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Full Length Research Paper

Optimized antibacterial measures against *Escherichia coli* O157:H7 and *Staphylococcus aureus*

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Varied mixtures of different concentrations 1, 1.5 and 2% of acetic (AA), lactic (LA), propionic (PA) and formic (FA) acids at 1:1 ratio were spray- washed on inoculated meat to evaluate their efficacy in reducing loads of *Escherichia coli* O157:H7 and *Staphylococcus aureus* on meat tissue. It was found that increasing the concentration of the used organic acids increased the bacterial lethality proportionally. And significant difference ($P<0.05$) was observed in the lethal effect of different mixtures and concentrations of the used organic acids. As a novel combination, FA treatments as combinations with AA, LA, and PA, especially FA with LA, reduced bacterial loads greatly, up to 3 logs cfu/ml and eradicated inoculated bacteria, *E. coli* O157:H7 and *S. aureus* completely within 3-6 days. This reduction was higher than that incurred by other combinations. Significantly, higher log reductions by the used organic acids were obtained for *S. aureus* than for *E. coli* O157:H7. It was concluded that the combination of LA and FA treatment was a highly promising, feasible, and economical method of decontaminating meat surface from both *E. coli* O157:H7 and *S. aureus* bacteria. Moreover, it is safe if compared with other approaches.

Key words: Antibacterial, *Escherichia coli*, *Staphylococcus aureus*, organic acid, sterilization, meat.

INTRODUCTION

Organic acids are weak acids that are commonly found in fruit juices and fermented foods (Luck and Jager, 1997). Organic acids have a long history of being applied as food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredients (Lin et al., 2007). Organic acids are generally recognized as safe (GRAS) antimicrobial agent and the dilute solutions of organic acids (1-3%) are generally without effect on the desirable sensory properties of meat when used as a carcass decontaminant (Smulders and Greer, 1998; Yoshikawa et al., 2007).

Various researchers indicated the antibacterial effect of different types of organic acids by usage different organic acids courtauld reduce the population of bacteria on meat surface; although several studies found that the reductions of bacteria were statistically significant, reductions were not sufficient enough to render meat safe (Shrestha and Min, 2004; Dubal et al., 2004; Ransom et al., 2003; Castillo et al., 2001). Therefore, researchers attempted to find new treatments which can increase the lethality effect of organic acids. To this end, organic acids were mixed with each other or with other antibacterial agents.

The effect of combination of organic acids with other antibacterial agents such as silver ions (Jo et al., 2007), copper (Beal et al., 2004) and hydrogen peroxide (Bell et al., 1997) have been studied. The results of these studies indicated stronger antibacterial effect compared with organic acids alone. However, these treatments might

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Abbreviations: AA, Acetic acid; **LA,** lactic acid; **PA,** propionic acid; **FA,** formic acid; **GRAS,** generally recognized as safe; **DW,** distilled water; **ANOVA,** analysis of variance.

Table 1. The different pair combinations of acetic, lactic, propionic and formic acids at 1, 1.5 and 2% concentrations.

Pair combinations of organic acids (abbreviated symbol)	Different concentrations of pair combinations (%)		
Acetic-Lactic (AALA)	AALA 1	AALA 1.5	AALA 2
Acetic-Propionic (AAPA)	AAPA 1	AAPA 1.5	AAPA 2
Acetic-Formic (AAFA)	AAFA 1	AAFA 1.5	AAFA 2
Lactic-Propionic (LAPA)	LAPA 1	LAPA 1.5	LAPA 2
Lactic-Formic (LAFA)	LAFA 1	LAFA 1.5	LAFA 2
Propionic-Formic (PAFA)	PAFA 1	PAFA 1.5	PAFA 2

have undesirable effects on health of humans caused by the residual traces of silver, copper and hydrogen peroxide on meat surface. Therefore, there is a grave need to formulate a strong antibacterial solution using multiple finely adjusted combinations of different organic acids without using hazardous elements. A recent study investigated the inhibitory effects of 17 organic acids (C-C fatty acids, sorbic, benzoic, phenylacetic, fumaric, succinic, lactic, malic and citric) on *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*; the highest inhibitory activities were observed for benzoic, citric, malic and sorbic acids. Moreover, certain combinations of these acids provided higher protection against tested bacteria (Skrivanova et al., 2010). Another study studied the effect of combination of different organic acids (tartaric, acetic, lactic, malic, and citric acids) on decontaminating meat from *Campylobacter jejuni*; they observed significant lowering of *C. jejuni* bacteria providing evidence that combinational organic acid treatment is the best choice (Birk et al., 2010).

To the best of our knowledge, there was no study investigated a large number of organic acids combinations at different concentrations for controlling different *E. coli* and *S. aureus* on meat all at once. Pre-vious studies focused on limited treatments for controlling other bacteria in which results were inconsistent because of the extensive variations in the experimental conditions. Accordingly, this study compared the antibacterial effect of different two-acids combinations of acetic (AA), lactic (LA), propionic (PA), and formic (FA) acids at 1:1 ratio on *E. coli* O157:H7 and *S. aureus* inoculated on meat in order to investigate, compare and adjust the optimal antibacterial effect of these treatments on the inoculated *E. coli* O157:H7 and *S. aureus* on meat at 4±1°C.

