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Interactions of Organic Acids with *Salmonella* Strains from Feedlot Water-Sprinkled Cattle

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Abstract

Organic acids are commonly used as a carcass wash to remove bacterial loads during food production. In this study the interactions of four organic acids with 145 *Salmonella* strains comprised of six different serovars from feedlot water-sprinkled cattle were studied. The pH was determined at the molar MICs (MIC$_M$s) of the *Salmonella* strains. The concentrations of the undissociated and dissociated organic acids were calculated at the MIC$_M$s of the *Salmonella* strains using the Henderson-Hasselbalch equation. Since all *Salmonella* strains behaved similarly to the different organic acids, the results of the six different *Salmonella* serovars Anatum, Cerro, Gaminara, Kentucky, Meleagridis and Muenster hide and feces strains were treated as a single group for each organic acid, acetic, citric, lactic and propionic acid. Bacterial inhibition was not solely dependent on pH or on the undissociated organic acid species, but was correlated with the dissociated organic acid species. A small drop in the concentration of the dissociated organic acids may result in a large number of bacteria escaping disinfection. Therefore, an organic acid carcass wash may not provide the expected elimination of surface bacteria if the concentration of dissociated organic acid is not carefully controlled. We suggest to maintain a concentration of dissociated propionic, acetic, lactic and citric acids of 15, 16, 17 and 20 mM, respectively, when carrying out a carcass wash with these organic acids to remove *Salmonella enterica* strains.

Keywords

Introduction

Salmonella enterica is a major pathogen causing infections from foodborne sources and is a threat to human health in the United States [1] as well as throughout the world [2]. The Centers for Disease Control and Prevention (CDC) has estimated that each year *Salmonella* causes one million foodborne illnesses in the United States alone, with 19,000 hospitalizations and 380 deaths [3]. *Salmonella* ranks only 2nd in the list of the top five bacterial pathogens contributing to domestically acquired foodborne illnesses in the United States [4]. However, the CDC has linked over 9 million cases of foodborne illness to 31 major pathogens [5]. Of these 31 pathogens, 15 pathogens are primarily associated with 95% or more of the total economic burden of foodborne illnesses, hospitalizations and deaths, and *Salmonella* ranks number one in this list of these 15 pathogens [4]. An analysis of the risk of *Salmonella* illness per serving of beef, lamb, pork, and poultry suggests there is a similar risk of illness from the four different meats [6]. The top ten *Salmonella* serovars isolated from commercial ground beef over a period from July 2005 to June 2007 were Montevideo, Anatum, Muenster, Mbandaka, Agona,

Cerro, Meleagridis, Typhimurium, Dublin and Kentucky [7]. These ten *Salmonella* serovars also have been associated with outbreaks of foodborne illness: Montevideo [8, 9], Anatum [10], Muenster [11], Mbandaka [8], Agona [12], Cerro [13, 14], Meleagridis [15], Typhimurium [16-18], Dublin [19, 20] and Kentucky [21, 22], to list a few instances.

Strategies used to control bacteria in the food chain continuum include the use of biocides as antiseptics and disinfectants [23]. The strategies to control pathogens must be comprehensive, following food products from the farm to the table [24]. An important step in processing beef is to acid wash the carcasses. Organic acids are used to perform the acid wash. The organic acids generally used are acetic [25, 26], citric [27], lactic [25-28] and propionic acids [26]. The acid wash step is very important to remove the bacteria carried along on the carcasses. Bacteria not removed from the carcass may turn up in sliced or ground meat. Therefore, the acid wash step should be studied. Recently, the *Salmonella* serovars Anatum, Montevideo and Kentucky were identified in cattle feces during a study to determine the prevalence of *Salmonella enterica* in South Texas beef cattle feedlots [29]. These *Salmonella* serovars are among the top 10 *Salmonella* serovars isolated from commercial ground meat [7], and can be important players in outbreaks of foodborne illness [8-10, 21, 22].

Researchers have often thought that bacterial inhibition by organic acids was dependent solely on pH or the undissociated acid species [30-32]. But the specific mechanisms by which pH and organic acids inhibit bacteria are not completely understood [33]. We have previously evaluated *Escherichia coli* O157:H7 strains from cattle carcasses, feces and hides and ground beef [34], *Pseudomonas aeruginosa* veterinary isolates [35], and the organic acid inhibition was clearly associated with the dissociated acid species. The disintegration of the LPS layer can be caused by a fully dissociable acid [36]. In a recent study of the big six non-O157 Shiga toxin-producing *E. coli* (non-O157 STEC) [37], overall, the dissociated acid concentrations were more closely correlated with the molar minimum inhibitory concentrations (MIC$_M$s). However, both the dissociated acid form and the undissociated acid form may play a part in bacterial inhibition [36].

