

PERSISTENCE OF *SALMONELLA* SPP. ON CHICKEN SKIN AFTER EXPOSURE TO AN ITALIAN MARINADE*

THOMAS P. OSCAR^{1,3} and MANPREET SINGH²

¹*U.S. Department of Agriculture
Agricultural Research Service
Room 2111, Center for Food Science and Technology
University of Maryland Eastern Shore
Princess Anne, MD 21853*

²*Department of Poultry Science
Auburn University
Auburn, AL*

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ABSTRACT

A series of experiments with chicken skin was undertaken to determine the effect of an Italian marinade on persistence of Salmonella spp. during refrigerated storage and marinating. Chicken skin was inoculated with 0.4 to 3.7 log of multiple antibiotic resistant strains of Salmonella Typhimurium (n = 3), Kentucky (n = 1) or Hadar (n = 1). Chicken skin was then exposed to the Italian marinade for 4 or 24 h at 6C to simulate normal marinating conditions of consumers. The persistence of Salmonella spp. on chicken skin was reduced (P < 0.05) by the Italian marinade with a greater reduction observed at 24 h than at 4 h of marinating. As expected, the persistence during marinating increased as a function of the initial number of Salmonella inoculated. In general, the effect of the Italian marinade on persistence was similar among the five strains of Salmonella tested.

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³Corresponding author. TEL: 410-651-6062; FAX: 410-651-8498; EMAIL: thomas.oscar@ars.usda.gov

PRACTICAL APPLICATIONS

Marinades contain organic acids (acetic, lactic) that decrease meat pH and help to reduce or eliminate pathogenic bacteria, such as *Salmonella*, from the product. Inclusion of other ingredients in marinades, such as salts and spices that have antimicrobial properties, helps to further reduce or prevent persistence of *Salmonella*. Results of this study with an Italian-style marinade indicate that consumers should marinate chicken in the refrigerator for 24 h rather than 4 h to maximize the benefit of the Italian marinade on reducing the risk of exposure to *Salmonella* that might contaminate and persist on chicken purchased in the retail marketplace.

INTRODUCTION

The poultry industry has strived to bring a safe protein source for meat consumers in the U.S.A. Through the development of Hazard Analysis Critical Control Points programs and more stringent U.S. Department of Agriculture guidelines, the reduction of microbes has been identified as a key goal of the poultry industry. Pathogenic bacteria are the most common cause of foodborne illness, with poultry being identified as one of the most common reservoirs of *Salmonella* spp. (D'Aoust 1989). An estimated 1.4 million people are sickened each year as a result of salmonellosis (Mead *et al.* 1999), with poultry and poultry products being implicated as the primary source of infection (Castillo *et al.* 2008). The increased demand for convenience foods has resulted in expansion of the processed meat and poultry industry. This has led to the development of further processed poultry products that includes items like battered pieces, breaded and pre-cooked cold cuts or marinated portions (Baker and Bruce 1989).

Marinating of meats is a practical method that has been used for many years to reduce the aging time required for meat tenderization. Marinades are a common method of poultry preparation and can be made from a variety of ingredients that offer both flavor and protection against pathogenic bacteria. Marinades alter the pH and color of meats, enhance flavor, improve tenderness and juiciness, and increase the weight of saleable product because of the retention of added water (Bjorkroth 2005). Marinades often make use of organic acids, salts, and plant extracts and oils that can offer a protective measure when incorporated with safe processing measures, ensuring a safe product for the consumer. When used in conjunction with other processes such as irradiation, an additive antimicrobial effect can be achieved (Mahrouf *et al.* 2003). The retail shelf life of refrigerated marinated chicken products is approximately 10 days. Treatment with marinades will increase the shelf life

of various meats and poultry products by preventing the growth of spoilage organisms. This is based on low pH, high sodium chloride concentration and the various spices in the marinade.

