Inhibitory Effects of oxalic acid on Listeria monocytogenes, Salmonella Enteritidis and Escherichia coli O157:H7 inoculated onto Chicken Breast stored at 4°C

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Abstract

Oxalic acid was evaluated for its effectiveness in inhibiting growth of selected pathogens on raw chicken breasts. Inoculated chicken breasts were dipped in oxalic acid solutions (0, 0.5, 1.0, 1.5 and 2.0% w/v) for 10, 20, and 30 min, packed in oxygen-permeable polyethylene bags, and stored at 4°C. Oxalic acid residues were determined using HPLC method. Counts of pathogens on chicken breasts were determined on days 0, 2, 5, 7, 10, and 14 after storage. Maximum oxalic acid concentration in unwashed chicken breast was 36 mg/100g. Washing of chicken reduced oxalic acid concentration by 50%. Oxalic acid concentration in cooked breast was 2mg/100g which is quite lower than levels in vegetables and herbs, used in daily diets. Chicken meat treated with oxalic acid could therefore be safe for human consumption. Reduction by 2.87, 2.02 and 4.12 log CFU/g, of L. monocytogenes, S. Enteritidis and E. coli O157:H7 respectively was observed in treated samples. Counts of the pathogens in treated samples decreased compared to untreated samples during 14 days storage. Sensory evaluation of cooked oxalic acid treated samples was acceptable to consumers after 14 days of storage. It was evident that oxalic acid has great potential for decontamination of chicken carcasses.

Keywords: Decontamination; Oxalic acid; Poultry; Listeria monocytogenes, Salmonella Enteritidis; Escherichia coli O157:H7.

Introduction

Poultry meat is one of the most common foods that cause foodborne infection and intoxication in humans [1]. Epidemiological reports available have implicated poultry as a major source for human food poisoning outbreaks [2, 3]. Poultry slaughtered in modern poultry processing plants is reported to harbour as many as 27 different species of bacteria [1], some of which are known to be pathogenic to humans. These pathogens are present on feathers and skin of broilers which can enter into the processing plant [4]. In poultry, foodborne disease is caused by pathogens such as Salmonella, Campylobacter jejuni and Listeria monocytogenes which are of great concern globally [3, 5, 6]. Other frequently encountered pathogens such as E. coli O157:H7 and Staphylococcus aureus are also of great concern to the food industry as they are identified as high risk pathogens in temperature abused, ready-to-eat (RTE) pork and poultry-meat products with extended shelf life [7, 8].

Of late, researchers have developed numerous decontamination strategies such as the use of chemicals, organic acids, hot water rinsing and irradiation to reduce the number of pathogenic bacteria capable of causing human diseases via poultry. The use of chemicals and organic acids in reducing pathogens in poultry have proven to be beneficial [9-11].

Oxalic acid, a final metabolic product of plants, including radish, spinach, parsley, carrots etc., has been recognised to possess various functions such anti-browning, antioxidant, intrinsic heat tolerance and inhibition of peroxidase and polyphenol oxidase activities [12-14]. In addition, Huang et al. [12] observed that oxalic acid can effectively control pericarp browning of litchi and banana during postharvest storage. Previously, Anang et al. [15] reported the inhibitory effect of oxalic acid on spoilage microorganisms, including Pseudomonas spp. and those of the Enterobacteriaceae family, in raw chicken. Their results showed that oxalic acid was effective in reducing populations of major spoilage microorganisms on raw chilled chicken breast.

The objective of the present study was to evaluate the effectiveness of oxalic acid in inhibiting growth of Salmonella Enteritidis, E. coli O157:H7 and L. monocytogenes inoculated onto raw chicken breast. It is envisaged that the results obtained from the present study will provide further information on the effectiveness of oxalic acid as a decontamination product for raw poultry and its...
resultant benefit to consumers.