In this present study, we describe the interactions of organic acids with six *Salmonella* serovars obtained in an earlier study of water-sprinkling on the incidence of zoonotic pathogens in feedlot cattle [38]. The susceptibility of *Salmonella* serovars Anatum, Cerro, Gaminara, Kentucky, Meleagridis and Muenster to the organic acids acetic, citric, lactic and propionic acids were conducted here. Comparisons are shown of the MIC$_M$s *vs.* the pH, undissociated acid species and dissociated acid species.

Materials and Methods

Salmonella enterica serovars

Salmonella enterica serovars Kentucky (20), Meleagridis (7) and Muenster (19) were isolated from the feces and the *S. enterica* serovars Anatum (1), Cerro (9), Gaminara (1),

Kentucky (45), Meleagridis (13) and Muenster (30) were isolated from the hides of Charolais-crossbred heifers at a commercial feedlot in Lockney, TX, USA [38]. *Salmonella* strains were stored in glycerol and TSB at –80 °C until used. All cultures were then grown for 24 h at 37 °C on trypticase soy agar w/5% sheep blood BBL Stacker Plates (Becton, Dickinson and Company, Sparks, MD, USA).

Organic acid susceptibility testing

MICs for the organic acids were determined by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) [39]. MICs were determined as the lowest concentration of a compound that showed no visible growth of the organism [40]. The organic acid susceptibility studies were carried out similarly to earlier studies with other pathogens [34, 35, 37]. Briefly, 50 µL of each organic acid solution was diluted 1:2 across a 96-well U-bottom Greiner bio-one microplate ((#82050-626, VWR, Houston, TX, USA) through column 11, and column 12 was the positive control. Several well-isolated colonies were selected from a sheep blood agar plate culture. The growth was transferred to a 5 mL Sensititre® demineralized water tube and adjusted to a 0.5 McFarland Standard using a Sensititre® Nephelometer® (Remel, Lenexa, KS, USA). Then 50 µL of the bacterial solution was transferred into 11 mL Sensititre® Mueller-Hinton broth with TES buffer to give an inoculum of approximately 9×10^5 CFU/mL. Then, 50 µL of inoculum was added to each well of the 96-well plate. The plates were covered with a plastic adhesive sealing film, SealPlate® (EXCEL Scientific, Inc., Victorville, CA, USA) and incubated for 20 h at 37 °C. Growth in the wells was visually observed using a Sensititre® SensiTouch® (Remel, Lenexa, KS, USA). *Escherichia coli* ATCC 25922 was used as a control organism for the organic acid susceptibility testing.

Acetic acid was obtained from EM Science (Gibbstown, NY, USA). Citric acid and propionic acid were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Lactic acid was obtained from Alfa Aesar (Ward Hill, MA, USA). The organic acids were diluted with reverse osmosis water to make working solutions and then filter-sterilized using 0.2 µm × 25 mm syringe filters (No. 431224, Corning Inc., Corning, NY, USA). The following concentrations of organic acids were tested: Two-fold dilutions of acetic acid, 32–32,768 µg/mL; citric acid, 16–16,384 µg/mL; lactic acid, 8–8,192 µg/mL; and propionic acid, 32–32,768 µg/mL were used. The pH of the solutions was not adjusted.

pH determination of solutions in the wells at the MICs

The pH was determined by combining the solutions in 16 wells (1,600 µL) at the same MIC value for each organic acid and for all MICs. The pH was measured using an Orion 3 STAR benchtop pH meter with a ROSS Ultra, glass combination pH electrode (#8102BNOWP, Thermo Fisher Scientific, Chelmsford, MA, USA). Each pH determination at an MIC was conducted in triplicate.