Marinades incorporate the intrinsic properties of their ingredients to provide flavor and antimicrobial protection. Organic acids (acetic, lactic) are commonly used as marinade ingredients; by decreasing the pH, the product becomes less suitable for the growth of bacteria (Bjorkroth 2005). However, when testing different acids on chicken thighs and legs, Moutney and O'Malley (1965) found that the higher concentrations needed to achieve maximum bactericidal efficacy caused adverse effects to poultry meat quality. The addition of other ingredients in cooperation with acids is necessary to achieve a better marinade product. Organic salts are also used as marinade ingredients. Chloride and phosphate salts are commonly used in meat products to provide antimicrobial properties. Sodium chloride rinses can not only prevent the attachment of bacteria, but also effectively reduce the numbers of previously attached bacteria (Thomas and McMeekin 1981; Oscar 2008). Marinades also incorporate the use of essential plant oils. There exist many naturally occurring antimicrobial agents from plants and animals, and even other bacterial species (Sofos *et al.* 1998). Spices have long been used for flavoring and food protection. Eugenol, a clove extract, is effective against *Salmonella* Typhimurium (Aktug and Karapinar 1987). Also, clove oleoresin and pimento leaf oil have been shown to be effective at 4C in reducing the numbers of spoilage organisms on chicken, but with no effect on *Listeria monocytogenes*, a pathogenic organism (Carlos and Harrison 1999). The initial binding of bacteria to the meat surface involves ionic interactions (Firstenberg-Eden *et al.* 1978; Firstenberg-Eden 1981), and marinating can reduce the attachment of *Salmonella* to the chicken.

The objective of this research was to determine whether an Italian marinade affects the persistence of *Salmonella* spp. on chicken that is stored and marinated at a refrigeration temperature (i.e., 6C) under conditions found in the consumer's home.

MATERIALS AND METHODS

Salmonella

Chicken skin was inoculated with multiple antibiotic resistant strains of *S. enterica* serotype Typhimurium definitive phage type 104 (DT104; ATCC 700408, American Type Culture Collection, Manassas, VA) and multiple antibiotic resistant strains of *S. enterica* serotypes Kentucky, Hadar and Typhimurium (s172 and s173) that were isolated from poultry. Multiple antibiotic

resistant strains were used to facilitate the detection of *Salmonella* in the presence of native flora. Stock cultures of the *Salmonella* spp. were maintained at -70°C in brain heart infusion (BHI; Difco, Becton Dickinson, Sparks, MD) broth that contained 15% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO).

Chicken Skin

Chicken thighs were purchased weekly from local retail outlets. The skin was removed and spread on a cutting board, followed by freezing at -20°C for 15 min. A number 10-cork borer was used to cut circular pieces of skin (2.14 cm^2) for challenge studies. Freezing was done to facilitate the cutting of the skin.

Inoculation Cultures

Thawed stock culture ($5\ \mu\text{L}$) was added to BHI broth (5 mL) in an Erlenmeyer flask (25 mL) that was sealed with a foam plug. Cultures were incubated at 30°C for 23 h and 150 rpm to obtain stationary phase cells for inoculation. Before the inoculation of skin, cultures were serially diluted to 10^{-7} in buffered peptone water (BPW; Difco Laboratories). The concentration of *Salmonella* in the inoculation culture was determined by spiral plating (Whitley Automated Spiral Plater, Microbiology International, Frederick, MD) $50\ \mu\text{L}$ of the 10^{-6} and 10^{-7} dilutions onto the appropriate agar medium for each strain followed by incubation at 38°C for 24 h and automated counting of the colonies that formed (Protocol, Microbiology International). The concentration of *Salmonella* in the inoculation culture (i.e., $\sim 10.2\ \log\ \text{cfu/mL}$) and the dilution (i.e., 10^{-7}) inoculated was used to calculate the dose inoculated onto the chicken skin.

Marinating Simulation

Chicken skins were placed into individual wells of a 12-well tissue culture dish (Falcon® Multiwell™, Becton Dickinson, Franklin Lakes, NJ) and then spot-inoculated with $2\ \mu\text{L}$ of a 10^{-7} , 10^{-6} , 10^{-5} or 10^{-4} dilution of the appropriate *Salmonella* culture. Before application of marinade, inoculated skins were incubated at 22°C for 15 min to allow the attachment of *Salmonella*. Marinade ($\frac{1}{2}$ tsp or 2.5 mL) was applied or not, and then inoculated skins with and without marinade were stored at 6°C for 4 h or 24 h to simulate normal marinating and storage times used by consumers. The marinade completely covered the skin portion.

