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Efficacy of Antimicrobials Against Unstressed or Cold-Stress Adapted Salmonella Cells and Surrogate Enterococcus faecium on both Broiler Carcasses and Wings

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Efficacy of Antimicrobials Against Unstressed or Cold-Stress Adapted *Salmonella* Cells and
Surrogate *Enterococcus faecium* on both Broiler Carcasses and Wings

Lacey Lemonakis

Thesis submitted to the Davis College of Agriculture, Forestry and Consumer Sciences
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Key words: broiler, antimicrobials, *Salmonella*, *Enterococcus*, cold-stress

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Abstract

Efficacy of Antimicrobials Against Unstressed or Cold-Stress Adapted *Salmonella* Cells and Surrogate *Enterococcus faecium* on both Broiler Carcasses and Wings

Lacey Lemonakis

The aim of this study was to evaluate the microbial quality of broiler carcasses and efficacy of antimicrobials on unstressed or cold-stress adapted cells of *Salmonella* and *Enterococcus* on broiler carcasses and wings. Broiler carcasses and wings were processed at a small USDA-inspected slaughter facility, located in West Virginia. The first part of the study included 42 broiler carcasses that had been pre-enriched in Buffered Peptone Water then secondarily-enriched into Rappaport Vassiliadis following incubation. Secondary enrichment broth was then streaked on to Xylose Lysine Tergitol 4 (XLT-4) and HardyCHROM™ agar for *Salmonella* detection; and further confirmation was performed using an API 20E kit and Latex Agglutination test. Various petrifilms such as aerobic plate count (APC), *Escherichia coli* (ECC), total coliforms (TCC), and yeast/ mold were analyzed. APC, ECC/TCC and Yeast/ Mold counts were 2.61, 1.08, and 2.37 log₁₀ CFU/mL respectively on broiler carcasses. Thirty and forty percent of the carcasses were positive for detection of *Salmonella* spp. and *Escherichia coli* (0.48 to 1.70 log₁₀ CFU/mL) respectively. Study II included fresh broiler carcasses and wings; these meat products were inoculated with unstressed and cold-stress adapted (grown in 4 °C for 7 days) *Salmonella Typhimurium* ATCC 14028, *Salmonella Tennessee* ATCC 10722, and *Enterococcus faecium* ATCC8459 (roughly 5.5 to 6.5 log₁₀ CFU/mL). Carcasses and wings were later assigned to undipped (control) or dipped treatments, these treatments consisted of peroxyacetic acid (PAA; 0.1%), lactic acid (LA; 5%), lactic/ citric acid blend (LCA; 2.5%), and sodium hypochlorite (SH; 67-69 ppm). Dip treatments were 30 seconds with carcasses and wings, with wings having a 2 minute drying period following

dipping. Immediately following treatments, surviving bacteria were recovered onto selective and non-selective agar to analyze total *Salmonella* and *Enterococcus*. Carcasses had antimicrobials reduce cold-stress adapted cells (1.1 to 1.7 and 1.0 to 2.1 log₁₀ CFU/mL) more ($P<0.05$) than the unstressed cells (0.7 to 1.5 and 0.2 to 1.7 log₁₀ CFU/mL) for *Salmonella* and *Enterococcus*. As for wings, cold-stress adapted *Salmonella* cells were more sensitive ($P>0.05$) to antimicrobials (reduced by 0.9 to 1.5 log₁₀ CFU/mL) than the unstressed cells (reduced by 0.5 to 1.2 log₁₀ CFU/mL). However, unstressed and cold-stress adapted *Enterococcus* behaved similar, ($P>0.05$), with reductions ranging from 0.9 to 2.0 log₁₀ CFU/mL. *Salmonella* and *Enterococcus* reductions on carcasses and wings increased in the following order: SH≤LCA<LA<PAA, irrespective of unstressed or cold-stress adapted cells. Application of post-chilling, antimicrobial treatment could be a beneficial intervention to *Salmonella* on locally processed broiler carcasses and wings. With the results of the study, *Enterococcus faecium* could potentially be used as a surrogate for *Salmonella* when being used in in-plant validation studies.

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1.0 Literature Review

1.1 Impact on Food Safety and Consumers

Food-borne illnesses are increasing, creating a serious public health concern. Transmission of food-borne pathogens through the food chain is still an unresolved issue (Galvez et al., 2010). One of the major concerns focuses on the production and distribution of food from the farm to reaching the consumers household to ensure they are receiving wholesome products. Consumers have the right to receive wholesome products, so implementing any interventions to help reduce the risk of cross-contamination in food processing should be influenced. Potentially consumers acquire a food-borne illness when food is improperly handled or cross-contamination occurs. Symptoms most commonly affiliated with food-borne illnesses include cramping, diarrhea and vomiting. Of the many known food-borne pathogens, a select few continuously contaminate our everyday food supply, more specifically ready-to-cook poultry products. The quality and safety of poultry products are affected by the several steps throughout processing and production. As noted, *Salmonella* spp. are commonly associated with ready-to-eat poultry products (Schraff, 2011). Approximately 1.4 million people are reported with *Salmonella* in the United States annually (Schraff, 2011). In 2011 the CDC reported an estimated 11% of food-borne illnesses were affiliated with *Salmonella*. Schraff, (2011) estimated health related cost to food-borne illness was \$51 billion; whereas, \$4.43 million was related to *Salmonella*.

Industries are advancing technology to a food-borne disease outbreaks. Even with advances in food safety, there are still ongoing challenges. Implementing a more rigorous food safety program and endorsing studies to ensure products are safe for handling and consumption are approaches to eliminate these problems (Bianchini et al., 2014). With the USDA implementing a stricter pathogen reduction standard, it's important for poultry processors to apply new

interventions to control pathogens such as *Salmonella*. Food industry is routinely using ingredients that may pose a high risk for *Salmonella* contamination; this practice is a major concern for food safety programs (Bianchini et al., 2014). Despite the industry's efforts to take preventative measures, recalls and outbreaks continue to occur.

1.2 *Salmonella* spp.

Salmonella are a Gram negative, non-spore forming bacilli in the Enterobacteriaceae family (Jay et al., 2012; Fraser, 2017). Approximately 2,000 serotypes exist and are known to cause human disease (Jay et al., 2012). Optimal growth temperature of *Salmonella* ranges from 35-37°C, or body temperature (Fraser, 2017). As for optimal pH, growth is near neutral but a pH above 9.0 or below 4.0 could be detrimental to the cells (Jay et al., 2012).