Calculation of the ratio of undissociated to dissociated acids

The ratio of the dissociated acid to the undissociated

acid can be calculated when the pH is known by using the Henderson-Hasselbalch equation [41]:

$$pH = pK_a + \log ([A^-]/[HA])$$

Where the pK_a is $-\log_{10}$ of the acid dissociation constant (K_a), $[A^-]$ is the molar concentration of the conjugate base (or dissociated weak acid), and [HA] is the molar concentration of the undissociated weak acid. The Henderson-Hasselbalch equation can be rearranged to give the ratio of undissociated acid to the dissociated acid [31]:

$$ratio = [HA]/[A^-] = 1/10^{pH-pKa}$$

Therefore, when the pK_a of the acid in question and the pH of the solution are known, then the ratio of the undissociated acid to the dissociated acid can be calculated. The pK_a for acetic, citric, lactic and propionic acid is 4.75, 3.14, 3.86 and 4.87, respectively. If the molar concentration of the acid is known, then the concentrations of the undissociated acid and dissociated acid species can be calculated from the ratio.

Results

pH associated with *Salmonella* MICs against the organic acids

The *Salmonella* MICs and MIC_Ms against the organic acids are shown in Table 1. The MICs for acetic, citric, lactic and propionic acids are quite similar between the different *Salmonella* serovars, Anatum, Cerro, Gaminara, Kentucky, Melagridis and Muenster, and the MICs are nearly identical for the same serovars obtained from either cattle hides or feces. Since all *Salmonella* serovars behaved similarly for each organic acid tested, the recorded pH at the MIC_Ms for all strains (n = 145) were combined into a single group at each MIC for each organic acid. Figure 1 shows a graphical representation of the pH obtained at the MIC_Ms of the four organic acids with the 145 *Salmonella* strains. The number of strains at each MIC_M is shown next to each data point. The MIC_M for 97.2% of the strains against acetic acid occurred at pH 5.15. The MIC_M for 100% of the strains against propionic acid occurred at pH
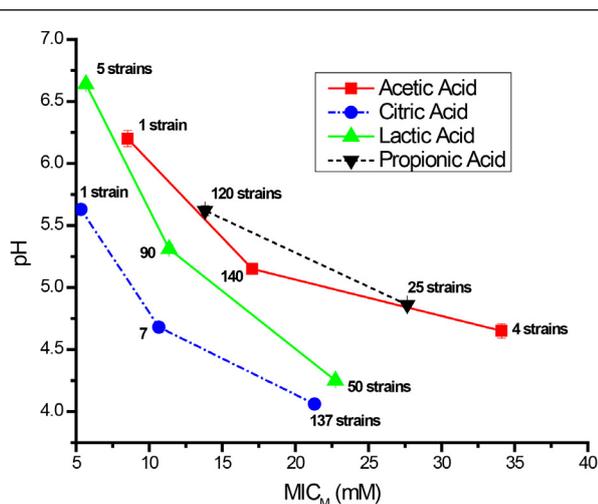

Figure 1: pH at the MIC_Ms of the 145 *Salmonella* strains in acetic, citric, lactic and propionic acids. The error bars represent the ± standard deviation of three pH measurements.

4.86. But the MIC_M for 100% of the strains against citric and lactic acids occurred at pH 4.06 and pH 4.25, respectively. This is almost a pH unit difference between the inhibition of 100% of the bacteria by acetic and propionic acid and the inhibition by citric and lactic acids.

The organic acids characteristic profiles of their interactions with the individual *Salmonella* serovars from hides and feces are shown in detail in the Supplemental Material (SM). The following SM figures show the pH *vs.* MIC_Ms in respect to acetic, citric, lactic and propionic acids against the animal hide strains from serovars Anatum (Figure S1), Cerro (Figure S2), Gaminara (Figure S3), Kentucky (Figure S4), Meleagridis (Figure S5) and Muenster (Figure S6), and against the animal feces strains from serovars Kentucky (Figure S7), Meleagridis (Figure S8) and Muenster (Figure S9).

Calculated concentrations of the undissociated organic acids at the *Salmonella* MIC_Ms