Marinade Preparation

The marinade was prepared by mixing one teaspoon or 5 mL of garlic powder and one level teaspoon of salt with one 16-oz (473 mL) bottle of

Italian-style salad dressing (Zesty Italian Dressing, Kraft Foods Global, Inc., Northfield, IL). The listed ingredients in the Italian-style salad dressing were: water, vegetable oil (soybean oil and canola oil), vinegar, high-fructose corn syrup, salt and less than 2% dried garlic, garlic, dried red bell peppers, dried onions, xanthan gum, spice, potassium sorbate, calcium disodium EDTA, lemon juice concentrate and oleoresin paprika.

Prevalence Assay

After marinating, individual chicken skins were transferred to individual stomacher bags (207-mL capacity, Whirl-Pak, Nasco, Fort Atkinson, WI) that contained BPW (40 mL). Samples were pulsified (Pulsifier® model PUL 100, Microbiology International) for 10 s, followed by incubation at 38C for 24 h. After incubation in BPW, the sample contents were mixed by manually massaging the bag for 15 s, and then the skin sample incubate (2 µL) was spot-inoculated onto XLH-CATS for *S. Typhimurium* isolates, XLH-NATS for *S. Kentucky* and XLH-TUGS for *S. Hadar*. All three were xylose lysine (XL) agar media that contained 25 mM of the buffering agent N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] or HEPES (H) and four of the following seven antibiotics (25 µg/mL each): chloramphenicol (C), ampicillin (A), tetracycline (T), streptomycin (S), novobiocin (N), sulfasoxazole (U) or gentamicin (G). All media supplements were from Sigma-Aldrich. It should be noted that since the *Salmonella* were pre-enriched in BPW for 24 h before spot inoculation onto selective media with multiple antibiotics, the assay allowed for recovery of injured cells and thus, decreases in prevalence were not over-estimates of food safety. The limit of detection of this assay is one viable cell per skin portion.

Experimental Designs

Eight experiments were conducted. The primary experimental designs were dose–response and two-by-two factorials. The dependent variable was *Salmonella* prevalence (%) and the independent variables were dose, time and strain. Three to five trials, each with a different batch of 10 or 12 chicken skins per treatment, were conducted per experiment.

Statistical Analysis

Dose–response experiments were analyzed by one-way analysis of variance (ANOVA), and when a significant effect ($P < 0.05$) of dose was detected, means were separated using Tukey's multiple comparison test. Two-by-two factorial experiments were analyzed by two-way ANOVA to determine the significance ($P < 0.05$) of main effects and the interaction of independent

variables. All statistical analyses were performed using version 5.0 of Prism® (GraphPad Software, Inc., San Diego, CA).

RESULTS

Experiment 1 (Fig. 1) examined the persistence of a low initial dose (0.4 log) of *S. Kentucky* on chicken skin exposed to Italian marinade for 24 h at 6C. Although the persistence of *S. Kentucky* on chicken skin was not affected ($P > 0.05$) by refrigerated storage for 24 h, marinating the chicken skin for 24 h at 6C completely eliminated all of the inoculated cells of *S. Kentucky*.

Experiment 2 (Fig. 2) examined the effect of initial dose on the prevalence of *S. Typhimurium* DT104 on chicken skin after 4 h of exposure at 6C to the Italian marinade. After 4 h of marinating, the persistence of *S. Typhimurium* DT104 on chicken skin was reduced ($P < 0.05$) at the two lowest (0.5 and 1.5 log) but not at the two highest (2.5 and 3.5 log) initial