Samples are obtained then cultured onto a selective media for *Salmonella*. Currently, laboratories use many variations of media for isolation of *Salmonella*. Xylose lysine tergitol 4 (XLT4) tends to be used widely for the isolation of non-typhi *Salmonella* spp. (XLT-4 AGAR, 1996-2016). For positive detection, colonies will appear black or yellow to red with black centers (XLT-4 AGAR, 1996-2016). This is caused by the production of hydrogen sulfide after incubation for 18- 24 hours (XLT-4 AGAR, 1996-2016). Further examination, morphology, color determination, and further testing for identification and differentiation of species are used (XLT-4 AGAR, 1996-2016). A more common testing panel is the API20E kit (XLT-4 AGAR, 1996-2016). The indicated kit provides conformation to identify which species of *Salmonella* is present.

1.3 Evidence of *Salmonella* in Poultry Products

Chickens are a natural host for pathogenic bacteria. *Salmonella* occur naturally in the intestinal tracts of broilers (Tamblyn and Conner, 1997). Poultry are asymptomatic carries of most

serotypes of *Salmonella* (McKee, 2012). Centers for Disease Control and Prevention (2014) reported that processed chicken products have been associated with numerous *Salmonella* outbreaks. Bacteria-related food safety concerns relate back to poultry meat that has not been properly cooked or to cross-contamination. These scenarios lead to foodborne disease outbreaks. Illnesses can arise from the moments broiler processed starts to the moment consumers receive their poultry products.

Salmonella contributes to 35.1% of illnesses related to poultry products (Batz et al., 2011). Among the thousands of serotypes of *Salmonella* spp., the top 3, causing food borne illness, are Enteritidis (19%), Typhimurium (14%), and Newport (10%) (Bourassa et al., 2015). Figure 1 presents the most common identified serotypes among meat and poultry classes. The Centers for Disease Control (2011) reported that *Salmonella* is responsible for 11% of food-borne illnesses; 35% of these require hospitalization and 28% result in death. Outside of illnesses related to food, cases can also arise from the laboratory setting. From 2010 to 2014, at least 158 cases of laboratory-associated infections with *Salmonella* Typhimurium ATCC 14028 were reported by PulseNet USA (Hu and Gurtler, 2017).

Within industries, there are methods applied to help provide safe food and steps to prevent cross contamination. One of the biggest concerns is reducing bacteria in the poultry products itself. Regulations and safety standards are presented by the USDA-Food Safety and Inspection Services (FSIS). USDA-FSIS requires a Hazard Analysis and Critical Control Point (HACCP) plan that must be implemented in food processing plants (Russell, 2005). HACCP is a proactive written plan for maintaining the control of potentially hazardous food at the critical point of food processing and preparation (Compliance Guideline, 2010). Certain criteria requirements are set and processing facilities are required to comply with these performance standards. That includes

successful conduct and monitoring the critical control points. Performance standards require routine testing for presence of *Salmonella* in the processing plants. New performance standards allow a certain percentage of positive bacteria present in a sampling period. A presence of positive samples for *Salmonella* must remain below an overall of 7.5% (McKee, 2012).

The food industry has been in continuous search of quick and cost-effective methods to help enumerate and identify microorganisms on food products and the equipment used to process food products (Russell, 2005). Since there is high positive rates of *Salmonella*, the poultry industry has set up standard post-harvest procedures for processors to follow. The major area of focus is processing which can be steps including de-feathering and evisceration. In further research, processing plants need to be closely monitored as to why evisceration is the leading factor in producing such high positive values of these two pathogen. This leads to the question of what steps can be implemented to reduce the high values.

Serotypes 2010	Number of Isolates	Percent of Total Positive	Percent of Analyzed Samples
Kentucky	208	45.41	3.05
Enteritidis	124	27.07	1.82
Typhimurium 5 -	27	5.90	0.40
Heidelberg	16	3.49	0.23
^a Typhimurium	14	3.06	0.21
^c 4,5,12:i:-	10	2.18	0.15
Johannesburg	6	1.31	0.09
Schwarzengrund	5	1.09	0.07
Senftenber	5	1.09	0.07
Berta	4	0.87	0.06
Braenderup	4	0.87	0.06
Thompson	4	0.87	0.06
^a Other serotypes	24	5.24	0.35
^b Unidentified	5	1.09	0.07
Total serotyped isolates	456		6.68
Not typed	2		0.03
^a Total positive	458		6.71
Total number of analyzed samples	6,828		

Figure 1. Profile of Serotypes Analyzed from PR/HACCP Verification Samples by Calendar Year. Broilers (1998-2005 'A' Set Samples; 2006-2010 All Samples) (*The percentages listed for total positive isolates may not equal the sum of the data in the Percent of Analyzed Samples column due to rounding. a. The ten most commonly isolated serotypes during a listed year are identified by name while less commonly identified serotypes are included in the —other serotypes category. When there is more than one serotype in tenth place, all serotypes in tenth place are listed. b. The —unidentified designation includes isolates for which a single specific serotype could not be determined including those that were solely named through antigenic formulas. c Prior to 2004, isolates fitting the designation in b were included in the —unidentified isolates category. d Typhimurium includes Typhimurium 5- (formerly Copenhagen). Note: For information on the most commonly identified serotypes causing human infection in the United States see CDC (2006). (Serotypes Profile of Salmonella Isolates from Meat and Poultry Products January 1998 through December 2010)

1.4 Introduction to poultry processing and approaches to pathogen control during processing

The safety and quality of poultry products is affected by the various steps throughout production. In the year 2006, the USDA-FSIS began a new initiative to reduce the levels of *Salmonella* in meat and poultry products (U.S. Department of Agriculture, Food Safety and

Inspection Service, 2006). *Salmonella* commonly occur in live poultry, plus contaminate the meat itself during the slaughtering area (McKee, 2012). Once the broilers are transferred out of the slaughter stages to other areas the broilers may carry bacteria and contaminate other equipment (McKee, 2012). A study by the USDA-FSIS reported that roughly 4% of broilers that enter the processing plant tested positive for *Salmonella*, but that number increases to 35% when leaving the processing facility (Lillard, 1989).

At processing plants, broilers undergo several processing steps before the final poultry product is complete. Those steps include stunning, exsanguination, scalding, picking, evisceration, and carcass chilling before packaging (Figured 2). The major concern during the processing is evisceration. During this procedure, edible and inedible viscera are removed from the carcass. The body cavity of the broiler is opened and the inside viscera are harvested. Thus can lead to cross-contamination from intestinal microorganisms spreading to other parts of the carcasses. In the removal of the internal organs, processors must take caution in removing the intestines and digestive tract. *Salmonella* are known to harbor and grow within these two areas of the broilers. If the gut is torn during the evisceration process, microbial contamination will occur, and will lead to cross-contamination of other broiler carcasses (Fanatico, 2003). After evisceration is complete carcasses are placed in a chiller tank. Before being placed in the chiller tank, carcasses must go through an inspection to determine if there is any fecal contamination. Any present fecal matter on the broilers must be sprayed with water or an antimicrobial mix to remove the matter.