MATERIALS AND METHODS

Organic acids

Three concentrations, 1, 1.5 and 2%, of organic acids that were used in pair combinations at 1:1 ratio were obtained by diluting glacial AA (100%), L-LA (90%), PA (99%) and FA (90%) (Merck, Germany) in sterile distilled water (DW) (Table 1).

Meat preparation

Fresh meat was obtained from a local butchery in Serdang, Selangor, Malaysia. Having been packed in sterile bags, the meat was transported to the laboratory in a cool box. The samples were prepared immediately after transferring meat to the laboratory. Several 10-gram pieces of meats were procured from freshly slaughtered cow.

Bacterial strains

Escherichia coli O157:H7 ATCC 888402 and *Staphylococcus aureus* ATCC 29247 were used. These strains were obtained from the American Type Culture Collection (ATCC, USA).

Organic acids treatment

Each species of bacteria was cultured on standard plate count agar (Merck, Germany) and was then incubated for 24 hours at 37°C. Afterward, bacteria were inoculated in a sterile DW and the concentration of bacterial cells was adjusted to 10^3 bacteria/ml.

The prepared 10 g pieces of meat were decontaminated by washing with hot sterile DW (80°C) for 30 s (Chowdhury et al., 2006); then they were kept for few minutes to reach room temperature. At this stage, about 10^3 bacteria/ml of *E. coli* O157:H7 and *S. aureus* (Benson, 1994) were inoculated individually on decontaminated meat by pouring and swabbing over meats surfaces (Dorsa, 1997). Subsequently, the inoculated meats with selected bacteria were kept for 20 min to allow attachment and adsorption of bacteria (Dubal et al., 2004).

After 20 min, the inoculated meat was spray washed with different combination of organic acids for 15 s individually (Bell et al., 1997). Once the inoculated meat was spray washed and drained, they were packed in sterile bags and were stored at 4±1°C. For each organic acid combination, two sets were treated as a replicate. However, some of the inoculated meats were kept as an inoculation controls.

Post-treatment microbiological analysis

Microbiological analysis was carried out immediately once the spray-washing of organic acids combinations was over till the 12th day of refrigeration. The surface pH of the treated samples was measured by using flat probe pH meter (Prescisa, Switzerland) at 0, 2nd, 6th and 12th days of storage. At this step, each piece of meat (10 g) was aseptically blended with 90 ml of sterile peptone water (Merck, Germany) in a laboratory blender (AOAC, 1990). Afterward, 1 ml of the blended treated samples of each inoculated meat with

Table 2. Log reductions of *E. coli* O157:H7 and surface pH of meat spray-washed with different treatments.

Combination of two acids	Log reduction pH	Concentrations		
		1%	1.5%	2%
AALA	Log10cfu/ml	1.36±0.5	1.49±0.5	1.68±0.5
	pH range	4.45-5.22	4.36-5.17	4.24-5.10
AAPA	Log10cfu/ml	1.25±0.5	1.38±0.5	1.51±0.5
	pH range	4.74-5.47	4.66-5.43	4.57-5.40
AAFA	Log10cfu/ml	1.66±0.5	1.91±0.5	3.18±0.5
	pH range	4.22-5.10	4.13-5.10	4.05-5.14
LAPA	Log10cfu/ml	1.29±0.5	1.44±0.5	1.59±0.5
	pH range	4.48-5.25	4.40-5.20	4.29-5.15
LAFA	Log10cfu/ml	3.18±0.5	3.18±0.5	3.18±0.5
	pH range	4.11-5.18	3.99-5.09	3.89-4.97
PAFA	Log10cfu/ml	1.59±0.5	1.76±0.5	1.94±0.5
	pH range	4.29-5.14	4.16-5.10	4.09-5.08

E. coli O157:H7 or *S. aureus* was transferred onto Petri dishes for pour plate culturing with standard plate count agar (Merck, Germany) individually.

Another 1 ml of each suspension was cultured as a duplicate. The Petri dishes were then incubated for 24 hours at 37°C. Next day, the colonies number for each sample was enumerated in each Petri dish as CFU ml^{-1}.

Statistical analysis

The bacterial population (CFU ml^{-1}) was obtained from four replicates performed on separate days and resulting means were converted to log_{10} CFU ml^{-1}. Differences between log_{10} CFU ml^{-1} of untreated beef carcass tissue and log_{10} CFU ml^{-1} of treated beef carcass tissue were calculated as log reduction (Bell et al., 1997; Bjornsdottir et al., 2006). Log reductions of treatments were compared by analysis of variance (ANOVA) test using the general linear models of SPSS 12.0 for windows, P value < 0.05 was considered as significant.