Figure 2 shows the distribution of Henderson-Hasselbalch calculated undissociated acid concentrations for acetic, citric, lactic, and propionic acids at the MIC_M of the 145 *Salmonella* strains. The undissociated acetic acid concentration at the MIC_M of 100% of the *Salmonella* strains tested was 19.0 mM. The undissociated citric acid concentration at the MIC_M of 100% of the *Salmonella* strains tested was 2.29 mM. The shaded band in Figure 2 shows the difference of 16.71 mM concentration whereby 100% of the strains would be inhibited by either the undissociated acetic acid or undissociated citric acid.
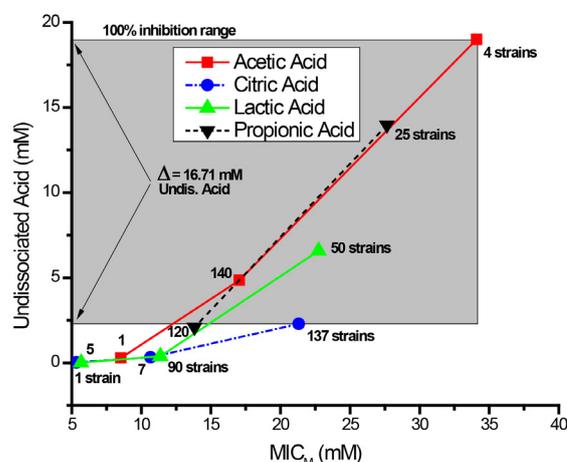

Figure 2: Concentrations of the undissociated acetic, citric, lactic and propionic acids at the MIC_Ms of the 145 *Salmonella* strains. The grey band shows a difference of 16.71 mM between the undissociated organic acids, acetic, citric, lactic and propionic acids required for disinfection of 100% of the *Salmonella* strains.

The following SM figures show the undissociated acid species *vs.* MIC_Ms in respect to acetic, citric, lactic and propionic acids against the animal hide strains from the individual serovars Anatum (Figure S10), Cerro (Figure S11), Gaminara (Figure S12), Kentucky (Figure S13), Meleagridis (Figure S14) and Muenster (Figure S15), and against the animal feces strains from serovars Kentucky (Figure S16), Meleagridis (Figure S17) and Muenster (Figure S18).

Table 1: Organic acid MICs and MIC$_M$s[a] for *Salmonella enterica* strains[b] for all serovars isolated from water sprinkled cattle.

| MIC[c] (µg/mL)	MIC$_M$ (mM)	Anatum	Cerro	Gaminara	Kentucky		Meleagridis		Muenster	
No. total Bacteria		1	9	1	45	20	13	7	30	19
Source		Hide	Hides	Hide	Hides	Feces	Hides	Feces	Hides	Feces
		No. of Bacteria at an Acid MIC								
Acetic Acid										
2048	34.1				1	1	1	1		
1024	17.05	1	9	1	44	19	12	6	30	18
512	8.53									1
Citric Acid										
4096	21.32	1	8	1	45	20	11	6	27	18
2048	10.66		1				2	1	2	1
1024	5.33								1	
Lactic Acid										
2048	22.74		7		18	4	6	3	3	
1024	11.37	1	2	1	27	14	7	4	26	17
512	5.68					2			1	2
Propionic Acid										
2048	27.65	1			8	3	4	3	4	2
1024	13.82		9	1	37	17	9	4	26	17

[a] MIC$_M$s = Molar MICs

[b] Strains were grown for 24 h at 37 ℃ on trypticase soy agar w/5% sheep blood, and the growth was transferred into Mueller-Hinton broth with TES buffer to give an inoculum of approximately 9 × 10⁵ CFU/mL. The inoculum (50 µL) was layered on top of 50 µL-acid solutions in each well of a 96-well U-bottom plate. The plates were covered with adhesive sealing film and incubated for 20 h at 37 ℃.

[c] MICs were visually determined using a Sensititre® SensiTouch®.

Calculated concentrations of the dissociated organic acids at the *Salmonella* MIC$_M$s

Concentrations of the calculated dissociated organic acids, acetic, citric, lactic and propionic acids at the MIC$_M$ of the 145 *Salmonella* strains are shown in Figure 3. The shaded band in Figure 3 indicates the molar dissociated organic acid concentrations required to produce the MIC$_M$s for 100% of the 145 *Salmonella* strains by all four acids. There is a difference in 5.36 mM concentration between the four dissociated organic acids that produce the MIC$_M$s for 100% of the 145 *Salmonella* strains. This difference results from a dissociated acid concentration of 19.03 mM for citric acid and a dissociated acid concentration of 13.67 mM for propionic acid. But there is only a concentration difference of 1.43 mM between the dissociated acetic acid and dissociated propionic acid concentrations that produced the MIC$_M$s for 100% of the 145 *Salmonella* strains.