In the processing industry many methods are used for reducing or eliminating bacteria on the birds. Facilities use a spraying mix of water and antimicrobial on-line reprocessing (OLR) or another method of inside-outside bird washer (IOBW). These two methods are generally used after an inspection of the carcass takes place. With this technique, approximately 1 log CFU/unit

bacterial reduction can be attained, although the results may not be consistent due to many factors, such as the amount of the carcass that is being covered with the spray mix or the pressure of the sprayer. Brashears et al., (2001) reported that high pressure sprayers might be unfavorable by forcing bacteria into the skin of the carcasses.

As for another technique, there are two approved methods used for chilling carcasses, including immersion ice water chilling and air chilling, which are widely used in the U.S. and European Union. Immersion is when the carcass are dipped in a tank that contains water chilled below 40°F (Compliance Guideline 2010). On the other hand, air chilling mists carcasses with water in a tight air chilled environment (Compliance Guideline, 2010). Most of the poultry processing plants in the United States use immersion chilling since it is more efficient. Immersion chilling consist of multiple tanks. There is a pre-chiller and a post-chiller tank. Pre-chiller is a term used when reducing the temperature of the carcasses after processing; whereas, a post-chilling tank is used as a dip treatment with antimicrobials to help decrease microbial loads (Compliance Guideline, 2010). These mixes used in post-dipping treatments contain organic acids or chlorinated products. Although facilities use different variations of antimicrobial washes, different interventions may or may not be applicable for use, meaning that specific amounts are allowed versus duration of contact time. Could the chilling process be the most effective approach to help inactivate foodborne pathogens, taking into consideration exposure time of the pathogens to the antimicrobial treatments?

Figure 2. Poultry slaughtering and processing: main steps. (2. Sectoral Economic and Energy Profiles, 2015)

1.5 *Enterococcus* spp.

Enterococcus genus belong to a group of microorganisms known as lactic acid bacteria (LAB) (Moreno et al., 2006). Bacteria of the genus *Enterococcus* or enterococci, includes more than 30 species (formerly the “faecal” or Lancefield group D streptococci) (Giraffa, 2002; Jay et al., 2005)). They are ubiquitous microorganisms, but have a predominant habitat in the

gastrointestinal tract of humans and animals (Giraffa, 2002). This potential foodborne bacterium is classified as a Gram positive, non-spore forming and facultative anaerobic cocci cells that occur singly, in pairs or in short chains, (Jay et al., 20012; Moreno et al., 2006; Bianchini et al., 2014). *Enterococcus* can grow in the presence or 6.5% NaCl, at a pH of 9.6 and the can survive heating at 60 °C for 30 minutes (Moreno et al., 2006). A select amount of species in this genus can grow in temperatures from 10 to 45 °C (Moreno et al., 2006). Various selection of media are recommended for culturing this bacteria, which include TSA agar and blood agar 5% (Enterococcus, Hardy Diagnostics, 1996-2016). Whereas for selective isolation, Bile Esculin Azide (BEA) and Mannitol Salt agar (Enterococcus, Hardy Diagnostics, 1996-2016). Growth during incubation last 18-24 hours (Enterococcus, Hardy Diagnostics, 1996-2016). Once grown on media, positive *Enterococcus* spp. appear to have a smooth, cream or white colonies (Enterococcus, Hardy Diagnostics, 1996-2016). After colonies appear positive on the media, further biochemical test can be conducted for confirmation. Those test consist of catalase-negative, PYR-positive and LAP-positive (Enterococcus, Hardy Diagnostics, 1996-2016).

1.6 Bacterial Pathogens used as Surrogates and Significance in Food Safety

U.S. Food and Drug Administration (U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2001) define a surrogate as “a non-pathogenic species and strain responding to a particular treatment in a manner equivalent to a pathogenic species and strain.” Indicator and surrogate microorganisms are used for a variety of uses and purposes in the food systems (Busta et al., 2003). This includes evaluating quality or the safety of processed or raw foods and validating the effectiveness of microbial control measures (Busta et al., 2003). In terms of evaluating the efficacy of given processing treatments in food systems, surrogate microorganisms are more commonly used in place of foodborne pathogens in challenge tests,

either done in a laboratory setting or in food manufacturing plants (Hu and Gurtler, 2017). Surrogate microorganisms with both characteristics and behaviors similar to a pathogen being evaluated can be extremely useful in decontamination steps throughout processing (Bianchini et al., 2014). The use of surrogates are a nonpathogenic microorganism with specific selected strains prepared in either a laboratory and inoculated into food products or organisms that can be naturally occurring (U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2001). A practical limitation of process validation, is that the actual pathogens can't be taken in to a food processing facility (Niebuhr et al., 2008). Foodborne pathogens must be handled in a level 2 biosafety facility with personnel who are appropriately trained for the scenario (Hu and Gurtler, 2017). The use of target pathogens in challenge studies can be ideal, but can be devastating if improperly handled. Mishandling of a pathogen can affect the researcher's safety, food safety, and even the safety of the processing environment (Hu and Gurtler, 2017).

Surrogates are generally selected from a population of well-known organisms that also have well-defined characteristics and a long history of avirulence which can easily be differentiated from other microflora (Kim and Harrison, 2009). When the laboratory is selecting a surrogate a review of the history of that specific surrogate is important when using it as a representation of the pathogenic microorganism (Greenberg et al., 2010). When choosing a surrogate for food intervention studies, the surrogate must justify support and documentation with regards to the target pathogen, food and intervention treatment being evaluated (Hu and Gurtler, 2017). The selection of appropriate surrogates is important in regards to specific interventions or the food process that is being imitated (Hu and Gurtler, 2017). Although it may not always be possible to use a surrogate that correlates with the pathogen being targeted in certain situations.