RESULTS

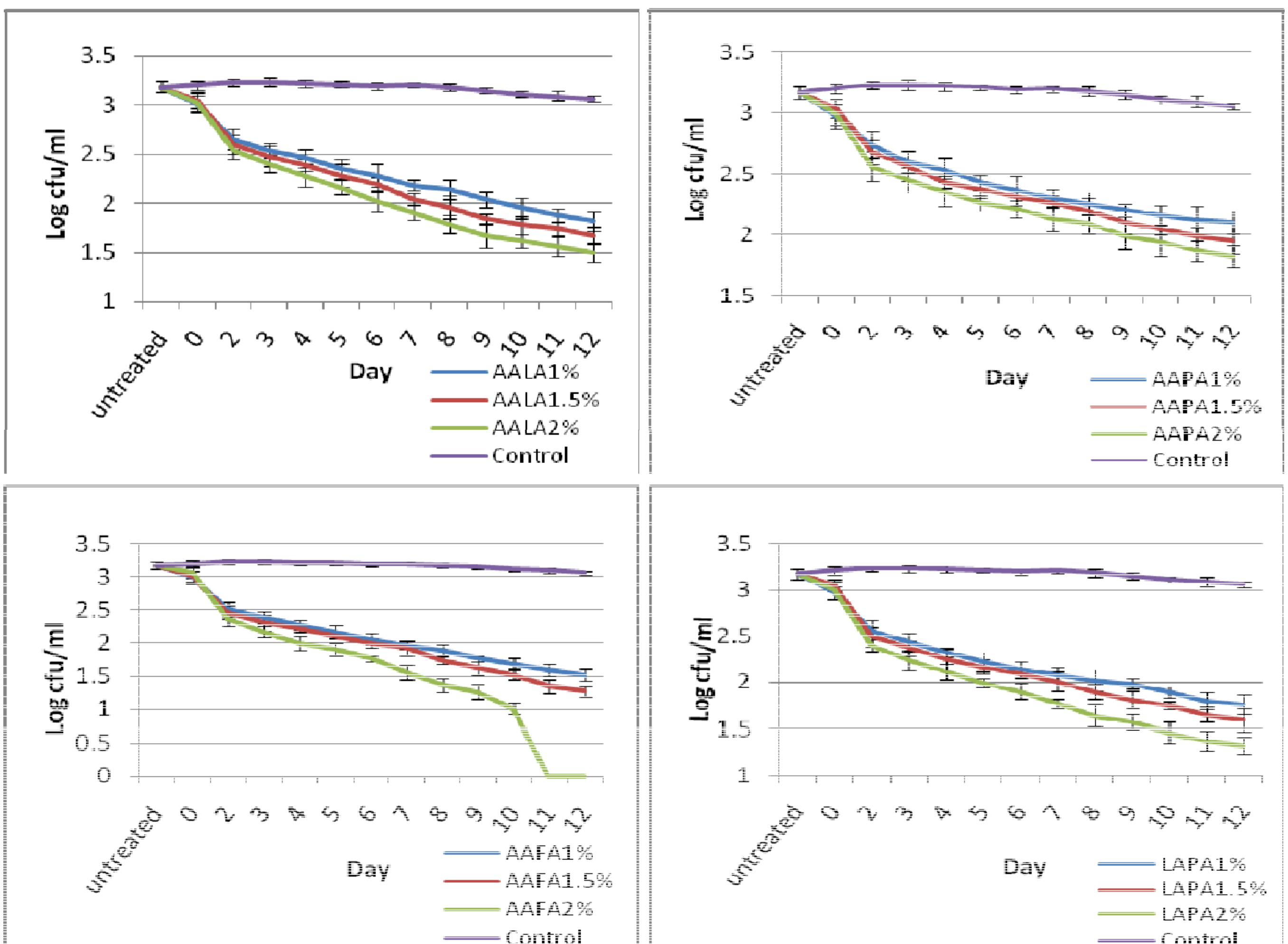
The initial surface pH of meat decreased directly after being spray-washed with organic acid treatments. With progress of storage, it gradually increased (Table 2 and 3) while the pH of untreated meat decreased. The population of *E. coli* O157:H7 (Figure 1 A-F) and *S. aureus* (Figure 2 A-F) decreased remarkably, but in different magnitudes, after being exposed to all treatments of organic acids (Table 2 and 3). The untreated meat showed no significant changes in the population of *E. coli* O157:H7 and *S. aureus* at pH ranges 6.18-5.17 and 6.12-4.86, respectively.

