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Effect of ultraviolet light, organic acids, and bacteriophage on *Salmonella* populations in ground beef



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ABSTRACT

This study investigated individual and combined effects of organic acids, bacteriophages, and ultraviolet light interventions on *Salmonella* populations in ground beef. Beef trim was inoculated with four *Salmonella* strains to result in a contamination level of 3.5 log CFU/g after grinding. Lactic (LA) and peroxyacetic (PAA) acids, bacteriophages (S16 and FO1a) (BA), and ultraviolet light (UV) were applied on fresh trim prior to grinding. Applications of individual or combined organic acids did not significantly decrease *Salmonella* populations in ground beef. Individual applications of BA and UV light decreased approximately 1 log CFU/g (P < 0.05). Combined applications of BA and UV provided an optimal decrease of 2 log CFU/g (P < 0.05). Organic acid applications do not reduce *Salmonella* populations in ground beef when applied on trim prior to grinding. Combined applications of UV and BA may be used in industry settings to improve *Salmonella* control in ground beef.

1. Introduction

According to the Centers for Disease Control and Prevention (CDC), it is estimated that *Salmonella* causes one million illnesses, 19,000 hospitalizations, and 380 deaths every year in the U.S. (CDC, 2016), whereas from 2000 to 2008, *Salmonella* was the leading foodborne pathogen causing the largest number of deaths (CDC, 2012). Due to this significant public health concern, the U.S. Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) released the *Salmonella* Action Plan, a combination of comprehensive strategies to improve robust food safety systems to reduce *Salmonella* contamination in meat and poultry products (USDA-FSIS, 2013).

Ground beef is a risk commodity since beef trim is sourced from different carcasses, suppliers, and production lots. If any of these sources are contaminated with *Salmonella* that survived interventions applied during processing, significant cross contamination may happen during mixing and grinding (Pohlman, Stivarius, McElyea, Johnson, & Johnson, 2002). Solutions used to decontaminate beef trim including organic acids and bacteriophage preparations are approved for use and have regulatory status in the U.S. (USDA-FSIS, 2017). Lactic acid is the most common organic acid used during meat processing to decontaminate products (Wheeler, Kalchayanand, & Bosilevac, 2014), whereas many processing plants also adopted oxidizing acids such as the peroxyacetic acid for carcass rinsing and application on beef trim. The application of bacteriophages as potential biocontrol agents is being widely considered as an alternative to improve Salmonella control in food processing (Zinno, Devirgiliis, Ercolini, Ongeng, & Mauriello, 2014). Bacteriophages can be used to specifically control Salmonella since its mode of action is based on recognition of specific receptors that are present on bacteria wall and other structures (Rakhuba, Kolomiets, Szwajcer Dey, & Novik, 2010). This allows targeting only specific bacteria without disrupting additional microbiota (Meaden & Koskella, 2013). Ultraviolet light (UV) application is gradually becoming more common in meat processing due its low cost and effectiveness. In the beef industry, UV application is commonly used as an antibacterial strategy during dry aging (Dashdorj, Tripathi, Cho, Kim, & Hwang, 2016). Ultraviolet light ranges in wavelength from 100 to 400 nm and is divided in three categories: UV-A (315-400 nm), UV-B (280-315 nm), UV-C (200-280 nm), and vacuum UV range (100-200 nm) (Lázaro et al., 2014), whereas UV-C in the range of 250-260 nm is lethal to bacteria, viruses, protozoa, mycelial fungi, yeasts, and algae. Although UV interventions are widely used for other foods, there are limitations associated to exposure time that leads to oxidation of myoglobin (Djenane, Sánchez-Escalante, Beltrán, & Roncalés, 2003) and consequently, detrimental effects on beef color.

Although significant efforts were made by regulatory agencies and private companies, additional strategies to control *Salmonella* in ground beef are still needed (USDA-FSIS, 2014). The objective of this study was to evaluate individual and combined effects of organic acids (lactic acid and peroxyacetic acid), bacteriophages, and ultraviolet light applied on

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inoculated beef trim prior to grinding on *Salmonella* populations in ground beef.