Materials and Methods

Preparation of bacterial inoculum

Cultures of *Listeria monocytogenes* (L55), *Salmonella Enteritidis* (S552) and *Escherichia coli* O157:H7 (E19 – characterised as a non-sorbitol fermenting), originally isolated from patients with foodborne illness, were obtained from the Malaysian Institute for Medical Research. The cultures were activated by transferring 0.05 ml of a frozen (-20°C) stock culture into 10 ml Tryptone Soy Broth (TSB) (Merek, Darmstadt, Germany) and incubated overnight at 35°C. This was then streaked onto Tryptone Soy Agar (TSA) (Merek, Darmstadt, Germany) and incubated for 24 h at 35°C. Working cultures were kept on TSA slants at 8°C and sub-cultured every 2 weeks. Bacterial inoculum was prepared according to the method described by Anang et al. [16] by transferring culture from the slants to 10 ml TSB and incubated at 35°C for 24 h. The 10 ml culture was later transferred to 990 ml TSB and incubated for 18 h. The inoculum was subsequently diluted with fresh TSB on the day of inoculation to yield c.a. 10^8-10^9 CFU/ml of *L. monocytogenes* (L55), *S. Enteritidis* (S552) and *E. coli* O157:H7 (E19).

Inoculation of chicken breast

Fresh, raw chicken breasts (approximately 150 g each) were procured immediately after slaughter. The samples were placed on ice and transferred to the laboratory within 20 min and stored at 4°C for no longer than 2 hours before being used. Each chicken breast was decontaminated, according to the method described by Anang et al. [16] and Greer and Dilts [17], by dipping in 70% ethanol, passed through a flame of a Bunsen burner and allowed to cool. The decontaminated chicken breasts were dipped in suspension of *L. monocytogenes*, *S. Enteritidis* or *E. coli* O157:H7 (10^8 CFU/ml) at room temperature (25°C) for 15 min. Ratio of chicken breast to culture suspension was 1:2 (w/v), which allowed complete immersion of the chicken breast. Initial counts of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 in chicken breast immediately after dipping in the inoculum suspension ranged from log 6.8- log 7.5 CFU/g. Thereafter, the chicken breasts were kept at room temperature for 20 min to allow for inoculum attachment. Inoculated chicken breast (150g and 25°C) was subsequently dipped in 200ml of 0 (control – sterile distilled water), 0.5, 1, 1.5 or 2% of oxalic acid (w/v) for 10, 20 or 30 min [13]. Chicken were completely immersed in the treatment solutions without stirring or agitation. After the oxalic acid dipping, the chicken breast samples were placed in a sterile plastic colander to drain the excess water (control) or oxalic acid solution, and individually placed in Low Density Polyethylene (LDPE) bags (oxygen transmission rate at 25°C). Chicken breasts were subjected to microbial analysis after treatment (day 0) and storage for 2, 5, 7, 10 and 14 days at 4°C. The experiment was repeated twice. In each experiment, two chicken breasts from each treatment were analysed at each sampling time.

Microbiological sampling and analysis

A 25 g sample of breast muscle was excised using a sterile knife and transferred into a stomacher bag containing 225 ml of 0.1% sterile buffered Peptone water (Oxoid CM509) and homogenized using Lab-blender 400 (Seward Laboratory, London) stomacher for 45 s. Serial dilutions were prepared using 0.1% sterile buffered peptone water and inoculated onto duplicate agar plates using a Whiteley Automatic Spiral Plater (WASP2 – Don Whiteley Scientific Ltd., Shipley, West Yorkshire, U.K.). One hundred microlitre (100μl) of respective dilutions were inoculated onto agar plates. *Listeria monocytogenes* was enumerated on PALCAM *Listeria* selective agar base (PALCAM, Merck 1.1222.0001, Darmstadt, Germany) according to Van Netten, et al. [18], to which PALCAM *Listeria* selective supplement (PALCAM, Merck 1.11755, Darmstadt, Germany) was added. The plates were incubated at 35°C for 48 h before counting. *Salmonella* counts were determined by plating diluted samples on XLT4 agar base (Merek 1.13919) supplemented with Sodium tetradecyl sulfate solution (XLT4 agar supplement, Merck 1.08981.0100) and incubated at 37°C for 24 h. Enumeration of *E. coli* O157:H7 was carried out on Sorbitol Mackey Agar (CT-SMAC) with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) (Oxoid CM0981) supplemented with Cefixime Tellurite (Oxoid SR0172) and incubated at 37°C for 24h. The Whiteley Acolyte Automated Colony Counter (Synbiosis Beacon House, Cambridge, UK) was used for counting colonies on each plate.