The reduction of selected bacteria showed that they were sensitive to all treatments, namely AALA, AAPLA, AAFA, LAPA, LAFA, and PAFA, but the antibacterial effect of these combinations were different on each bacterium. For *E. coli* O157:H7, ANOVA of log reductions at the end of the storage period showed that LAFA and AAFA exerted the highest antibacterial effect when compared to AAPA, LAPA, AALA, and PAFA treatments (P<0.05). Analysis of log reductions of *E. coli* O157:H7 showed that the mean reductions of AAFA at 2% concentration and LAFA at 1, 1.5 and 2% concentrations were similar at the end of the storage period but they could be distinguished by three way interaction analysis (acid×concentration×day). Three-way interaction analysis showed that these treatments had different log reductions level on different days. The AAFA 2% caused complete log reduction, 3.18 log_{10} cfu/ml, on the 11th day of storage while LAFA at 1, 1.5 and 2% concentrations caused complete log reduction on the 7th, 6th and 4th days of storage respectively (Figure 1 A-F).

For *S. aureus*, ANOVA of log reductions at the end of the storage period showed that AAFA, LAFA, and PAFA exerted the highest antibacterial effect when compared to AALA, AAPA, and LAPA (P<0.05). The mean log reductions of *S. aureus* bacteria spray-washed with AAFA, LAFA and PAFA at 1, 1.5 and 2% concentrations, respectively were similar at the end of the storage period (P>0.05). However, they could be distinguished by the three way interaction analysis (acid×concentration×day). Three-way interaction analysis showed that these treatments had different log reductions levels on different days. Complete bacterial eradication was achieved by

Table 3. Log reductions of *S. aureus* and surface pH of meat spray-washed with different treatments.

Combination of two acids	Log reduction pH	Concentrations		
		1%	1.5%	2%
AALA	Log10cfu/ml	1.60±0.5	1.79±0.5	1.98±0.5
	pH range	4.37-5.29	4.26-5.27	4.18-5.26
AAPA	Log10cfu/ml	1.42±0.5	1.55±0.5	1.73±0.5
	pH range	4.63-5.53	4.50-5.41	4.41-5.46
AAFA	Log10cfu/ml	3.16±0.5	3.16±0.5	3.16±0.5
	pH range	4.12-5.30	4.00-5.15	3.89-5.03
LAPA	Log10cfu/ml	1.48±0.5	1.67±0.5	1.88±0.5
	pH range	4.38-5.34	4.27-5.24	4.15-5.23
LAFA	Log10cfu/ml	3.16±0.5	3.16±0.5	3.16±0.5
	pH range	4.07-5.24	3.93-5.09	3.81-4.97
PAFA	Log10cfu/ml	3.16±0.5	3.16±0.5	3.16±0.5
	pH range	4.18-5.35	4.06-5.22	3.95-5.10