2. Materials and methods

2.1. Salmonella strain recovery and inoculum preparation

A total of four Salmonella strains associated with previous outbreaks were used in the study. Multiple strains were used to ensure that interventions performed robustly against combinations of different genotypes. Strains included S. Infantis (ATCC 51741), S. Heidelberg (ATCC 8326), S. Newport (ATCC 27869), and a streptomycin resistant S. Enteritidis C (Se 13) were used in this study. Strains were obtained from the ATCC® and Micreos Food Safety B.V. (MICREOS Food Safety, Inc., Wageningen, The Netherlands). ATCC® strains were recovered by thawing freeze-dried pellets for approximately 2 min in water bath at 37 °C and subsequently transferring the entire content of the vial to a sterile test tube containing 5 mL of tryptic soy broth (TSB), which were incubated overnight at 37 °C. Individual recovered cultures were streaked on xylose lysine deoxycholate (XLD) agar plates. The S. Enteritidis C was directly recovered from a glycerol stock by streaking the content of the micro tube onto XLD agar plates supplemented with 500 μ g/mL of streptomycin. Plates were incubated aerobically at 37 °C to ensure that strains were live and viable. Initial cultures were prepared by suspending a single colony of each strain XLD plate in 10 mL of TSB. Tubes were incubated overnight with shaking at 37 °C. Individual inoculum was prepared by transferring 1 mL of each culture into Erlenmeyer flasks containing 40 mL of sterile TSB and incubated at 37 $^\circ\text{C}$ until optical density at 600 nm (OD_{600}) reached values from 0.5 to 0.6 McFarland units. Individual inoculum was diluted in 0.1% Buffered Peptone Water (BPW) prior to inoculation.

2.2. Sample preparation, experimental design, and bacterial inoculation

Fifteen (15) kg of beef trim (80% lean) from 5 different carcasses were procured from Wolfpack Meats, the University of Nevada, Reno (UNR) federally inspected harvesting and processing facility, and transported under refrigeration (4 °C) to the UNR's Meat Quality Laboratory. In order to precisely evaluate the effects of antimicrobial treatments regardless meat source, a composite sample was used. An aliquot of 1.5 kg was collected from the initial 15 kg batch by selecting random pieces and screened for Salmonella spp. to ensure that meat was not contaminated. Subsequently, 10 batches of 1.2 kg (total 12 kg) were subsampled from the 13.5 kg remaining. Later, a total of 10 samples (replications) for each treatment (12 treatments) were generated by subsampling 100 g from each 1.2 kg batch (12 treatments, 10 replications per treatment, total of 120 samples) and assigned to a completely randomized design. The following model was used: $Y_{ii} = \mu + \alpha_i + \varepsilon_i j$, where Y_{ii} was Salmonella count, μ was the grand mean across the treatments included in the experiment, and α_i was the effect of treatment from the grand mean specific to the i levels, which included Control not inoculated (CO), Control Inoculated (COI), Lactic Acid (LA), Peroxyacetic Acid (PAA), Ultraviolet light (UV), Bacteriophages (BA), LA + PAA, LA + UV, LA + BA, PAA + UV, PAA + BA, and UV + BA.

Beef trim was inoculated with a bacteria cocktail comprising all four *Salmonella* strains to yield approximately 3.5 log CFU/g in ground beef. Briefly, 2 mL of the inoculum was uniformly pipetted on beef trim surfaces whereas the same volume of sterile 0.1% BPW was pipetted on CO samples. Prior application of antimicrobials, inoculated samples were kept at room temperature for 30 min to allow bacterial attachment.

2.3. Application of antimicrobial treatments

Lactic acid (2-hydroxypropionic acid, 87.5–88.5%, w/w), PAA (Ethaneperoxoic acid, stabilized, < 43%), and BA (S16 and FO1a

Table 1

Killing efficiency of bacteriophage preparation (phages S16 and FO1a) for four Salmonella
strains (Yeh et al., 2017).