The following SM figures show the dissociated acid species *vs.* MIC$_M$s in respect to acetic, citric, lactic and propionic acids against the animal hide strains from serovars Anatum (Figure S19), Cerro (Figure S20), Gaminara (Figure S21), Kentucky (Figure S22), Meleagridis (Figure S23) and Muenster (Figure S24), and against the animal feces strains from serovars Kentucky (Figure S25), Meleagridis (Figure S26) and Muenster (Figure S27).
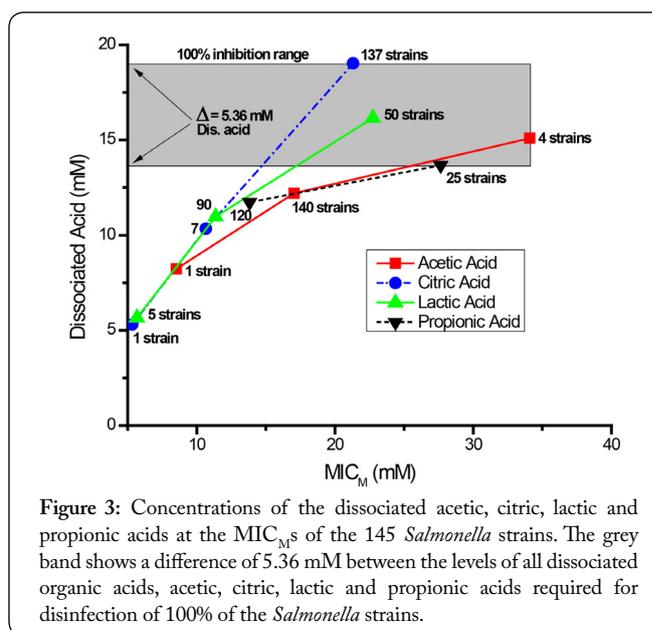
Figure 3: Concentrations of the dissociated acetic, citric, lactic and propionic acids at the MIC$_M$s of the 145 *Salmonella* strains. The grey band shows a difference of 5.36 mM between the levels of all dissociated organic acids, acetic, citric, lactic and propionic acids required for disinfection of 100% of the *Salmonella* strains.

Discussion

When *Salmonella* strains were subjected to acetic and propionic acids, the MICs for 99.3% and 100% of the strains occurred at a pH of approximately 5.2 and 4.9, respectively.

A pH of approximately 4.1 and 4.2 was measured at the MICs of 94.5% and 96.6% of the strains when they were subjected to citric and lactic acids, respectively. This nearly 1 pH unit difference in pH at the MICs of the two acid groups indicate that the MICs of these organic acids were not solely dependent on the pH of the acids, as has been suggested by others [31], but rather some other aspect of these acids. If pH were the primary factor in inhibition of the bacteria, then one would expect the MICs for these same bacteria for all the different acids would be at the same pH value, but that is not the case. The *Salmonella* strains demonstrated similar behavior against the organic acids as did *E. coli* O157:H7 [34], non-O157 STECs [37] and *P. aeruginosa* [35], except the pH values for the *E. coli* O157:H7 strains were somewhat lower, possibly because O157:H7 strains are known to have multiple acid-resistance systems to protect them from extreme acid stress [42]. After comparing the study here of *Salmonella* strains to the previous three studies, *E. coli* O157:H7 [34], the non-O157 STECs [37] and *P. aeruginosa* [35], we observed a trend beginning to develop. The MIC_Ms of 100% of the *Salmonella* strains with acetic acid was at pH 4.9, 98.6% of non-O157 STECs with acetic acid was at pH 4.79 [37] and the MIC_Ms of 99% of the *P. aeruginosa* strains with acetic acid was at pH 4.9 [35]. Since *E. coli* O157:H7 has glutamate- and arginine-dependent acid-resistance systems to protect it from extreme acid stress [42], we see more acid-tolerance in *E. coli* O157:H7. The MIC_Ms of 99.3% of the *E. coli* O157:H7 with acetic acid were somewhat lower at pH 4.29, but citric and lactic acids had a profound effect resulting in the MIC_Ms of 98.2% of the *E. coli* O157:H7 strains at pH 3.95 and pH 3.73, respectively [34]. The MIC_Ms for the major portion of the *Salmonella* strains here with citric and lactic acids were also observed about 1 pH unit lower at pH 4.1 and pH 4.2. *Salmonella enterica* also has efflux systems, and efflux pumps or overexpressed efflux pumps can contribute too many classes of chemical resistance [43]. Also, *S. enterica* has been shown to display arginine-dependent acid-resistance [44]. The MIC_Ms for 100% of the non-O157 STECs with citric and lactic acids were at pH 4.08 and pH 3.98, respectively [37]. However, the MIC_Ms for 98% of the *P. aeruginosa* strains with citric and lactic acids were at pH 4.53 and pH 5.28, respectively [35]. The result obtained for *P. aeruginosa* [35] may well be different since *P. aeruginosa* is known to utilize lactate [45, 46].

