumerated at each time interval by spread plating onto two types of media. The first type was a nonselective medium (TSA) for injured and uninjured cell recovery of both species, and the second type was a selective medium (Baird-Parker Agar (BPA) or xylose lysine deoxycholate (XLD) agar) for recovery of uninjured *S. aureus* or *Salmonella* Rissen. All plates were incubated at 37°C for 24 h. Colonies on duplicate plates were counted, and percentage of survival was calculated with 100% viability representing the colony counts of the cultures just prior to treatments of organic acid salts and freeze-thawing challenge. The number of injured cells was calculated by subtracting the number of colonies on a selective medium from those on a nonselective medium (Jay et al., 2005). Three replications were performed for each organic acid salt and freeze-thaw treatment.

**Statistical analysis**

Data were analyzed using analysis of variance to determine if significant differences (P≤0.05) existed between values and Duncan’s multiple range test to compare means between treatments.

**RESULTS**

**Minimum inhibitory concentration of sodium lactate, sodium acetate and potassium sorbate**

The MIC values indicated that most tested bacteria were more resistant to sodium acetate than potassium sorbate and sodium lactate at almost all pH levels (Table 1). The pH had a minor influence on the MIC values of sodium acetate, but not for the other salts. Mostly, sodium lactate and potassium sorbate provided greater inhibition to all tested bacteria compared to sodium acetate at almost all pH levels. At low pH, sodium lactate was more effective at inhibiting most tested bacteria than at high pH. At pH 4.5-5.0, sodium lactate at low concentration (23.9-47.8 mg/ml) was inhibitory to most tested bacteria. Potassium sorbate was more effective at inhibiting some tested bacteria at pH 6.0-6.5 than sodium lactate.
and sodium acetate. *E. coli* was the most sensitive strain to sodium lactate and potassium sorbate at pH 4.5, while *L. monocytogenes* was the most susceptible bacterium to sodium lactate at pH 4.5-6.0. Compared with the other bacteria, sodium acetate at pH 4.5 and 5.0-7.0 showed a greater inhibitory effect to *Y. enterocolitica* with MIC of 13.1 and 52.5 mg/ml, respectively.

**Survival of Salmonella Rissen and Staphylococcus aureus in raw pork treated with sodium lactate, sodium acetate and potassium sorbate in combination with freezing and thawing**

Based on the MIC values, sodium lactate (1.9 mg/g), sodium acetate (8.4 mg/g) and potassium sorbate (2.6 mg/g) were applied to raw pork prior to freezing and thawing. After 48h freezing, the number of survival cells of *S. aureus* in frozen pork with sodium lactate was significantly less than the number in pork treated with other organic acid salts (P<0.05) (Figure 1a). However, the number of injured cells in pork treated with sodium acetate and potassium sorbate was higher than in pork treated with sodium lactate (Figure 1b). Viable cells of *Salmonella* Rissen in all treatments rapidly decreased after 12 h of freezing and thawing. Among all treatments, the number of viable *S. Rissen* cells in frozen pork containing potassium sorbate was the lowest (Figure 1c). After 12-h freezing, the injured cells of *S. Rissen* in almost all treatments increased (Figure 1d), but no significant differences were observed between treatments (P>0.05). Compared to the control, potassium sorbate and sodium lactate provided a synergistic effect with freezing and thawing that lowered the survival rate of *S. Rissen* and *S. aureus*, respectively (Figures 1a and 1c). In most cases, the number of viable cells of *S. Rissen* in pork was less than the number in

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S. aureus, while the number of S. Rissen injured cells was higher than the number of S. aureus injured cells in pork after 72h freezing and thawing.

**DISCUSSION**

Sodium lactate, a weak organic acid salt was effective in inhibiting most tested bacteria especially at low pH. Undissociated acid molecules are able to penetrate rapidly through the bacterial cell membrane, dissociate and acidify the cell interior. When the internal pH of cells decreases below a certain threshold value, cellular functions are inhibited (Shelef, 1994). Sodium lactate dissociates into uncharged acid molecules, anions and cations. Accumulation of anions is responsible for the toxic effect of acids at low pH (Russell, 1992). Moreover, the inhibitory effect of sodium lactate was probably due to its chelating properties and ability to reduce the aw (Shelef, 1994). Lactate is able to chelate a large portion of the metallic nutrient ions, depleting the cell of its essential nutrients (Stratford, 2000). In the present study, the MICs of sodium lactate at pH 4.5-5.5 were lower than the MICs at pH 6.0-7.0. This is in agreement with Houtsma et al. (1996) who found that the MIC values of sodium lactate against most
tested bacteria decreased at low pH, except for \textit{S. aureus}.