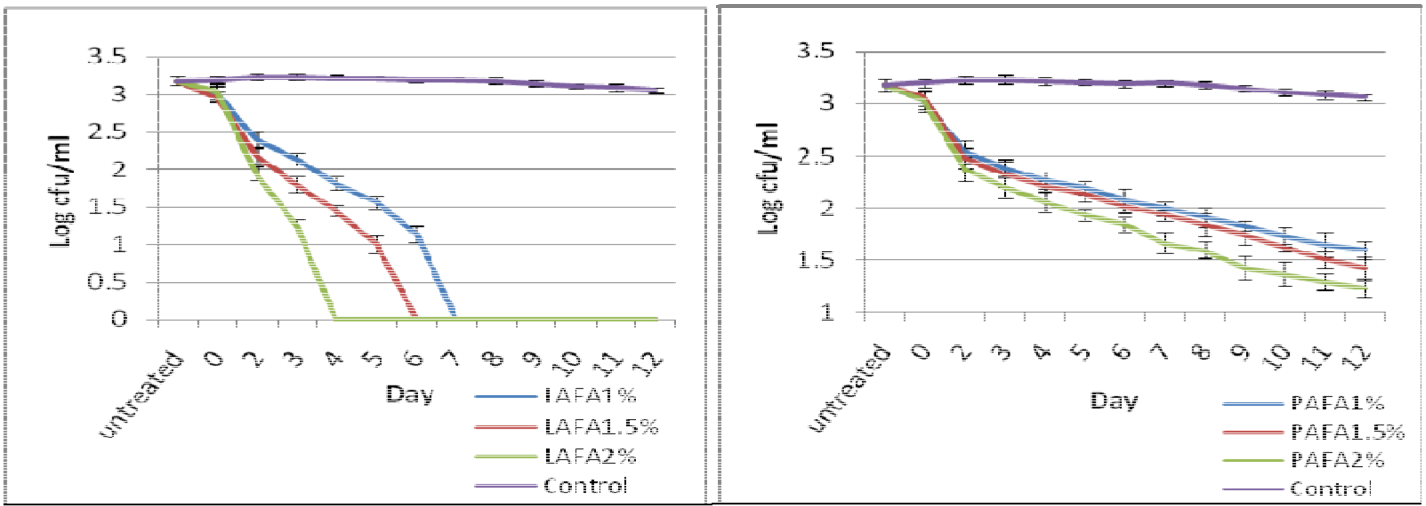
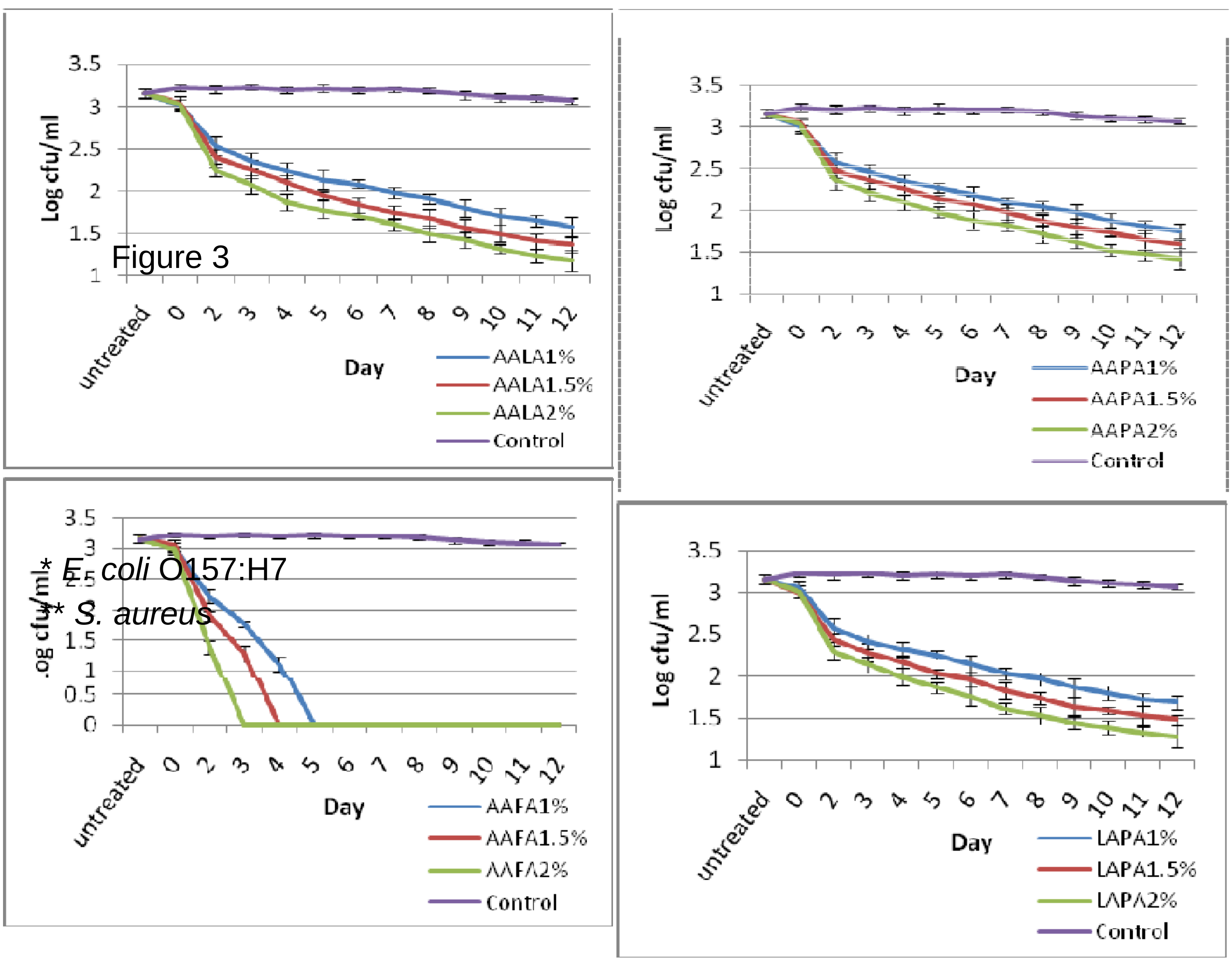


Figure 1. Log reduction of *E. coli* O157:H7 exposed to AALA (1-A), AAPA (1-B), AAFA(1-C), LAPA (1-D), LAFA (1-E), PAFA (1-F) stored for 12 days. A progressive lowering of *E. coli* O157:H7 number was detected over time.