As previously noted, surrogates are used to replace pathogenic bacteria used in a challenge test. When looking at past studies, appropriate measures are used when selecting a surrogate in today research. Although a researcher must carefully select specific strains of surrogate bacteria to provide a proper margin of safety but also must be careful in not selecting a surrogate that is over resistant (Friedly et al., 2008). *Enterococcus faecium* has been used as a surrogate in a number of studies pertaining to thermal lethality (Bianchini et al., 2014). In a study by Jeong et al., (2011), *Enterococcus faecium* was used as a surrogate for *Salmonella* spp. in validating dry heat process in almonds. This microorganism, *Enterococcus faecium*, is generally selected due to its nature and similarities with *Salmonella* spp., which are commonly found in poultry products. *Enterococcus faecium* is widely used in food due to its high thermal resistance (Hardie and Whiley, 1997; Morrison et al., 1997). *Enterococcus faecium* has the ability to grow in a wide range of temperatures (Murray 1990; Devriese et al., 1993) with a presence of salt and in lower pH values (San Perez et al., 1982; Giraffa et al., 1997). Channaiah et al., (2016) used *Enterococcus faecium* in a study and demonstrated greater thermal resistance compared with *Salmonella* during bun baking and could serve as a conservative surrogate to validate thermal process lethality in commercial bun baking operations. In a study conducted by Enache et al., (2015), it was reported that *E. faecium* is a conservative but appropriate surrogate for *Salmonella* under testing conditions. A study conducted by Niebuhr et al., (2008), concluded from their study that bacteria, either individually or collectively, have the potential to be used to validate meat processes for the reduction of *Salmonella*. Use of surrogates in future studies will be beneficial to not only researchers but to industries as well.

1.7 Antimicrobial Intervention Approaches for Processing

Poultry processing facilities are trying to control or even improve the safety of food products by decontaminating the carcasses or products during or at the end of production (Bolder, 1997). Laury et al. (2009) reported that antimicrobials are commonly used in the industry to reduce pathogen loads. Bolder (1997) specified that antimicrobial interventions are applied to poultry products to both physically and chemically reduce numbers of pathogens such as *Salmonella* spp. in U.S. processing plants and to prevent human foodborne infections. Dickson and Anderson (1992) stated that a variety of antimicrobial treatments for broiler carcasses have been investigated, with a primary focus on those that are to be practical and effective.

Antimicrobials help reduce microbial contamination on carcasses and inhibit the cell survival and reproduction of bacteria. For the antimicrobials to be effective in the facilities, they first must be approved by the USDA-FSIS, and must show efficacy against microorganisms. Compliance Guideline (2010), notes that the antimicrobial treatments must contain a certain concentration and must have a specific contact time between the wash and the carcasses. Treatments must also take into consideration the cost effectiveness and minimal adverse effect on the quality of final products. A few examples of antimicrobials approved for processing facilities are chlorine, inorganic acids, and organic acids (Bodler, 1997). This includes acetic and lactic acid, chickxide (lactic/ citric acid blend), and peracetic acids (Bolder, 1997). The industry has such a variation of treatments processors are using. More processing plants tend to use chlorine and organic acids since they are more effective. However, most antimicrobials have not been studied under mobile processing slaughter conditions (Killinger et al., 2010).

One of the most common antimicrobial treatments for decontamination of poultry is chlorine, it's used because it is inexpensive, safe, and easy to used (White, 1998; Northcutt and Jones, 2004).

When chlorine is the treatment, it must be mixed in with water at optimal temperatures and contain a certain concentration. In addition to chlorine in the poultry processing water, the USDA-FSIS requires a certain concentration, used in parts per million, allows levels of 20-50 ppm (USDA-FSIS, 2003). Efficacy of chlorine for bacterial reduction will decrease with an increase in pH and the organic load (Byrd and McKee, 2005). However when comparing chlorine to other treatments, no other treatments have been able to replace chlorine (Northcut et al., 2007). Chlorine used at maximum level of 50 ppm, around a 1-log microbial reduction is seen (James et al., 1992). Although chlorine levels are not to exceed 50 ppm, *Salmonella* spp. were eradicated from poultry carcasses with treatments of 300-400 ppm but not with 50 ppm with chlorine treatment (Teotia and Miller, 1975; Bautista et al., 1997). However, McKee (2011) stated that for chlorine to be effective, there must be at least a contact time of 1 to 1.5 hours. A study done by Northcutt et al., (2007) recovered 6.3 and 3.9 log₁₀ CFU/mL on control carcasses for *Campylobacter* and *Salmonella*; after washing with sodium hypochlorite *Campylobacter* and *Salmonella* populations recovered were 1.6 and 2.4 log₁₀ lower than the control carcasses. Chlorine concentration levels will vary in the industries when comparing it to acid antimicrobial treatments.

Acid is used to destroy bacteria by penetrating through the cell and disrupting their cell membrane (Mani-Lopez et al., 2012). The acid molecule dissociates, this then further acidifies the cells interior, destroying the bacteria cells (Marriott, 2006). Organic acids have been investigated due to their bactericidal activity and because they are generally recognized as safe (GRAS) (Dickson 1992; Izat et al., 1989; Thomson et al., 1967). These specific types of acids are known for antimicrobial properties and their treatments have continuously been used throughout poultry processing.

In studies, organic acid treatments are found to be more effective before the bacteria are attached to the surface of the carcasses. Acid concentrations that had $\geq 4\%$ were required to kill ≥ 2 log reduction of cells that were attached to broiler skins (Tamblyn and Conner, 1997). As previously noted, acids have been combined with other antimicrobial treatments in order to help lower the levels while assuring the efficacy of bacterial reduction. McKee (2011) conducted a survey and found that the most prevalent chemical used in post-chill applications in the industry was peracetic acid (PAA). This is a mix of an organic acid, which acetic (acetic acid) and an oxidant mixed (hydrogen peroxide) (Mani-Lopez et al., 2012). In regards of reduction, this antimicrobial treatment (PAA) reported a greater than a 3-log reduction when compared to using other individual antimicrobials (Bauermeister et al., 2008). Chen et al., (2014) found that 0.007 and 0.1% PAA had the greatest reductions on *Salmonella* and *Campylobacter*, of approximately 1.5 and 1.3 log- reductions. Although in a study by Laury et al., (2009) found that spraying carcasses for 5 seconds with Chicxide had resulted in a reduction of 1.3 log CFU/mL, whereas immersing 5 seconds resulted in a 2.3 log CFU/mL reduction. Lactic acid has shown effectiveness as an antimicrobial intervention and is commonly used (Hamby et al., 1987; Hardin et al., 1995). Anang et al., (2007) used a 10 minute time with 2% lactic acid dip and observed a 1.17 log-reduction of *Salmonella enteritidis* on chicken breasts. However, further evaluation of organic acids may help provide an economical and effective mean in controlling bacteria during poultry processing (Tamblyn and Conner, 1997).

Reducing microbial contamination may better be achieved through chemical applications during post-chilling as opposed to pre-chilling interventions. There has been an increase in poultry processing facilities that are using post-chilling antimicrobial interventions (McKee, 2011). Postchill application is one of the newer strategies used in the poultry processing industry, this

application is applied immediately after the products leave the primary chiller (McKee, 2012). This method is used as a last resort to defend against pathogens before broilers leave the chilling system. This technique has a short time-period and higher concentrations of antimicrobials and is less expected to affect the quality of the final product and its quality. Furthermore, post-chilling has advantages over pre-chilling since the carcasses will be the cleanest, since the organic matter on the carcasses are lower during this period. In theory, respectively, bacteria can be greater reduced more effectively.