The 100% inhibition range for all four undissociated organic acids, acetic, citric, lactic and propionic for the 145 *Salmonella* strains extends from 2.29 mM undissociated citric acid to 19.0 mM undissociated acetic acid. This is a difference of 16.71 mM across the four acid species. There appears to be no correlation as to the concentration of undissociated acids with the MIC_Ms for the 145 strains. These results are consistent with results obtained with *E. coli* O157:H7 [34] and with the non-O157 STECs [37]. The undissociated acetic, lactic and citric acids had a difference in concentration of 47.77 mM for 98.3% of the 344 *E. coli* O157:H7 strains at their MIC_Ms [34]. The undissociated acetic, citric, lactic and propionic acids had a difference in concentration of 14.07 mM for 98.6% of the 138 non-O157 STEC strains at their MIC_Ms [37]. In both of these previous studies, as in this study with *Salmonella*, there

was no indication of a correlation between the concentrations of undissociated acid with the MIC_Ms.

There was a relatively small dissociated acid concentration window for the MIC_Ms of the 145 strains of *Salmonella* for all four organic acids tested. This window had a difference in concentration of 5.36 mM and ranged between 13.67 mM dissociated propionic acid to 19.03 mM dissociated citric acid. This result was very similar to that observed for *E. coli* O157:H7 [34]. The dissociated acetic, citric, lactic and propionic acids had a difference in concentration of 5.44 mM at the MIC_Ms of 98.3% of the 344 *E. coli* O157:H7 strains tested [34]. The concentration window ranged between 13.8 mM dissociated propionic acid to 19.36 mM dissociated lactic acid. The results observed here with *Salmonella enterica* are also very similar to that observed for the non-O157 STECs [37]. The dissociated acetic, citric, lactic and propionic acids had a difference in concentration of 6.19 mM at the MIC_Ms of 100% of the 138 non-O157 STEC strains tested [37]. The concentration window ranged between 12.93 mM dissociated lactic acid to 19.12 mM dissociated citric acid. Also, the dissociated acetic and citric acids had a very small difference in concentration at the MIC_Ms of 98% of the 175 *P. aeruginosa* strains tested [35]. Only a difference in concentration of 0.26 mM was observed for the dissociated acetic and citric acids at the *P. aeruginosa* MIC_Ms, which ranged between 9.98 mM dissociated acetic acid to 10.24 mM dissociated citric acid [35].

It is abundantly clear that the dissociated organic acids have a good correlation with the MIC_Ms for *E. coli* O157:H7 [34], *P. aeruginosa* [35], non-O157 STECs [37], and for the 145 *Salmonella* strains in this study isolated from feedlot water-sprinkled cattle. However, it has been thought that both the dissociated acid form as well as the undissociated acid form may play a role in inhibiting bacteria [36]. We suggest that during the use of the organic acids tested here to control *Salmonella enterica* strains, a dissociated acid content of about 15, 16, 17 and 20 mM propionic, acetic, lactic and citric acids, respectively, be maintained during use of organic acid carcass wash procedures, except for the use of lactic acid and *P. aeruginosa* [35]. Lactic acid is not an appropriate acid to use against *P. aeruginosa* [35].

Conclusion

Inhibition of *Salmonella* strains was not solely dependent on pH or the concentration of undissociated organic acids. The concentration of dissociated acetic, citric, lactic and propionic acids correlated well with the MIC_Ms of 100% of the 145 *Salmonella* strains. A small drop in the concentration of the dissociated organic acids may result in a large number of bacteria escaping disinfection. Therefore, an organic acid carcass wash may not provide the expected elimination of surface bacteria if the concentration of dissociated organic acid is not carefully controlled. A concentration of approximately 15, 16, 17 and 20 mM dissociated propionic, acetic, lactic and citric acids, respectively, is suggested to be maintained when disinfecting the *Salmonella* serovars studied here.

Acknowledgements

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