Determination of oxalic acid residue in treated chicken breast

Oxalic acid residues in treated chicken breasts were determined using a modified method described by Savage et al. [19]. After dipping in various concentrations of oxalic acid and draining excess solution for 5 min, the samples were divided into 2 groups. One group of samples was washed in distilled water for 2 min with mild rubbing before extraction while the other group remained unwashed.

Oxalic acid residue was also determined for oven roasted (160°C for 30 min) chicken breast dipped in 0.5 and 1.5% oxalic acid for 10 min. The representative samples of chicken breast meat were dried to constant weight at 105°C for 2 h to determine dry matter for calculating the oxalic acid content per 100 g of fresh weight.

Extraction of oxalic acid: After dipping in the various solutions of oxalic acid, chicken breast were allowed to drain off excess solution and then divided into 2 groups. One group of chicken breast was washed in distilled water before extraction while the other group was not washed. Oxalic acid residue was also determined for oven roasted (160°C for 30 min) chicken breast that was dipped in 0.5 and 1.5% oxalic acid for 10 min. One hundred grams (100g) of oxalic acid treated chicken breast (taken from the surface, 3 mm thick) was cut in smaller pieces, freeze dried for 24 hrs, using the Labconco Freezene 1 litre benchtop freeze dry system (Labconco, Missouri, USA) and ground to fine sample size using ceramic piston and mortar. One gram (1g) of the ground freeze-dried oxalic acid treated chicken breasts were weighed into 250 ml beakers and 50 ml of 2M HCl added. Each beaker was placed in a water bath at 80°C for 15 min. The extract was allowed to cool to room temperature, and then transferred quantitatively into a 100 ml volumetric flask and made up to 100 ml volume with 2M HCl. The extracts were later centrifuged at 3000 rpm for 2 min and 10 ml of the supernatant filtered through a 0.45 μm cellulose acetate membrane (Sartorius AG, Goettingen, Germany). A 10μl of extract was analysed using a Shimadzu Chromatographic System (Shimadzu Corporation, Japan), consisting of Shimadzu LC-6A Isocratic/Gradient Pump, Shimadzu SPD-6A UV/VIS Spectrophotometric detector set at 214 nm. Chromatographic separation was performed using an
Aminex HPX-87H 300 x 7.8 mm organic acid analysis column (Bio-Rad Laboratories, Ca., USA) with isotropic elution set at 0.6 ml/min with 0.008 M sulphuric acid as mobile phase. Before use, the mobile phase was filtered through 0.45 µm membrane and degassed using a vacuum. A known standard was prepared in the range of 1 to 70 mg/100 ml by dissolving oxalic acid in 50 ml 2M HCl. The solution was quantitatively transferred to 100 ml volumetric flasks and made up to 100 ml volume using 2M HCl. All the standard solutions were filtered through 0.45 µm cellulose acetate membrane filter prior to analysis. Each HPLC analysis of the standards and samples were performed in triplicate.

Statistical Analysis

Statistical analysis of the data obtained was analysed using the SAS software (version 9.4) (SAS Institute Inc., Cary, N.C.). Analysis of variance was carried out at 95% significance level (P < 0.05) and Duncan’s t test was used for the means.

Results and Discussion

Oxalic acid residue in treated chicken breast

Oxalic acid residues in chicken breast dipped in 0.5-2.0% oxalic acid solutions for 10, 20 or 30 min are shown in Table 1. The highest residual amount of 35.6 mg/100g was observed when the chicken breast was dipped in 2.0% oxalic acid for 30 min. This amount which is equivalent to 0.04% (based on 85% dry matter of chicken breast) is quite low compared to the reported values in vegetables and herbs used in daily diets including parsley (1.70%, dry weight basis), chives (1.48%), spinach (0.97%), beet leaves (0.61%), carrots (0.50%), and radish (0.48%) [20]. Washing of chicken with water significantly reduced oxalic acid residues by 50% or more (Table 1). In the cooked chicken breast (160°C for 30 min.) treated with 0.5% and 1.0% oxalic acid, the oxalic acid residues were found to be 1.94 mg/100g and 2.0 mg/100g, respectively.