1.8 Conclusions

The abnormally high numbers of foodborne pathogens present on broiler carcasses is increasing every year. A target of 2-log reduction in bacterial counts to help eliminate or reduce *Salmonella*, most common pathogen on poultry products. Considering *Salmonella* continues to be a major cause food borne illness, despite the USDA regulations have become stricter. In the poultry processing industry, frequent contamination is occurring. Industries are trying to better understand what can be done to prevent these pathogens from spreading. Despite the variety of options available, studies have shown that antimicrobial treatments, may be the most suitable intervention in the processing plants.

In more recent years, some intervention strategies have been implemented. Post-chilling decontamination tanks have been able to provide a substitute for pathogen reduction during poultry processing (McKee, 2011). While the efficacy of antimicrobials has been validated against *Salmonella*, further studies can incorporate chillers to identify if it will be beneficial for the industries as well as for the consumers.

2.0 Study Objectives

Objective 1: Evaluating the prevalence of *Salmonella* spp. and aerobic plate counts (APC), *Escherichia coli*, total coliforms (TCC), and yeast and molds on broiler carcasses from a small, local, USDA- Inspected Facility in West Virginia

Objective 2: Evaluating the efficacy of commercial antimicrobials against *Salmonella* cells that were either unstressed or cold-stressed and non-pathogenic surrogate *Enterococcus faecium* on broiler carcasses and wings

3.0 Introduction

The Centers for Disease Control and Prevention (2011) estimated that each year in the United States, approximately 48 million people are affected and suffer from food-borne illnesses. Contaminated poultry meat is one of the most common food products that creates a public health concern. *Salmonella* and *Campylobacter* spp. are the two food-borne pathogens most commonly associated with poultry meat products (Nagel et al., 2013). Of these two foodborne pathogens, *Salmonella* is more common (Scallan et al., 2011). Out of all the illnesses attributed to poultry, *Salmonella* is responsible for 35% of illnesses (Batz et al., 2011). *Salmonella* spp. associated with poultry meat account for an estimated 9.4 million illnesses a year, leading to 55,961 hospitalizations and 1,351 deaths annually in the United States (Scallan et al., 2011).

Since 2011, new performance standards have been settled by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) in response to baseline studies that require routine testing (USDA-FSIS, 2010). These performance standards require routine testing for *Salmonella* in all processing plants to ensure that positive samples remain below an average of 7.5% (USDA-FSIS, 2010). Studies conducted by Scheinberg et al., (2013) and Trimble et al., (2013) evaluated the presence of foodborne pathogens in chicken products and verified the presence of *Salmonella* spp. ranged from 28-45%. Other studies can evaluate the prevalence of these foodborne pathogens and lead to best practices to prevent contamination during processing and packaging.

Bacterial contamination of poultry carcasses during processing is undesirable, but unavoidable (Tamblyn and Conner, 1997). With high percentages of *Salmonella* present,

processing plants need to reduce or eliminate these pathogens before these products reach the consumers (Tamblyn and Conner, 1997). Although, there is no generally accepted and applicable method to reduce these specific pathogens causing illnesses, slaughtering facilities must somehow prevent such bacteria contamination (Bolder, 1997). Eliminating or decreasing pathogen counts, especially *Salmonella* in meat products post-harvest, can be resolved by different physical as well as antimicrobial treatments suggested by Mani-Lopez et al., (2011). Processing plants use various antimicrobial treatments ranging from a simple water wash to the application of organic acids, or mixes of various acids, and chlorine treatments. Commercial treatments such as lactic acid, peroxyacetic acid, and sodium hypochlorite are just a few antimicrobials approved by USDA-FSIS for use in decontaminating poultry carcasses (USDA-FSIS, 2017). Current, available data on industry scale poultry processing demonstrates that various commercial antimicrobials control *Salmonella* in poultry meat processing (Oyarzabal, 2005; McKee, 2011, 2012; Russel, 2012).

Recently, pathogen-control interventions during processing have changed and will continue to change with new laboratory advances. Real poultry processing facilities do not allow the use of pathogens for validity testing due to the biosafety concern. The use of surrogates and the fact that they are a helpful approach was considered by Bianchini et al., (2014) and Boney and Moritz (2016) to study the behavior and see if *Enterococcus faecium* mimicked *Salmonella* and can be used in future studies. The studies reviewed focus on various antimicrobial treatments or strategies that combine approved antimicrobials with pre- and post-chilling environments to eliminate specific foodborne pathogens on broiler carcasses and produce safe products for consumers. The objective of the study is to determine prevalence of *Salmonella* spp., APC's, TCC's, *E. coli*, and yeast and molds. The second part of the study evaluates the antimicrobial efficacy of peroxyacetic acid, lactic acid, lactic/ citric acid blend, and sodium hypochlorite against

unstressed and cold-stressed adapted *Salmonella* and non-pathogenic *Enterococcus faecium* on broiler carcasses and wings.

4.0 Materials and Methods

4.1 Microbial Quality and Welfare of Broiler Carcasses

A study consisted of 42 fresh then frozen broiler carcasses obtained from a small West Virginia Department of Agriculture Meat and Poultry Inspection Division facility (MPID). Broilers carcasses were processed by a USDA-FSIS facility in the state of Maryland. Whole chickens were transported to the state of West Virginia, where whole chickens were sold at the local Morgantown, WV Farmers Market. Whole chickens were purchased and delivered in coolers back to the Food Microbiology lab at West Virginia University. The broiler carcasses were stored at -25 °C, they were thawed at 4 ° for 48-72 hours for analysis. Following thawing, whole carcasses were placed in 400 mL of Buffered Peptone Water (BPW, Hardy Diagnostics, MD, USA) and vigorously shaken for 60 seconds in a sterile poultry rinse bag (Nasco, Fort Atkinson, WI, USA) (Scheinberg et al., 2013). The rinsate was plated on 3 M[®] APC, *E. coli*/TCC, and yeast/ mold petrifilms (3 M Microbiology Products, St. Paul, MN, USA) for enumeration of the total aerobic bacteria, generic *E. coli*, coliforms, and fungi. Petrifilms were hand counted after incubation at 25 °C for 72 hours (APC), 35 °C for 48 hours (*E. coli*/ TCC), and 25 °C for 120 hours (yeast/mold).