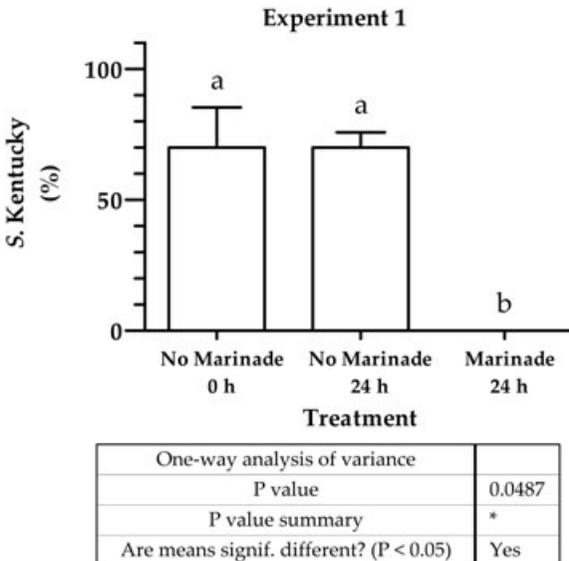


FIG. 1. EXPERIMENT 1 – PERSISTENCE OF A LOW INITIAL DOSE (0.4 log) OF *SALMONELLA* KENTUCKY ON CHICKEN SKIN EXPOSED TO ITALIAN MARINADE FOR 24 h AT 6C. BARS ARE THE MEAN ± SEM OF THREE SEPARATE TRIALS WITH 10 CHICKEN SKINS PER TREATMENT PER TRIAL. BARS WITH DIFFERENT LETTERS DIFFER AT $P < 0.05$

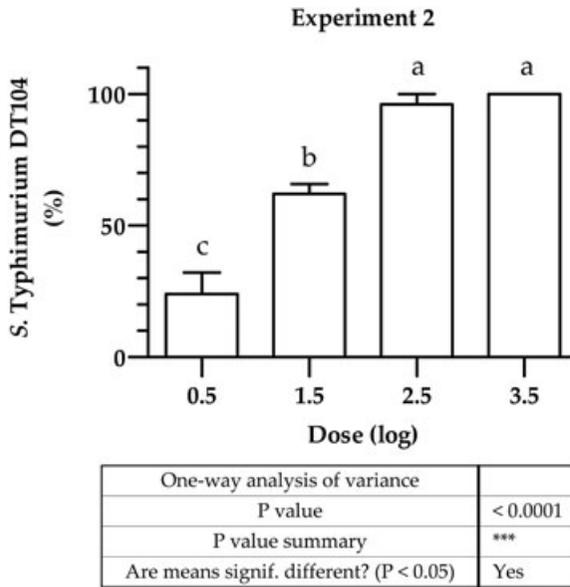


FIG. 2. EXPERIMENT 2 – PERSISTENCE OF log DOSES (0.5 TO 3.5) OF *SALMONELLA* TYPHIMURIUM DT104 ON CHICKEN SKIN EXPOSED TO ITALIAN MARINADE FOR 4 h AT 6C. BARS ARE THE MEAN \pm SEM OF FIVE SEPARATE TRIALS WITH 10 CHICKEN SKINS PER TREATMENT PER TRIAL. BARS WITH DIFFERENT LETTERS DIFFER AT $P < 0.05$

doses. In contrast, when marinating time was increased to 24 h in Experiment 3 (Fig. 3), the persistence of *S. Typhimurium* DT104 was reduced ($P < 0.05$), regardless of initial dose, but with the extent of the reduction, as expected, being inversely related to initial dose.

Experiment 4 (Fig. 4) directly compared the persistence of *S. Typhimurium* DT104 with the persistence of *S. Hadar* on chicken skin after exposure to the Italian marinade for 24 h at 6C. The results of this experiment indicated that marinating the inoculated chicken skins in Italian marinade for 24 h at 6C reduced ($P < 0.05$) the persistence of both strains of *Salmonella* by a similar extent, and that in the absence of marinade, no reduction in persistence was observed.

In Experiments 5 to 8 (Figs. 5 to 8), the persistence of low initial doses of four strains of *Salmonella*, three of which were isolated from poultry, on chicken skin marinated for 4 h or 24 h at 6C, was examined. In all four experiments, the persistence of *Salmonella*, regardless of strain, was reduced ($P < 0.05$) to a greater extent at 24 h than at 4 h of marinating and in the absence of marinade; persistence was close to 100%.