Based on the predicted LD₅₀ in rats (of 375 mg/kg) [21] and extrapolating from this dose for a person weighing 150 lb (68.1 kg), consumption of 25.5 g of oxalic acid is required for an LD₅₀. Based on the rat model considered, it was concluded, an individual would have to consume about 71.63 kg of the 2.0% oxalic acid treated chicken breast (unwashed after treatment) to have 25.5 g oxalic acid required for an LD₅₀. Hence, treating chicken breast with oxalic acid at the current level proposed in the study, and compared to oxalic acid contents in vegetables and herbs used in daily diets, it was concluded that the available amounts contain in the chicken breast after treatment would be safe for human consumption.

Listeria monocytogenes, Salmonella Enteritidis and Escherichia coli O157:H7 growth during storage of chicken breast

Table 2 shows the log-reduction in L. monocytogenes, S. Enteritidis and E. coli O157:H7 inoculated onto chicken breast and treated with different concentrations of oxalic acid. According to Blackburn and McCarthy [22], the use of SMAC-based media may lead to overestimated count of E. coli O157:H7. Report from the work by Clavero and Beuchat [23], Sage and Ingham [24] and Singh et al. [25] have indicated poor recovery of injured cells and therefore underestimation of numbers of viable E. coli O157:H7 when SMAC was used. In addition, previous studies conducted using the methods described by Blackburn and McCarthy [22] observed no difference in injured E. coli O157:H7 cells during organic acid decontamination [26, 27]. The result for Salmonella Enteritidis reduction was less when compared to L. monocytogenes which has maximum reductions of log 2.0 CFU/g at the highest concentration after dipping in oxalic acid.

Table 3 presents the result of L. monocytogenes counts in inoculated chicken breast treated with oxalic acid during storage at 4°C. A decrease in Listeria monocytogenes was recorded until day 2 for samples treated with 0.5 and 1.0% oxalic acid solutions for 10, 20 or 30 min. However, Listeria counts on chicken breast dipped in 0.5, 1.0 or 1.5% oxalic acid solution for 10, 20 or 30 min were ca. 1.79-3.24 log CFU/g less on day 2 compared to those before treatment. Listeria counts on chicken breast treated with water (control) decreased slightly on day 2 of storage but increased relatively fast throughout the storage time. There was a significant reduction in Listeria monocytogenes counts when chicken breasts were treated with 2% oxalic acid for 10 min compared with 20 or 30 min until day 7 of storage. However, a significant decrease in Listeria monocytogenes was observed between the 3 dipping times after

| Oxalic acid concentration (%) | Dipping duration (min) | Oxalic acid residue in chicken (mg/100g) |
|-----------------------------|------------------------|----------------------------------------|
|                             | Unwashed chicken       | Washed chicken                         |
| 0.5                         | 10                     | 8.97¹*                                 |
|                             | 20                     | 9.76¹*                                 |
|                             | 30                     | 11.29¹*                                |
| 1.0                         | 10                     | 10.07¹*                                |
|                             | 20                     | 11.29¹*                                |
|                             | 30                     | 12.46¹*                                |
| 1.5                         | 10                     | 13.50¹*                                |
|                             | 20                     | 20.72¹*                                |
|                             | 30                     | 28.66¹*                                |
| 2.0                         | 10                     | 16.16¹*                                |
|                             | 20                     | 22.60¹*                                |
|                             | 30                     | 35.64¹*                                |

Mean values in the same row bearing different superscripts are significantly different from each other (P ≤ 0.05).

*Oxalic acid residue of treated chicken breast oven-roasted (160°C for 30 min.)