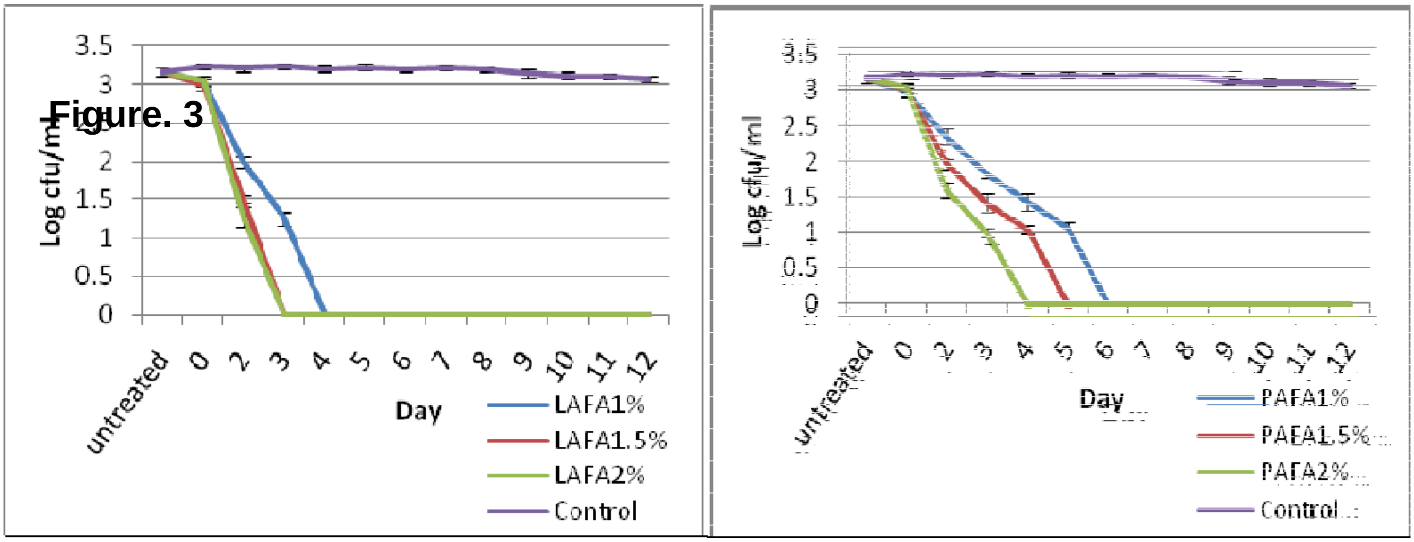


Figure 2. Log reduction of *S. aureus* exposed to AALA (2-A), AAPA (2-B), AAFA (2-C), LAPA (2-D), LAFA (2-E), PAFA (2-F) stored for 12 days. A progressive lowering of *E. coli* O157:H7 number was detected over time.

AAFA at 1, 1.5 and 2% on 5th, 4th and 3rd, LAFA at 1% on 4th, 1.5 and 2% on 3rd, PAFA at 1, 1.5 and 2% on 6th, 5th and 4th day of storage respectively (Figure 2 A-F). Therefore, besides the nature of organic acid used, the concentration of organic acid was found to play an important role to govern the magnitude of log reduction of the treated bacteria. ANOVA of log reduction of *E. coli* O157:H7 and *S. aureus* showed that there was significant difference among 1, 1.5 and 2% concentrations of each organic acid used (P<0.05). The findings of the current study showed that the antibacterial effect of the used organic acids at concentration 2% was higher than at 1.5% which was in turn higher than at 1% (P<0.05).

The log reductions of *E. coli* O157:H7 was compared with that of *S. aureus* after being exposed to various treatments. It was shown that most of log reductions in *S. aureus* bacteria were greater than these of *E. coli* O157:H7 especially for PAFA, and AAFA treatments (Figure 3).

DISCUSSION

The main goal of this study was to investigate the antibacterial effect of different combinations of various organic acids in order to find the best spray-wash treatment that could decrease microbial loads of bacteria quite efficiently on beef tissue.

The current study revealed that various organic acids exerted different lethal effects on the selected bacterial population as well as various bacterial species showed different sensitivity to the same mixtures of organic acids. This indicated that diverse factors might affect the antibacterial activity of organic acids. Chaveerach et al. (2002) revealed that the degree of bactericidal activity of different organic acids on the bacterial cell would most probably depend on the acid concentration, structure of the acid, and capacity of a cell to alkalinize the cytoplasm.

The reduction rate of *E. coli* O157:H7 and *S. aureus* was proportional to the type and the concentration of each treatment. ANOVA for log reduction of *E. coli* O157:H7 and *S. aureus* showed that there was a significant difference between 1, 1.5 and 2% concentrations of each organic acid.

Log reductions analysis showed that the increase in the concentration of organic acids resulted in increasing the antibacterial effect of organic acids. These findings were similar to that of another study (Anderson and Marshall, 1990) which investigated the reduction in the microbial population of *E. coli* and *S. Typhimurium* exposed to 1, 2 and 3% concentrations of lactic acid. They found that population reduction of *E. coli* was more evident by increasing the concentration of lactic acid. Another study also observed that 4% concentration of acetic and lactic acids caused stronger reduction effect on population of bacteria than 2% concentration (Conner et al., 1997).

However, another study did not find significant correlation between concentration of organic acid and bacterial reduction, but they found that the fine adjust-ment of certain organic acids with certain other acids might lead to more striking reductions in bacteria than changing concentration of any given organic acid (Cheung et al., 2010).

The findings of several recent studies showed that the combination of antibacterial agents have stronger antibacterial effect than each one alone (Birk et al. 2010; Cheung et al., 2010; Skrivanova et al., 2010). Dubal et al.