The remaining rinsate, approximately ~390 to 395 mL, was incubated at 35 ° for a 24 hour period. From the incubated solution, one tenth of the pre-enrichment was transferred to 10 mL Rappaport Vassiliadis (RV, Hardy Diagnostics, MD, USA). RV tubes were placed into a 42 °C incubator for 24 hours. From the RV tubes, a loop-full of incubated enrichment was streak plated onto Xylose Lysine Tergitol 4 (XLT-4) and HardyCHROM[™] agar (Hardy Diagnostics, MD, USA). Plates were placed into the incubator at 35 °C for 24 hours. After incubation, 1-3 presumptive *Salmonella* colonies the XLT-4 and HardyCHROM[™] were confirmed using the Oxoid

Salmonella Latex Agglutination test kit (Oxoid Ltd, Basingstoke, Hampshire, UK). Another confirmation test, API 20E test kit (BioMerieux, Durham, NC, USA) was used. *Salmonella typhimurium* ATCC 14028 was used as the positive control for the biochemical and immunological tests.

4.2 *Salmonella* and *Enterococcus* inoculum preparation

Salmonella Typhimurium ATCC 14028, *Salmonella* Tennessee ATCC 10722, and *Enterococcus faecium* ATCC 8459 were used in this study. Each *Salmonella* strain were maintained on Xylose Lysine Tergitol 4 (XLT-4) agar (Hardy Diagnostics, MD, USA). Whereas, the *Enterococcus* strains were maintained on Bile Esculin agar (Hardy Diagnostics, MD, USA). These two agars were stored at 4 °C. Regarding preparation of unstressed and cold-stress cells, the procedures are described by Shen et al., (2010). Unstressed cells, single colonies of each *Salmonella* and *Enterococcus* strains, were inoculated into 10 mL of Tryptic Soy Broth (TSB; Hardy Diagnostics, MD, USA) and incubated at 35 °C for 24 hours. Subcultures and 0.1 mL of the 24 hour suspension were added to 10 mL of TSB and incubated for an additional 24 hours at 35 °C. To prepare the cold-stress cells, previous subculture was triplicate-washed in a 10 mL tube of 0.1% Buffered Peptone Water (BPW; Becton Dickinson and Company, Sparks, MD, USA), the mix was re-suspended in 10 mL of TSB and stored in a refrigerated setting at 4 °C for 7 days. Previously, two *Salmonella* cultures were combined, and this mixture was centrifuged (VWR Symphony 4417, VWR International, Radnor, PA; 5,000 × g, 15 minutes). Following centrifugation, a duplicate wash was done with 0.1% BPW to remove any broth remaining in the tube. Cultures were centrifuged once more and re-suspended in 0.1% BPW. *Enterococcus* cultures were prepared similarly. This preparation resulted in a final inoculum suspension of ~ 8 log CFU/mL of *Salmonella* and *Enterococcus*.

4.3 Broiler carcasses and wings collection and inoculation

Broiler carcasses and skin-on wings were collected from the same processor that is a USDA-MPID in Maryland. An average of 800 pasture-raised cage-free birds and each year. Processing included: stunning, exsanguination, single stage static scalding, evisceration on stainless-steel table, and a wash bath. The wash was composed of an antimicrobial agent, a citric- and lactic-acid based blend, before the carcasses were placed in a static container with ice water for 24 hours. In terms of the skin-on wings, they were manually removed by cutting them off the carcasses. As required by the USDA- MPID, the whole carcasses and wings were kept in a cooler, transported back to the West Virginia University Food Microbiology lab, where they remained in the walk in freezer for 24-72 hours until the experimentation. Broiler carcasses and skin-on wings were thawed at 5 °C in a walk-in cooler for 48-72 hours depending on thawing time. Carcasses and wings were randomly assigned to a treatment and were inoculated with unstressed and cold-stress adapted *Salmonella* and *Enterococcus* based on the experiment procedure noted by Scott et al., (2015a). As for the carcasses and wings, both sides (all surface area) were spot inoculated with 5 drops of 200 µL of the bacterial suspension. Mixture was spread over both sides of the carcasses and wings using an L spreader (L shaped spreader, Hardy Diagnostics, MD, USA). Immediately after inoculation, carcasses and wings remained under a biohazard hood at ambient temperature for 20 minutes. This allowed time for cell attachment to the skin surface. Bacterial suspension was also spread plated on to agars. Plates were counted to identify what the final target inoculum. Final target inoculation on carcasses and wings ranged from ~ 5.5 to 6.5 log CFU/mL.

4.4 Antimicrobial treatment of broiler carcasses and wings

Inoculated broiler carcasses and wings were left untreated (control), followed by four treatments: peroxyacetic acid (PAA, 0.1%, pH 3.0, 15.7 °C. Birko, CO, USA), lactic acid (LA,

5%, pH 2.0, Birko, CO, USA), lactic and citric acid blend (LCA, 2.5%, Chicxide, Birko, CO, USA), and sodium hypochlorite (SH, free available chlorine, 67-69 ppm, pH 9.41, 14.4 °C, Birko, CO, USA). Treatment included immersing four wings into a 3-liter solution and three carcasses into a 10-liters solution. Dip treatments of antimicrobial solutions were applied agitation (~500 rpm) for 30 second periods. Carcasses were not drained, whereas wings received a 2-minute period to drain. Tested concentrations of all antimicrobials, except, chlorine, was the highest level allowed in the USDA-FSIS Directive 7120.7 (USDA-FSIS, 2017). Antimicrobial concentrations for PAA, LA, LCA were calculated per the supplier's fact sheet. In regarding chlorine concentrations, treatment concentration based off SH concentration used during poultry processing, which is 50 ppm of free chlorine; however, it is unclear whether the initial chlorine or residual concentration. This is concerning due to the instantaneous reaction between the chlorine and organic matter. Consequently, a slightly higher concentration of chlorine, ranging from 67-69 ppm was used. Residual free chlorine concentrations were examined and noted. Carcasses ranged from 11.8 to 28.0 ppm for wings, after 20 second dip treatment, based on the chlorine measurement using the DPD-method (Shen et al., 2013).

4.5 Microbial analysis

Total aerobic plate counts, for both *Salmonella* and *Enterococcus* populations were analyzed. Following antimicrobial treatment, carcasses were rinsed in 400 mL of BPW, supplemented with 0.1% sodium thiosulfate (Fisher Scientific, Springfield, NJ, USA). Carcasses were individually placed into sterile chicken-rinse bags, they were vigorously shaken (manually) for a 60 second period. Individual wings were placed in a sterile Whirl-Pak® (Nasco. Fort Atkinson, WI, USA), containing 150 mL of BPW supplemented with 0.1% sodium thiosulfate. Each bag containing a wing was vigorously shaken for 60 seconds. Broiler carcasses and wings

were shaken to ensure cells can detach from skin surface to determine survival or reduction of unstressed or cold-stress adapted cells. Following rinsing, BPW mixtures were plated onto four different media. Tryptic Soy Agar (TSA) was used for aerobic plate counts, XLT-4 and HardyCHROM™ agar was used to grow *Salmonella*; whereas Bile Esculin agar grew *Enterococcus*. Plates were incubated for 24 hours before counting using the counter in the laboratory.