In this study, potassium sorbate provided good inhibition of some tested bacteria at pH 6.0-6.5. Potassium sorbate has activity at a maximum pH of 6.0-6.5 (Sofos and Busta, 1981). However, potassium sorbate showed effective inhibition of some tested bacteria such as \textit{E. coli} at pH 4.5-5.5. This pH range was close to its pKa (4.75). At this pH, 50\% of the sorbate is undissociated (Sofos and Busta, 1981). The inhibitory mechanism of sorbate may have been due to its undissociated form which can enter into the cell, dissociate and inhibit enzyme activities such as enolase, lactate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, \( \alpha \)-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase and aspartase (Kabara and Eklund, 1991).

Surviving cells of \textit{S. aureus} and \textit{Salmonella} Rissen greatly decreased in frozen pork containing sodium lactate and potassium sorbate, respectively. This may have been due to their antimicrobial activities. Tompkin \textit{et al.} (1974) reported that 0.1\% potassium sorbate prevented total microbial growth as well as \textit{Salmonella} and \textit{S. aureus} growth in uncured, cooked sausage at 27°C. In addition, Rice and Pierson (1982) reported inhibitory activity of potassium sorbate against \textit{Salmonella} in frankfurter sausages. Vacuum-packed poultry pieces and muscle fillets treated with potassium sorbate were also reported to increase shelf life as a result of the antimicrobial activity of the potassium sorbate (McMeekin \textit{et al.}, 1984). Growth inhibition of \textit{S. aureus} in pork sausages treated with sodium lactate in combination with nisin was reported by Scannell \textit{et al.} (1997). Although viable counts of \textit{Salmonella} Rissen and \textit{S. aureus} in frozen pork decreased, a number of cells still survived. This was caused by the fact that acetate and lactate are bacteriostatic rather than bactericidal (Jay \textit{et al.}, 2005), and the concentration of organic acid salts at MIC values may not have been sufficient to damage all bacterial cells in the raw pork. Shelef (1994) reported that the antimicrobial agent in foods was not as effective as in broth when used at the same concentration. This was in agreement with Drosinos \textit{et al.} (2006) who indicated that addition of MIX 1 (lactic acid, sodium acetate and potassium sorbate) and MIX 2 (potassium lactate and potassium acetate) prevented the lactic acid bacterial growth in modified MRS, but not in meat product. Moreover, meat composition including protein and fat and some components that are cryoprotectants may protect microorganisms from destruction during freezing. EL.-Kest and Marth (1992) stated that bacterial cells were not affected by freezing in the presence of cryoprotectants such as amino acid, carbohydrates, glucose and calcium.

In this experiment, the number of survival cells of \textit{Salmonella} Rissen and \textit{S. aureus} in pork decreased, while the number of injured cells increased as freezing time increased. A decreasing in the number of these bacteria may not have been only caused by antimicrobial activity of the organic acid salts but also have been a result from the effects of freezing and slow thawing. The cells may have been injured by the formation of ice crystals or exposure to high concentrations of solutes. Ray (1996) stated that almost all water in food is frozen when the temperature is reduced to -20°C. In this condition, ice crystals formed extracellularly and intracellularly, and concentrated solutes in unfrozen water outside the cell affected the viability of microorganisms in that food. The difference in osmotic pressure between the freezing medium and the supercooled cytoplasm results in the movement of water from the inside to the outside of the cells, causing cell dehydration. Microorganisms that are tolerant to dehydration will be resistant to freezing injury (Lowry and Gill, 1985). Therefore, it is possible that three groups of cells, survival cells, injured cells and dead cells may exist in meat after freezing and thawing. Merryman (1966) suggested that
during slow freezing (slow cooling rate at <1°C min⁻¹), microorganisms resistant to dehydration during increasing concentration of solutes will be relatively tolerant to freeze injury. Freeze injury has been demonstrated by loss of viability due to the leakage of cellular materials, increased sensitivity to antimicrobial agents, increased nutritional requirement, extended lag phase and increased sensitivity to radiation (Ray, 1996).

The results of this study indicated that freezing and thawing in combination with organic acid salts were effective in eliminating the pathogenic bacteria in raw pork. Yoon et al. (2004) also reported that addition of a mixed solution of potassium lactate and sodium diacetate decreased the number of survival cells of L. monocytogenes Scott A in smoked salmon stored at -20°C for 10 months.

Using organic acid salts in combination with freezing and thawing is capable of decreasing the number of viable pathogenic bacterial cells in raw pork, thereby enhancing microbiological safety of pork and pork products. However, addition of organic acid salts at concentrations higher than the MIC values is recommended in order to eliminate the pathogenic bacteria effectively.

LITERATURE CITED