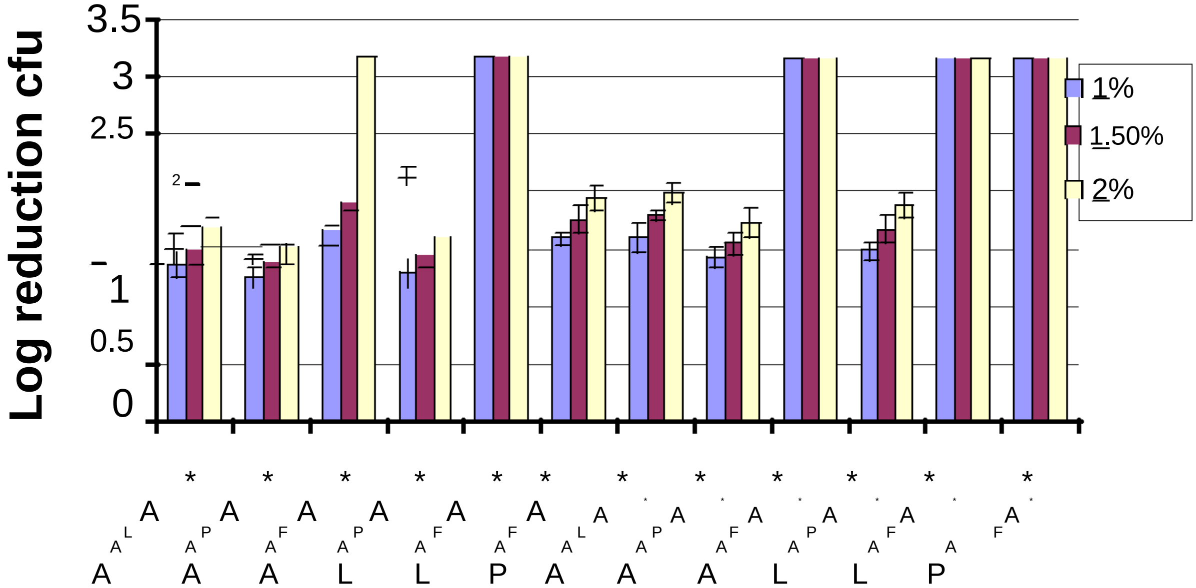


Figure 3. Log reductions of final bacterial eradication of *E. coli* O157:H7 and *S. aureus* exposed to 1, 1.5 and 2% concentrations of AALA, AAPA, AA FA, LAPA, LAFA and PAFA. Most of the used organic acids combinations eradicated both bacteria in range 1.2 to 1.99 log_{10}CFU reduction after 12 days of treatment. However, AAFA 2% and LAFA at 1, 1.5 and 2% showed complete bacterial eradication of *E. coli* O157:H7 on 10^{th}, 6^{th}, 5^{th} and 3^{rd} days of storage respectively. AAFA, LAFA and PAFA at 1, 1.5 and 2% concentrations showed complete bacterial eradication of *S. aureus* on 4^{th}, 3^{rd}, 2^{nd}, 3^{rd}, 2^{nd}, 2^{nd}, 5^{th}, 4^{th} and 3^{rd} days of storage.

(2004) found that the spray-wash of contaminated meat with combination of 1.5% acetic and 1.5% propionic acids had better lethal effect on *S. aureus, L. monocytogenes, E. coli* and *S.* Typhimurium in contrast with lactic acid 2%. Another study indicated that combination of acetic and hydrogen peroxide had greater reduction effect on the population of *E. coli, Listeria innocua* and *Salmonella wentworth* than each one alone (Bell et al., 1997). It was also found that the combination of 2% lactic acid and 2% acetic acid reduced population of bacteria on beef more than each one alone (Goddard et al., 1996). Neverthe-less, most of these studies were limited in terms of the scale of used combinations and the extent of compa-risons made to elect the best formulated combination of antibacterial organic acids. On the other hand, in the current study, three concentrations of six combinations of organic acids were tested against two highly pathogenic bacteria namely *E. coli* O157:H7 and *S. aureus*. For E. coli O157:H7, formic acid in combination with lactic acid, LAFA at 1, 1.5, and 2%, was found to be far the best combination to exert prompt and complete eradication of bacteria within only 7, 6, and 4 days of storage respectively while other tested combi-nations, except for AAFA 2%, failed to induce complete eradication of bacteria after 12 days of storage at 4°C. For *S. aureus*, formic acid combinations with other organic acids showed again the highest antibacterial effects. LAFA, AAFA, and PAFA succeeded to eradicate bacteria completely within 3 to 6 days while other combinations failed to do so.

Hence, LAFA exerted the best reduction effect on two of most problematic bacteria in meat industry and public health, *E. coli* O157:H7 and *S. aureus* namely, *E. coli* O157:H7 and *S. aureus* bacteria, with more than 3 log reduction in just 3-6 days. As far as our knowledge, this is the first study investigated the powerful antibacterial effect of lactic and formic acids combination and this is the first report demonstrates and publishes such findings. The main reason of the stronger antibacterial effect of treatments with formic acid might be attributed to the fact that formic acid is the shortest chain organic acid, which could be beneficial for its diffusion into the cell and cause acidification of the cytoplasm (Chaveerach et al., 2002; Östling and Lindgren, 1993; Skirvanova et al., 2006).