Strain	Bacteriophage application	Average CFU (4 plates)	Killing efficiency (%)
ATCC 51741	Control ^a 10 ⁹ PFU/ml	126.75 1.25	99.0
ATCC 8326	Control 10 ⁹ PFU/ml	334.75 3	99.1
ATCC 27869	Control 10 ⁹ PFU/ml	160 1.25	99.2
Se 13	Control 10 ⁹ PFU/ml	398.50 5.25	98.6

^a No bacteriophage applied.

phages) were provided by commercial suppliers. Stock solutions of LA, PAA, and BA were diluted in potable water to simulate industry practices. Organic acids were diluted to reach 5% and 400 ppm for LA and PAA, respectively. Titer of the bacteriophage stock solution was determined to be at 10^{11} PFU/mL by following the methodology described by Adams (1959), modified by Yeh et al. (2017). Phage killing efficiency for each strain tested in this research was previously described by Yeh et al. (2017) (Table 1). Bacteriophage stock solution was diluted in potable water to reach a final titration of 10⁹ PFU/mL. Potable water used in this study was previously tested to ensure it was free of Salmonella spp. and chlorine. Organic acid and BA applications were performed by pipetting 5 mL of each solution on trim surface. Application of UV was performed by using a table top, stainless steel rotating drum prototype, measuring 80 cm tall \times 56 cm wide \times 51 cm long (Revco Systems, Meridian, ID, U.S.A.). The drum was equipped with an array of two internal 110 V 16-in. UV-C Emitters (Steril-Aire, Inc. Burbank, CA, U.S.A.) at a wavelength of 254 nm at 23 °C with light intensity of $800\,\mu\text{W}/\text{cm}^2$.The height of the UV-C emitters was adjusted $10\,\text{cm}$ above beef trim during the irradiation treatment while tumbling was performed for 2 min at 12 rpm. Total exposure time to UV was approximately 30 s based on the set up of the emitters (1/4 of the total)area of the tumbler). When combining different applications, UV was applied prior to LA, PAA, and BA, whereas organic acids were applied prior to BA.

2.4. Sample grinding

After antimicrobial treatments were applied, samples were covered and stored at 5 $^{\circ}$ C for 1 h and 30 min prior to grinding. Samples were ground twice using table top electric grinders (model 33–0201-w, Weston, China). A total of 12 electric grinders were individually assigned to be used for individual treatments.

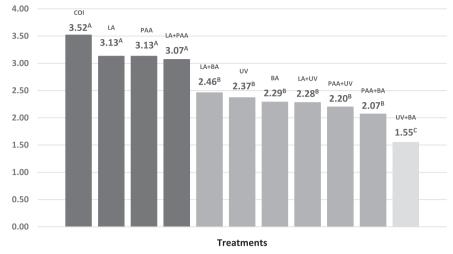
2.5. Salmonella enumeration

A 25 g aliquot from ground samples was collected and individually placed in sterile bags. Samples were paddle-blended with 225 mL of sterile 0.1% BPW using a Stomacker (Model 400 circulator, Seward, London, UK) at 200 rpm for 2 min. *Salmonella* counts were determined by plating recovered bacteria on XLD agar plates. Briefly, 10 mL of the homogenate was centrifuged at $10,000 \times g$ for 6 min and the supernatant was discarded to avoid plating phages. Bacterial pellets were resuspended in 10 mL of BPW, vortexed, and serially diluted. Dilutions were plated onto XLD agar plates in duplicate, incubated overnight at 37 °C, and typical *Salmonella* colonies were enumerated (CFU/g).

2.6. Statistical analysis

The experiment was arranged as a completely randomized design as described in item 2.2. Data were analyzed using the GLIMMIX

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procedure of SAS[®] 9.3 package (SAS Institute, Inc., USA). When significance ($P \le 0.05$) was indicated by ANOVA, means separations were performed by using the LSMEANS and DIFF functions.