4.6 Data analysis

Studies were repeated twice; 4 wings and 3 carcasses per treatment replication were used. This approach brought the total of 8 wings per treatment and 6 samples for carcasses per treatment. Experiments were conducted on a completely randomized 5×2 factorial design. Five treatments with two stress-type cells, unstressed and cold-stressed account for this design. A Mixed Model Procedure of SAS (version 9.2, SAS, Cary, NC) was used. Analyzing the survival and reduction of *Salmonella* and *Enterococcus*. The experimental model included the data analyzing individual factors such as antimicrobial treatment, cell stress type, and their interactions. Comparing the level of reduction between the *Salmonella* and *Enterococcus* and their responses to the different antimicrobial treatments, the Mixed Model Procedure used individual factors of the bacterial medium, the treatments used, the cell stress type, and all possible interactions of the study. Reduction data was determined by using a reduction ratio of $\log_{10} (N_0/N)$. N_0 states the average control plate counts, whereas the N accounts for the plate count of each individual antimicrobial treated sample. The means were compared using $\alpha = 0.05$ significance level determined by Tukey HSD.

5.0 Results and Discussion

Table 1 shows the populations recovered on APC, *E. coli*/ TCC, and yeast/molds from 42 samples. These results are hygienic indicators of the microbial quality of whole raw carcasses. The APC is ranged from 2.1 to 3.4 log CFU/mL with an average of 2.6 log CFU/mL; whereas, the population of TCC ranged from 0.5 to 2.3 log CFU/mL with an average of 1.1 log CFU/mL. Compared to a previous study done on whole chickens from a farmers' market and supermarket in Pennsylvania, investigators found that APC and TCC populations ranged from 2.6 to 4.2 and 0.8 to 1.5 log CFU/g, respectively (Scheinburg et al., 2013). Total populations of yeast/molds ranged from 1.6 to 4.0 log CFU/mL, averaging 2.4 log CFU/mL. As a potential food safety hazard, yeast/molds may produce a mycotoxins that may have carcinogenic and immunosuppressive effects. Generic *E. coli* is used to assess potential fecal contamination on processed meat and poultry products per the USDA-FSIS (Scheinberg et al., 2013). In the current study, approximately 30% (12/42) of carcasses sampled tested positive for *E. coli*, with range of 0.5 to 1.7 log CFU/mL; this level is comparable to previous studies of Scheinberg et al., (2013) and Northcutt et al., (2006). Although levels of APC and *E. coli*/TCC on theses carcasses from the USDA-MPID are not greater than the previous study, it is still a concern for small broiler processors to apply a post-harvest interventions to further reduce background microflora that remains on broiler surfaces.

5.1 *Salmonella* spp. prevalence on broiler carcasses.

A total of 42 broiler carcass samples were used and evaluated for the presence/ absence of *Salmonella* spp. using API 20E strips with biochemical profile 6704752 (Levy et al., 2014). Further confirmation of *Salmonella* was done with the Latex Agglutination test. The frequency of *Salmonella* detection (29%) in this study is lower than a previous study that surveyed carcasses

from a small USD-IF in the southeastern United States; 43% were positive for *Salmonella* with 8% positive from Cornish Cross and 78% positive from Red Ranger birds (Trimble et al., 2013). Current results are comparable to findings from a study conducted in Pennsylvania where 28% were *Salmonella* positive from the farmers' market and 20% were positive from local organic farms at the supermarkets (Scheinberg et al., 2013). Occurrence of *Salmonella* present on small, locally processed broilers may be attributed to lack of farm management, regulatory guidelines, insistence on minimal antimicrobial application, and a single static chilling tank. Our results provide an important preliminary baseline dataset for frequency of detection for *Salmonella* on USDA-IF broilers produced in West Virginia. This data justifies development of post-harvest control strategies that use a post-chilling dipping treatments mixed with an antimicrobial agent.

5.2 Efficacy of antimicrobials to inactivate aerobic plate counts and *Salmonella*

Total aerobic plate counts were determined using TSA (Table 1). Aerobic plate count for untreated, unstressed, and cold-stressed adapted *Salmonella*, inoculated on whole broiler carcasses and wings, ranged from 6.0 to 6.3 log CFU/mL. Carcasses with antimicrobial treatments showed a main effect ($P=0.001$) despite the type of stress ($P=0.947$), and an interaction treatment and the cell stress type. Although for wings, the individual factors analyzed and the interactions were statistically significantly ($P<0.05$). A difference of 0.2 to 0.3 log units in various comparison is not considered microbial relevance (Scott et al., 2015b). Of the various antimicrobials tested, reductions in aerobic plate counts ranged from 0.7 to 2.3 log CFU/mL on carcass and 0.6 to 1.7 log/CFU/mL on wing samples inoculated with unstressed or cold-stressed cells. Samples treated with 0.1% PAA had the greatest ($P<0.05$) reduction of aerobic plate counts on carcasses and wings. This difference ranged from 1.7 (unstressed) to 2.3 (cold-stressed) on carcasses; whereas, wings

ranged from 1.4 (unstressed) to 1.7 (cold-stressed) log CFU/mL, respectively. Other treatments, LA, LCA, and SH notably, had similar reductions on aerobic plate counts; results ranged from 0.7 to 0.9 and 0.6 to 1.1 log CFU/mL for all carcasses and wings.

The efficacy of antimicrobial treatments was based on *Salmonella* recovery from XLT-4 and HardyCHROM™, Table 2 and 3, respectively. Antimicrobial treatment reduced ($P<0.05$) *Salmonella* on carcasses and wings for stressed microorganisms. Interactions between antimicrobial treatments and stress cell type showed significance ($P<0.05$) on the HardyCHROM™ agar, but showed no significance on the XLT-4 agar. Cold-stress adapted *Salmonella* cells were more sensitive ($P<0.05$) to antimicrobial treatment compared to unstressed cells on both broiler carcasses and wings. As an indication of *Salmonella* load, control broiler carcass counts ranged from 5.4 to 5.6 log CFU/mL of both unstressed and cold-stress adapted *Salmonella*.