Moreover, the initial surface pH of meat for LAFA treat-ment showed the lowest pH value compared with other treatments. Therefore, the low pH, which is attributed to the release of high number of proton ions in aqueous environment, reflected a considerable synergistic effect resulted by using these two acids. This synergistic effect resulted in the strongest lethal effect on *E. coli* O157:H7 and *S. aureus*. It is noteworthy to mention that 2 and 3 % concentrations of FA, AA, PA, or other acids alone did not lead to a complete eradiation of either *E. coli* O157:H7 or *S. aureus* bacteria within 12 days of storage [data not shown]. Accordingly, since the individual acids, AA, FA, and PA, acted much weaker than the combi-nations, LAFA, PAFA, and AAFA, the most probable mechanism for the very high antibacterial effect of these combinations is synergistic rather than additive. There-fore, adjusting the optimal synergistic effect of different combinations of the organic acids can result in a more effective and faster sterilization. Moreover, it leads to a safer sterilization due to the shorter period required for the sterilization and due to the lower concentrations used of the synergized combinations of organic acids.

However, the mechanism of the observed synergistic effect of the used organic acid combinations is still unknown (Chaveerach et al., 2002; Malicki et al., 2004; Birk et al., 2010). Nevertheless, some hypotheses could be postulated for the stronger lethal effect of combined organic acids in comparison with each one alone. The stronger lethal effect of organic acids combination maybe due to the release of more proton ions by acids in aqueous environment when compared with each one alone or maybe acids when are combined together, the resulting suspension possesses mixture of different structures of acid molecules which helps each pair of acids to compensate for the inherent deficit of the other which thereby augments the inoculating power of the combination (Birk et al., 2010; Skrivanova et al., 2010). Another hypothesis, maybe the most acceptable, the amount of non-dissociated form of organic acids is increased in the aqueous environment when the organic acids are combined together. The antibacterial effect of the organic acids was found to be caused mainly by the non-dissociated form of organic acids (Dibner and Buttin, 2002; Ricke, 2003). Two studies reported individually that a short chain organic acids such as acetic, lactic and citric acids possesses higher bactericidal activity than the non-organic acids such as hydrochloric acid and that bactericidal activity of the organic acids depends mainly on their non-dissociated form (Eklund, 1983; Brocklehurst and Lund, 1990) . The non-dissociated organic acids can passively diffuse through a bacterium's cell wall and once internalized into the neutral pH of the cell cytoplasm, they dissociate into anions and protons, both of which exert an inhibitory effect on bacteria (Ricke, 2003; Nursey, 1997; El-ziney et al., 1997; Carpenter and Broadbent, 2009) leading to the disruption of proton motive force and the inhibition of the substrate transport mechanisms (Carpenter and Broadbent, 2009). Collectively, these actions of organic acids can negatively affect cell viability.

Both of Gram-negative and Gram-positive bacteria are problematic microorganisms in meat industry and public health. The findings of the current study indicated that Gram-positive bacteria, *S. aureus*, were more sensitive to organic acids than Gram- negative bacteria, *E. coli* O157:H7. Similarly, pervious reports revealed that Gram-positive bacteria were more sensitive to organic acids than Gram-negative bacteria. It was observed that lactic acid was 2 to 5 times more efficient in inhibiting the growth of *L. monocytogenes* than enterobacteria (Östling and Lindgren, 1993). It also was reported that the effect of organic acids was more pronounced in cultures of *Clostridium perfringens* than *E. coli* and *Salmonella* sp (Skirnova et al., 2006) . Therefore, the organic acid treatments against Gram negative bacteria have not been satisfactorily successful. Interestingly, the current study showed that 1, 1.5, and 2% of LAFA and 2% of AAFA exerted a complete eradication of *E. coli* O157:H7 population, which is one of the sterilization-resistant Gram negative bacteria. This provided evidence that carefully optimized and selected combinations of organic acids can solve the problem of Gram negative resistance towards sterilizing processes using either individual organic acids or the non-optimized organic acid combinations.

Conclusion

Taken together, the population of *E. coli* O157:H7 and *S. aureus* decreased remarkably after spray-washing with AALA, AAPA, AAFA, LAPA, LAFA and PAFA treatments. Among the treatments, these involved formic acid, showed remarkably stronger lethal effect than others on *E. coli* O157:H7 and *S. aureus*. LAFA showed the best antibacterial effect on both bacteria. In addition, these results indicated that *S. aureus* was more sensitive to organic acids than *E. coli* O157:H7. Collectively, it was concluded that the combination of lactic and formic acids treatment is a feasible and economical method of decontaminating meat efficiently from two of most trouble making pathogens namely, *E. coli* O157:H7 and *S. aureus* without any adverse effects on human health.

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