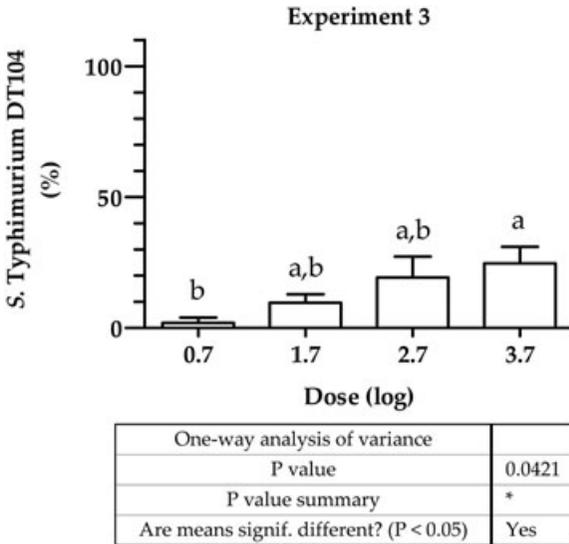
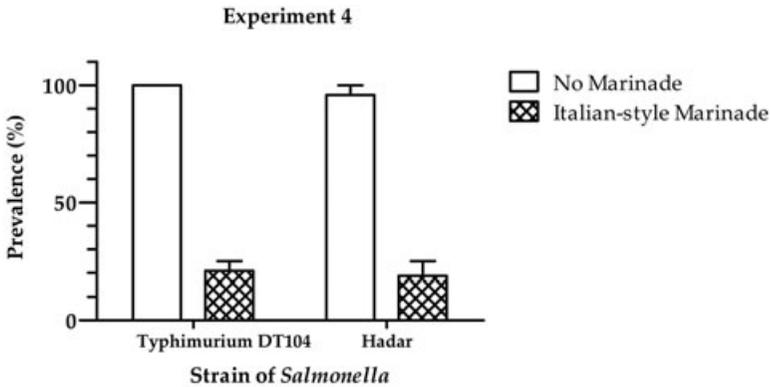


FIG. 3. EXPERIMENT 3 – PERSISTENCE OF log DOSES (0.7 TO 3.7) OF *SALMONELLA* TYPHIMURIUM DT104 ON CHICKEN SKIN EXPOSED TO ITALIAN MARINADE FOR 24 h AT 6°C. BARS ARE THE MEAN \pm SEM OF FIVE SEPARATE TRIALS WITH 10 CHICKEN SKINS PER TREATMENT PER TRIAL. BARS WITH DIFFERENT LETTERS DIFFER AT $P < 0.05$

DISCUSSION

The initial number of *Salmonella* on the chicken carcass is low, typically less than 30 cells per carcass (Surkiewicz *et al.* 1969). Therefore, in the majority of experiments in the current study, a low initial dose of *Salmonella* (<10 cells per skin portion) was inoculated onto chicken skin so as not to perturb the natural ecology of the skin, and thus provide a more realistic assessment of the efficacy of the Italian marinade for improving food safety. After exposure to the marinade, inoculated skin portions were incubated in a large volume (40 mL) of BPW to dilute out the antimicrobial ingredients in the marinade and to allow injured cells of *Salmonella* to recover before detection by drop-plating onto selective media with multiple antibiotics. In designing the selective media with multiple antibiotics, the antibiotic resistant phenotype of each strain of *Salmonella* was considered and a specific combination of antibiotics was used, which allowed the test strain to grow but at the same time prevented the native flora on the chicken skin from growing on the drop plates. The selection of serotypes was based on a recent study showing that multiple antibiotic resistant strains of *S. Typhimurium* and *S. Kentucky* are predominant



Two-way analysis of variance		
Source of Variation	% of total variation	P value
Interaction	0.02	0.8154
Marinade	96.33	< 0.0001
Strain	0.16	0.4773

FIG. 4. EXPERIMENT 4 – PERSISTENCE OF A LOW INITIAL DOSE (0.6 log) OF *SALMONELLA* TYPHIMURIUM DT104 AND A LOW INITIAL DOSE (0.8 log) OF *SALMONELLA* HADAR ON CHICKEN SKIN EXPOSED TO ITALIAN MARINADE FOR 24 h AT 6C. BARS ARE THE MEAN \pm SEM OF FOUR SEPARATE TRIALS WITH 12 CHICKEN SKINS PER TREATMENT PER TRIAL

serotypes found on freshly processed chicken on the Delmarva Peninsula (Parveen *et al.* 2007).