3. Results

No Salmonella growth was observed on plates from CO samples. Control inoculated samples vielded 3.52 log CFU/g after inoculation with the cocktail containing all four Salmonella strains. Effects of individual and combined treatments on Salmonella populations after grinding are presented in Fig. 1. Application of antimicrobial treatments on beef trim prior to grinding significantly affected bacteria loads in ground beef. Individual applications of organic acids (LA and PAA) as well combination of both (LA + PAA) did not significantly decrease Salmonella counts when compared to COI samples (3.13, 3.13, and 3.07 log CFU/g for LA, PAA, and LA + PAA, respectively). When antimicrobial combinations of LA + BA, LA + UV, PAA + UV, and PAA + BA were applied, reductions ranging from approximately 1 to 1.5 log CFU/g were observed (2.46, 2.28, 2.20, and 2.07 log CFU/g for LA + BA, LA + UV, PAA + UV, and PAA + BA, respectively). Individual applications of UV (2.37 log CFU/g) and BA (2.29 log CFU/g) also reduced Salmonella by 1.2 CFU/g. However, the magnitude of the antimicrobial effect of individual effects of UV and BA, as well as combinations of LA + BA, LA + UV, PAA + UV, and PAA + BA, was statistically similar. No differences were observed when comparing linear combinations of organic acids plus UV versus organic acids plus BA (P = 0.56). Application of UV combined with BA on beef trim prior to grinding led to an optimal reduction of 2 log of Salmonella in ground beef when compared to COI samples (1.55 and 3.52 log CFU/g for BA and COI, respectively).

4. Discussion

Lactic acid is one of the most common organic acids used for the decontamination of animal carcasses and trim (Castillo et al., 2001). Antimicrobial effects of LA are based on the disruption of the cell regulation forcing the bacterium to spend significant amount of energy to maintain its interior optimal pH (Foster, 1995). This change in metabolism and radicals produced by acid stress damage cellular mechanisms leading to directly inactivation of bacteria (Desriac et al., 2013). Carlson et al. (2008) demonstrated that LA solutions at 10% applied on live cattle reduced *Salmonella* by at least 1.5 log on hides. When applied on chicken skin, LA led to a similar decrease of *Salmonella* spp. (Chaine, Arnaud, Kondjoyan, Collignan, & Sarter, 2013). Özdemir et al. (2006) showed lower reductions of 0.7 log when immersing inoculated beef steaks in 2% LA solutions. Harris, Miller,

Fig. 1. Least square means (log CFU/g) of Salmonella populations in ground beef followed by application of food safety interventions on intact trim.

Lonergan & Brashears (2006) showed that LA applications at 2 and 4% on beef trim decreased Salmonella by approximately 1.5 log on trim and in ground beef, whereas the lower values were sustained overtime during refrigerated and frozen storage. Conversely, Stivarius, Pohlman, McElyea, and Waldroup (2002) did not observe significant reduction of Salmonella in ground beef by previously treating trim with 5% of lactic acid. This is probably due to the ability that Salmonella has to adjust to acid environments and survive in not favorable pH conditions (Mani-López, García, & López-Malo, 2012). Burin, Silva Jr, and Nero (2014) showed that under acid stress conditions, Salmonella may develop a significant acid tolerance behavior by increasing the expression of genes including rpoS, nlpD and clpP. These genes regulate the bacteria protection ability against cell damage caused by acid stress (Hengge-Aronis, 2002; Lange, Fischer, & Hengge-Aronis, 1995). Therefore, LA treatment was not effective against the four strains selected for this study due to the ability of Salmonella to develop this acid tolerance.