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Table 2. Log-reduction of *Listeria monocytogenes*, *Salmonella* Enteritidis and *E. coli* O157:H7 inoculated onto chicken breast and treated with different concentrations of oxalic acid for 10, 20 and 30 min.

| Oxalic acid concentration (%) | Dipping duration (min) | Log CFU/g |
|------------------------------|------------------------|-----------|
|                              | L. monocytogenes | S. Enteritidis | E. coli O157:H7 |
| 0.0                          | 10                | 0.51        | 0.05     | 0.17    |
|                              | 20                | 0.55        | 0.01     | 0.23    |
|                              | 30                | 0.48        | 0.06     | 0.16    |
| 0.5                          | 10                | 1.52        | 0.75     | 3.33    |
|                              | 20                | 1.81        | 0.90     | 3.32    |
|                              | 30                | 2.06        | 0.78     | 3.44    |
| 1.0                          | 10                | 1.55        | 0.84     | 3.66    |
|                              | 20                | 2.51        | 1.11     | 3.83    |
|                              | 30                | 2.31        | 1.37     | 3.9     |
| 1.5                          | 10                | 1.87        | 1.28     | 3.67    |
|                              | 20                | 2.57        | 1.41     | 3.86    |
|                              | 30                | 2.64        | 1.83     | 3.99    |
| 2.0                          | 10                | 2.49        | 1.32     | 3.68    |
|                              | 20                | 2.87        | 1.78     | 3.94    |
|                              | 30                | 2.67        | 2.02     | 4.12    |

Table 3. Counts of *Listeria monocytogenes* (log CFU/g)

| Oxalic acid concentration (%) | 10-min dip | 20-min dip | 30-min dip |
|-------------------------------|-----------|-----------|-----------|
|                               | 0  | 2  | 5  | 10 | 14 | 0  | 2  | 5  | 10 | 14 | 0  | 2  | 5  | 10 | 14 |
| 0.0                           | 6.79 | 6.53 | 7.43 | 7.66 | 8.16 | 8.63 | 6.74 | 6.55 | 7.54 | 7.71 | 8.28 | 8.64 | 6.95 | 6.92 | 7.20 | 7.71 | 8.35 | 8.64 |
| 0.5                           | 5.93 | 5.66 | 6.59 | 6.71 | 6.72 | 6.74 | 5.63 | 5.02 | 5.55 | 5.93 | 6.16 | 6.54 | 5.39 | 4.83 | 4.59 | 5.55 | 5.55 | 6.53 | 6.68 |
| 1.0                           | 5.90 | 5.61 | 6.44 | 6.52 | 6.45 | 6.51 | 4.94 | 4.82 | 5.38 | 5.47 | 6.03 | 6.35 | 5.14 | 4.96 | 4.84 | 5.19 | 5.40 | 6.16 | 6.76 |
| 1.5                           | 5.58 | 5.40 | 5.68 | 5.81 | 5.83 | 5.08 | 4.88 | 4.91 | 5.78 | 4.95 | 5.12 | 4.81 | 4.33 | 4.62 | 4.66 | 4.67 | 4.35 |
| 2.0                           | 4.96 | 4.82 | 5.50 | 5.76 | 5.59 | 5.68 | 4.58 | 4.31 | 4.57 | 4.65 | 4.81 | 4.98 | 4.78 | 4.21 | 4.40 | 4.61 | 4.61 | 4.75 |

1 Mean values in the same column bearing different superscripts are significantly different from each other (P ≤ 0.05).
2 Mean values in the same row under same day bearing different superscripts are significantly different from each other (P ≤ 0.05).

storage for 10-14 days. After day 14 of storage, *Listeria* counts on oxalic acid treated chicken breast were 1.9-3.89 log CFU/g less than on control breasts. In addition, at the end of the 14 day storage *Listeria monocytogenes* counts on chicken breast treated with 2.0% oxalic acid for 20 or 30 min were less than 5 log CFU/g. The optimum concentration of oxalic acid and dipping time, for decontamination of *Listeria monocytogenes*, is the combination of 2% and 30 min of dipping. However, 1.5% oxalic acid at 30 min dipping time achieved similar results.