Results from this refrigeration study indicated that although the persistence of different strains of *Salmonella* on chicken skin was similar in the absence and presence of an Italian marinade, reductions in *Salmonella* persistence on chicken skin with native flora depended upon the initial dose of *Salmonella* inoculated, as well as the length of time the chicken skin was marinated. As expected, as the initial dose of *Salmonella* increased, persistence in the presence of the Italian marinade increased. A consistent finding was that marinating the chicken skin for 24 h produced a far greater reduction in *Salmonella* persistence than marinating the chicken skin for only 4 h. In fact, in Experiments 1, 3, 6 and 7, the reduction of *Salmonella* persistence was close to 100%, indicating that the Italian marinade was capable of eliminating low initial doses of *Salmonella*. These results suggest that consumers should marinate chicken purchased at retail markets for 24 h rather than for 4 h to maximize *Salmonella* reduction and food safety.

Although *Salmonella* levels on freshly processed chicken are low, improper storage of chicken after processing can lead to rapid growth of *Salmonella* to high and infectious levels. Therefore, two experiments were

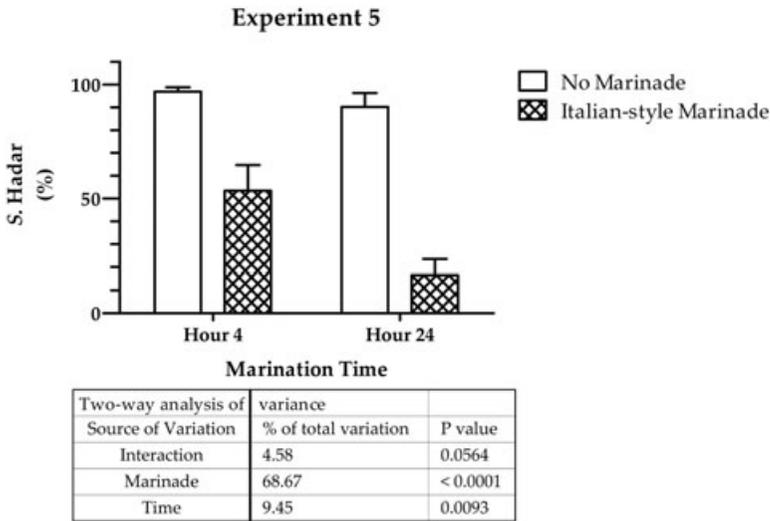


FIG. 5. EXPERIMENT 5 – PERSISTENCE OF A LOW INITIAL DOSE (0.5 log) OF *SALMONELLA* HADAR ON CHICKEN SKIN EXPOSED TO ITALIAN MARINADE FOR 4 h OR 24 h AT 6°C. BARS ARE THE MEAN \pm SEM OF FIVE SEPARATE TRIALS WITH 12 CHICKEN SKINS PER TREATMENT PER TRIAL

conducted in which the effect of the Italian marinade on persistence of higher initial doses (>1 to 3.7 logs) of *Salmonella* was evaluated. In the first experiment (Experiment 2), no reduction in *Salmonella* prevalence after 4 h exposure to the Italian marinade was observed for initial doses of *Salmonella* of 2.5 and 3.5 log. The initial dose of *Salmonella* at which the Italian marinade was capable of causing a 50% reduction in the persistence or prevalence was only between 0.5 and 1.5 log at 4 h of marinating. In contrast, in the second experiment (Experiment 3), large reductions in *Salmonella* persistence after 24 h of exposure to the Italian marinade were observed at all initial doses (0.7 to 3.7 log) tested. The initial dose of *Salmonella* at which the Italian marinade was capable of causing a 50% reduction in prevalence was >3.7 log. This indicated that 24 h of marinating was much more effective than 4 h of marinating in reducing the risk of exposure to *Salmonella* from contaminated chicken that has been temperature-abused after processing.