Peroxyacetic or peracetic acid is an equilibrium solution of acetic acid, hydrogen peroxide, and water. Its antimicrobial properties are based on the oxidation of the outer membrane of the bacteria cell due to the release of active oxygen (Liberti & Notarnicola, 1999). The oxygen oxidizes sulfhydryl and sulfur bonds in proteins and enzymes which react with other oxidized metabolites. This leads to a disruption of the chemiosmotic function of the lipoprotein cytoplasmic membrane compromising transport of essential compounds through dislocation or rupture of cell walls (Baldry & Fraser, 1988; Leaper, 1984). Intracellular PAA may also impair bacterial biochemical pathways, transport through membranes, and affect intracellular solute levels (Fraser, Godfree, & Jones, 1985). Bauermeister, Bowers, Townsend, and McKee (2008) demonstrated that levels of 200 ppm of PAA significantly reduced Salmonella count in poultry carcasses when solution was added into chilling water. Effects of PAA applications on Salmonella loads in beef were demonstrated by Ellebracht et al. (2005), who submerged trim in solutions of 200, 500, and 1000 ppm and reported maximum reduction of 1 log when applying the first two concentrations, respectively. Conversely, Guerrero-Beltrán and Barbosa-Cánovas (2004) reported that PAA had little effect on the numbers of aerobes, coliforms, Escherichia coli, and were less effective than 4% lactic acid applications when treating beef carcasses. In addition, King et al. (2005) reported that concentrations up to 600 ppm of PAA did not reduce Salmonella loads when sprayed onto beef carcasses. Salmonella oxidative stress resistance may be related to gene expression regulation when the bacteria is exposed to environments with high concentrations of reactive oxygen species (ROS) (Buchmeier et al., 1997). Hérbrard, Viala, Méresse, Barras, and Aussel (2009) suggested that genes encoding catalases KatE, KatG, and KatN, and alkyl hydroperoxide reductases AhpC and TsaA are able to degrade ROS such as hydrogen peroxide. This is in agreement with our findings and suggests that strains used in our study were resistant to PAA at 400 ppm.

Antimicrobial effects on UV are based on the ability that the wavelength has to damage DNA of microorganisms. The light damages DNA molecules by cross-linking neighboring thymine and cytosine (pyrimidine nucleoside bases) in the same strand, producing cyclobutyl pyrimidine dimers and consequently, blocking DNA transcription and replication (Guerrero-Beltrán & Barbosa-Cánovas, 2004). When damage is too extensive, the cell undergoes to apoptosis (Escalona, Aguayo, Martínez-Hernández, & Artés, 2010; Hijnen, Beerendonk, & Medema, 2006). Wallner-Pendleton, Sumner, Fronging, and Stetson (1994) reported approximately 1.8 log decrease of nalidixic-acid resistant Salmonella typhimurium after applying UV on whole broiler carcasses. Additionally, UV applications led to 80.5% reduction of Salmonella on chicken skin (Sumner, Wallner-Pendleton, Froning, & Stetson, 1996). When applied onto pork skin and muscle surfaces, UV intensity at 1000 μ W/cm⁻² reduced 2 and 4.6 log of Salmonella senftenberg loads, respectively (Wong, Linton, & Gerrard, 1998). In beef short plates, UV irradiation at 275 µW/s for 60 s eliminated 97% of bacteria including Pseudomonas, Micrococcus, and Staphylococcus species (Stermer, Lasater-Smith, & Brasington, 1987). Although several research reports demonstrated significant UV efficiency to control and eliminate bacteria in many food sources (Graça, Santo, Quintas, & Nunes, 2017; Lim & Harrison, 2016; Mansor, Shamsudin, Adzahan, & Hamidon, 2014; Mukhopadhyay, Ukuku, juneja, & Fan, 2014), Gayán, Serrano, Raso, Álavarez, and Condón (2012) observed UV resistance of Salmonella Typhimurium STCC 878 and suggested that this strain may have a more efficient DNA repairing system. In our study, intermittent exposure of beef trim for approximately 30 s during tumbling for 2 min reduced Salmonella loads in ground beef by 1 log. Possibly, longer exposure periods could extent the antimicrobial effect of UV. However, our goal was to apply a low dosage of UV to avoid possible photo-oxidation and consequently avoid compromising color or sensory attributes. Although these quality attributes were not studied in this research, it is expected that the dosage of 800 μ W/cm² intermittently applied on beef trim for 30 s does not negatively affect color and other quality attributes. Lázaro et al. (2014) previously demonstrated that dosages up to 1950 μ W/cm² do not promote relevant changes on quality indicators of chicken breast.