With regard to *E. coli* O157:H7, treating chicken breast with oxalic acid for 10 to 30 min caused a significant reduction in *E. coli* O157:H7 counts and also retarded its growth during the 14 days of storage at 4°C (Table 5). However, *E. coli* O157:H7 counts on chicken breast dipped in water (control) continued to increase throughout the 14 days storage and reached a maximum of between 8.10 and 8.21 log CFU/g at the end of storage. Dipping chicken breast in oxalic acid for 30 min resulted in significantly greater reductions in *E. coli* O157:H7 counts compared to 10 or 20 min dipping, *E. coli* O157:H7 counts on 1.5% oxalic acid treated chicken breast for 30 min and 2.0% oxalic acid treated chicken breast for 20 or 30 min remained less than 4 log CFU/g throughout the 14 days storage. At the end of the 14 days storage, *E. coli* O157:H7 counts on oxalic acid treated chicken breast were 3.01-4.33 log CFU/g less than counts on water treated chicken breasts which were above log 8.0 CFU/g. *Salmonella* counts were reduced significantly to 2% oxalic acid and dipping time, for decontamination of *E. coli* O157:H7 independent of time of dipping. From the results, the optimum concentration of oxalic acid and dipping time, for decontamination of *E. coli* O157:H7, is the combination of 2% at 20 min. However, 1.5% oxalic acid at 20 min dipping time seem to achieve similar results. 

There are several published reports on the use of organic acids such as lactic acid for reducing the microbial counts in poultry, meat and in swine [16, 28-30]. Reduction in *Listeria monocytogenes*...
counts on oxalic acid treated chicken breast was relatively similar to earlier reports by Anang et al. [16] and Gonçalves, et al. [31] in chicken breast treated with up to 4% lactic acid. The authors had reported a 2.87 log CFU was observed when chicken breast was dipped in 2% oxalic acid at 25°C for 20 min. Greer and Dilts [17] treated pork lean tissue with 3% lactic acid and observed 1-log reduction in L. monocytogenes counts. Furthermore results from their study, after storage for 7 days, a maximum of 2 log reduction in Listeria counts was observed when pork lean tissue was treated with 3% lactic acid at 55°C. In another study, Ozdemir et al. [30] reported a 0.69 and 1.09 log reduction in L. monocytogenes counts in beef treated with 1 and 2% lactic acid, respectively. These reductions in Listeria counts were relatively lower than those reported in the present study, which were 1.55 and 2.49 log CFU/g (1 and 2% oxalic acid). Further from their study, after 5 days of storage (at 4°C) reductions in Listeria counts (by 2.16 and 2.54 log, respectively) were also observed. Whereas in this study, after 5 days of storage, the log reduction in Listeria count was at a maximum of 2.26 and 3.05 log at 1 and 2% levels of oxalic acid, respectively. Additionally, organic acids such as acetic acid have also been indicated to be effective against Listeria monocytogenes inoculated in beef [10, 29, 32].

Results of the present study indicate that oxalic acid is more effective in killing and inhibiting S. Enteritidis and E. coli compared to lactic and acetic acid. Beef inoculated with S. Typhimurium treated with 2% acetic acid, showed a log reduction of 1 unit [33]. Podolak et al. [34] also reported a log reduction of 0.2–0.8 units in Salmonella counts when beef was treated with 1% lactic or acetic acid at 55°C. Anang et al. [16] observed just above one log reduction in E. coli O157:H7 inoculated chicken breast treated with up to 4% lactic acid. The authors had observed a complete inhibition of Salmonella Enteritidis when chicken breast was dipped in oxalic acid, the maximum log reduction in Salmonella count was 2.02 whilst the maximum log reduction in E. coli count was more than doubled, at a value of 4.12. Counts of S. Typhimurium DT104 and non-O157:H7 enterohaemorrhagric E. coli inoculated onto beef surface were equally reduced in the range of 2-3 log UTCD when acetic and oxalic acid spray [37].

The difference in the effect of oxalic acid against Salmonella Enteritidis and E. coli O157:H7 in the present study will, however, needs further evaluation.

Results from the present study clearly demonstrated that treating chicken breast with oxalic acid could be beneficial in the decontamination of poultry contaminated by certain common foodborne pathogens. The results also indicate the possible delay in growth or inhibition of the pathogens during subsequent storage at 4°C. Further commercial application of oxalic acid as a preservative is warranted.

Acknowledgments

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