The traditional approach to evaluate the efficacy of food chemicals for pathogen reduction is to inoculate a food sample with a high and nonecological dose (6 to 9 log) of the test pathogen, followed by exposure of the food to the chemical and quantification (D-values) of the log reductions achieved over time. However, since this approach creates an artificial ecology where the test pathogen outnumbers the native flora of the food, it is likely to result in an

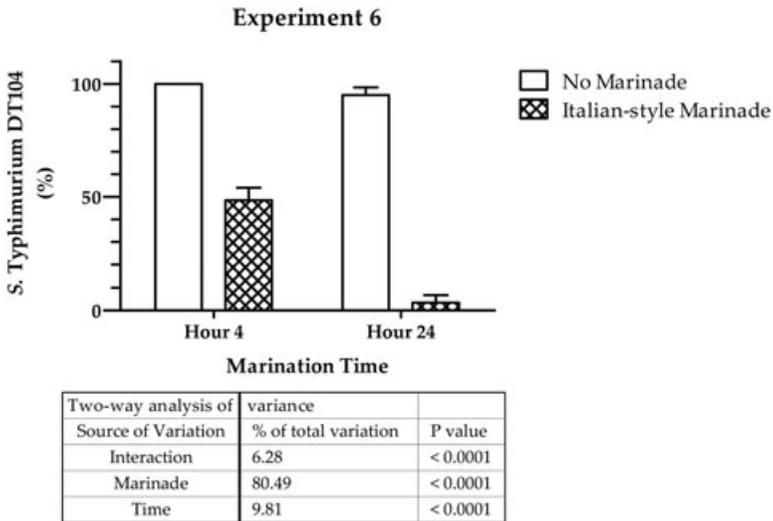


FIG. 6. EXPERIMENT 6 – PERSISTENCE OF A LOW INITIAL DOSE (0.6 log) OF *SALMONELLA* TYPHIMURIUM DT104 ON CHICKEN SKIN EXPOSED TO ITALIAN MARINADE FOR 4 h OR 24 h AT 6C. BARS ARE THE MEAN \pm SEM OF FIVE SEPARATE TRIALS WITH 12 CHICKEN SKINS PER TREATMENT PER TRIAL

over-estimate of efficacy by creating an artificial situation where the probability of the food chemical finding and killing the pathogen is artificially increased. Therefore, an alternative approach to evaluating food chemical efficacy for food safety was developed (Oscar 2008; this study) in which ecologically relevant doses (0 to <4 logs) of *Salmonella* are inoculated onto chicken skin with native flora and then food chemical efficacy is evaluated using a prevalence assay that takes advantage of the multiple antibiotic resistance phenotype of the test strains to form a low-cost and technically easy method for evaluating food safety improvements. In a previous study (Oscar 2008), this new approach to food chemical testing was used to show that application of kosher salt followed by rinsing was an effective processing procedure for reducing *Salmonella* persistence on chicken skin during kosher processing of poultry. In the present study, this novel method for food chemical testing was used to demonstrate the efficacy of an Italian marinade for reducing *Salmonella* persistence on chicken skin marinated for 4 h or 24 h at 6C.

There have been other studies that have reported decreases in bacterial populations on poultry meat in response to marinating for 24 h. Perko-Makela *et al.* (2000) conducted a study on the survival of *Campylobacter* on marinated chicken products and reported a 2.4 log decrease of *Campylobacter* after 24 h