Bacteriophage applications have become a suitable food safety intervention for the food industry due to the unique ability of infecting and lysing specific bacterial cells (García, Martinez, Obeso, & Rodriguez, 2008). Various studies demonstrated the effects of bacteriophages on Salmonella in different food matrixes. Zinno et al. (2014) reported up to 3 log cycles reductions of Salmonella loads in liquid-eggs, chicken breast and ground chicken. Bacteriophage applications were also efficient in decreasing several Salmonella strains in poultry carcasses and parts (Bielke et al., 2007; Fiorentin, Vieira, Barioni, & Embrapa, 2005; Higgins et al., 2005; Sharma, Dhakal, & Nannapaneni, 2015 and Sukumaran, Nannapaneni, Kiess, & Sharma, 2015). In addition, Yeh et al. (2017) reported a consistent 1 log reduction of Salmonella loads in four meat matrixes (beef, pork, chicken, and turkey) when applying bacteriophages on trim and thighs prior to grinding. Host inactivation by bacteriophages occurs within the first few hours after phage application (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003) although Bigwood, Hudson, Billington, Carey-Smith, and Heinemann (2008) and Shao and Wang (2008) suggested that inactivation of the host may also be related to optimal temperature application. Hungaro, Mendonça, Gouvea, Vanetti, and de Oliveira Pinto (2013) reported improved bacteriophage activity in temperatures ranging from 25 °C to 37 °C. This is possibly related to bacterial metabolism, which is optimum in such temperatures. However, applications at higher temperatures are not possible in meat processing settings due to the cold environment. Previous research, showed that the antimicrobial effect of bacteriophages is significant in meat processing temperatures (Fiorentin et al., 2005; Guenther, Herzig, Fieseler, Klumpp, & Loessner,

2012; Yeh et al., 2017). Sengupta and Chattopadhyay (2013) suggested that bacteria remain alive in cold temperatures and also continue its metabolism, which allows bacteriophage attachment and replication. The activity of phages used in this experiment (S16 and FO1a) was previously described by Marti et al. (2013) and Lindberg and Holme (1969), respectively. Briefly, the S16 binds to the outer membrane protein C (ompC) whereas the FO1a binds to the terminal *N*-acetylglucosamine residue present on the outer lipopolysaccharide core of the membrane. Both bacteriophages are able to lyse a broad range of strains since ompC and the *N*-acetylglucosamine residue are very common structures on membranes of *Salmonella* strains. In our study, both phages were effective in performing their antimicrobial activity at meat processing temperatures.

Applications of food safety interventions based on different modes or mechanisms of action are effective to decrease bacteria due to continuous exposure of the cells to different stressing factors (Mikš-Krajnik, Feng, Bang, & Yuk, 2017). Previous research demonstrated that UV and bacteriophage combinations were effective in reducing *Listeria monocytogenes* in chicken breast filets (Yang, Sadekuzzaman, & Ha, 2017). However, bacteriophages must be always applied after UV since the light intensity may damage bacteriophage DNA and lyse the cell.

5. Conclusion

Applications of lactic acid at 5% and peroxyacetic acid at 600 ppm on beef trim do not decrease *Salmonella* populations in ground beef. Individual applications of UV (254 nm) at 800 μ W/cm² for approximately 30 s and bacteriophages S16 and FO1a at 10⁹ PFU/mL reduced *Salmonella* loads by approximately 1 log CFU/g. When combined, UV and bacteriophage reduced 99% (2 log cycles) of *Salmonella* in ground beef. Implementation of combined applications of UV and bacteriophage interventions is feasible for the meat industry since antimicrobial spraying and tumbling methods are commonly practiced in meat industry settings. This approach may improve multiple hurdles' systems and decrease *Salmonella* incidence in ground beef.

Conflict of interest

The authors declare no conflict of interest associated with this research.

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