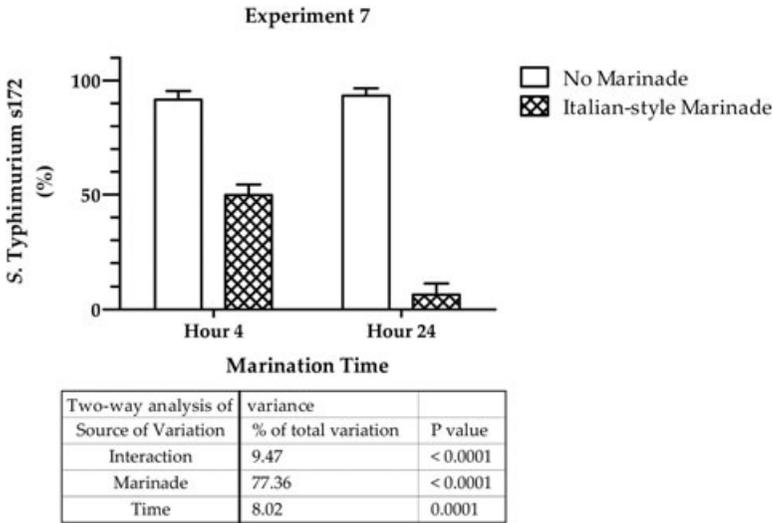


FIG. 7. EXPERIMENT 7 – PERSISTENCE OF A LOW INITIAL DOSE (0.5 log) OF *SALMONELLA* TYPHIMURIUM S172 ON CHICKEN SKIN EXPOSED TO ITALIAN MARINADE FOR 4 h OR 24 h AT 6°C. BARS ARE THE MEAN \pm SEM OF FIVE SEPARATE TRIALS WITH 12 CHICKEN SKINS PER TREATMENT PER TRIAL

of marinating, which is in concordance with results from this study. Another study conducted by Mahrour *et al.* (2003) reported that only one out of five marinated samples of chicken legs were positive for *Salmonella* after 24 h, as opposed to three out of five positives in the control chicken legs. Thus, the finding that marinating for 24 h reduces persistence of *Salmonella* on chicken is not unique to this study.

CONCLUSIONS

Results from the present study indicate that marinating of chicken with an Italian-style marinade can reduce the persistence of *Salmonella*, and that the extent of the reduction in persistence is much greater at 24 h than at 4 h of marinating and depends on the initial dose of *Salmonella* present. At a low initial dose (<10 cells per skin portion) of *Salmonella*, marinating for 24 h not only reduced but in some cases completely eliminated the risk of consumer exposure to *Salmonella*. As value-addition and shelf-life extension of poultry products is increasing along with consumer demands for convenience foods, it will be interesting to further study the effectiveness of other marinades and longer marinating times on *Salmonella* persistence at various storage temperatures.

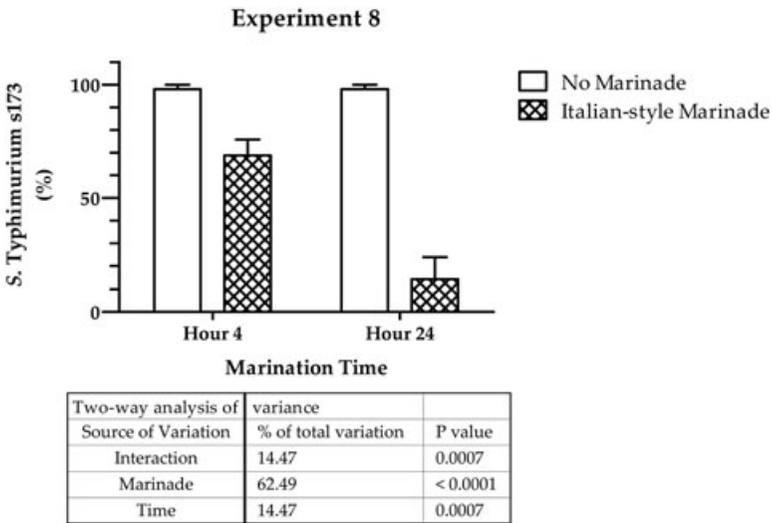


FIG. 8. EXPERIMENT 8 – PERSISTENCE OF A LOW INITIAL DOSE (0.5 log) OF *SALMONELLA* TYPHIMURIUM S173 ON CHICKEN SKIN EXPOSED TO ITALIAN MARINADE FOR 4 h OR 24 h AT 6C. BARS ARE THE MEAN \pm SEM OF FOUR SEPARATE TRIALS WITH 12 CHICKEN SKINS PER TREATMENT PER TRIAL

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