Unstressed *Salmonella* counts were obtained from broiler carcasses that had been treated with PAA, LA, LCA, and SH for 30 seconds. Reductions ranged from 0.9 to 1.7 log CFU/mL and 0.6 to 1.5 log CFU/mL, respectively, for counts determined with XLT-4 and HardyCHROM™ agars. The same treatments produced a greater ($P<0.05$) reduction of cold-stress adapted *Salmonella* cells on carcasses. Carcasses levels were 1.3 to 2.1 log CFU/mL on XLT-4 and 1.1 to 1.8 log CFU/mL on HardyCHROM™. In comparison, wings showed similar results; antimicrobial dipping treatments reduced ($P<0.05$) cold-stressed *Salmonella* cells; cold-stressed reductions ranged from 0.9 to 1.7 log CFU/mL and unstressed was comparable with 0.5 to 1.3 log CFU/mL. Cold-stress cells were more susceptible to interventions; these findings agree with previous studies carried out by Semancheck and Golden (1998) and Shen et al., (2011). This sensitivity is due to the increasing amount of unsaturated fatty acids across the cell membrane and the reduction of their melting point after adapting to cold-stress. Results suggested that the chilling process

generated cold-stress adapted pathogenic cells on poultry surfaces; this adaption to cold-stress might increase the efficacy of post chilling, antimicrobial dipping treatments.

Among antimicrobial treatments, PAA was the most effective ($P<0.05$) for reducing *Salmonella* on carcasses and wings (Table 3 and 4). Other treatments, lactic acid, lactic/citric acid blend, and sodium hypochlorite, produced similar results and were less effective compared to PAA. PAA had reduced unstressed cells by 1.5 log CFU/mL on HardyCHROM™ and by 1.7 log CFU/mL on XLT-4; reductions of 1.5 log CFU/mL on HardyCHROM™ to 1.7 log CFU/mL on XLT-4, in respects of carcasses (Table 3) and wings (Table 4). Sensitivity of cold-stressed cells to PAA was greater; the reduction level was increased to 1.8 (HardyCHROM™) and 2.1 (XLT-4) log CFU/mL on carcasses and 1.5 (HardyCHROM™) and 1.7 (XLT-4) log CFU/mL on wings. PAA, which is combination of peracetic acid and hydrogen peroxide, is approved by USDA-FSIS for application of poultry carcasses during post-chill dipping at concentrations no greater than 2,000 parts per million (ppm) (0.2%) (McKee, 2011). Nagel et al., (2013) reported that 0.1% PAA reduced nalidixic acid resistant-*Salmonella* by 2.3 log CFU/mL on broiler carcasses in a pilot plant, chilling tank at a 20 second dwelling time. A recent study from the same research group found that 0.1% PAA achieved a 1.5 log reduction of *Salmonella* on post-chilled ground chicken (Chen et al., 2014). Scott et al., (2015a) reported that 0.007% (700 ppm) PAA reduced *Salmonella* populations by 1/5 log on chicken wings after a 20 second dipping treatment. The aforementioned studies stated that PAA (0.04-0.1%), used during the post-chill dipping process, is the most effective antimicrobial to decontaminate *Salmonella* on poultry products. Even when comparing PAA to other commercially used products such as chlorine, lysozyme, and sulfuric acid. For small poultry producers in West Virginia and Pennsylvania, producers are more intrigued by use of PAA instead of chlorine to decontaminate foodborne pathogens.

In the current study, broiler carcasses and wings treated with LA (5%) had reduced unstressed *Salmonella* cells by 1.1 (HardyCHROM™) to 1.3 (XLT-4) log CFU/mL and 0.8 (HardyCHROM™) to 1.1 (XLT-4) log CFU/mL, respectively (Table 3 and 4). In terms of cold-stressed cells, higher ($P<0.05$) reductions of 1.7 log CFU/mL (on both HardyCHROM™ and XLT-4) and 1.4 (HardyCHROM™) to 1.7 (XLT-4) log CFU/mL were observed on inoculated broiler carcasses and wings, compared to unstressed cells. LA is currently used to reduce *Salmonella* on retail poultry and meats produced since USDA-FSIS issued a pathogen reduction standard for *Salmonella* in 1994 that allowed concentration $\leq 5\%$ for pre- and post-chilling of animal carcasses (Laury et al., 2009). An earlier study (Izat et al., 1990), found that 1% of LA in chilling and scalding water reduced *Salmonella Typhimurium* below the detectable limit. A study by Yang et al., (1998) sprayed 2% LA on carcasses, obtaining a 2 log-reduction of *Salmonella* per carcass. Killinger et al., (2010) also studied the efficacy of a 2% LA rinse on chicken wings. Authors observed a greater than 5 log-reduction in inoculated *Salmonella*. For the current study, an approximately 1-2 log-reduction of *Salmonella* was observed; meeting expectation since there was only a 30 second dip time. This was compared to another study by (Killinger et al., 2010), who used a 3 minute treatment.

In the present study and for unstressed *Salmonella* cells, LCA blend and SH treatments produced similar reductions ($P>0.05$) of 0.6 to 0.7 (HardyCHROM™) to 0.9 to 1.1 (XLT-4) log CFU/mL for carcasses; reductions of 0.5 (HardyCHROM™) to 0.8 to 1.0 (XLT-4) log CFU/mL was observed on wings compared to the controls (Table 3 and 4). In terms of treatment effects on cold-stressed cells, an additional reduction ($P<0.05$) of 0.3 to 0.8 log CFU/mL was observed on carcasses and wings using XLT-4 and HardyCHROM™ agar (Tables 3 and 4). Recently, the use of chlorine in the poultry chillers has declined due to the reactivity of chlorine with organic matter

(Shen et al., 2016). Chlorine treatments require a high concentration and a long contact time, generating gas and trichloramines, hindering export to Russia (Chen et al., 2014). In small poultry processing facilities, interest in using chlorine is slowly decreasing due to expectation for natural and organic processes. Chicxide is a newly commercial blend of lactic/ citric acids; approved for use in poultry meats with a maximum concentration allowance of 2.5% according to USDA-FSIS (USDA-FSIS, 2005). Laury et al., (2009) reported that spraying Chicxide, on broiler carcasses for 20 seconds, reduced *Salmonella* by 1.3 log CFU/mL; dipping treatments resulted in 2.3 log, *Salmonella* reduction. Furthermore, these authors indicated the Chicxide results were comparable to chlorine. The aforementioned study suggests that a lactic- and citric acid based antimicrobial agent could be beneficial in applying post-chilling treatments for small scale poultry processors.

5.3 Efficacy of antimicrobials to inactivate *Enterococcus faecium*

Response of unstressed and cold-stressed, adapted *Enterococcus faecium* to various antimicrobial treatments was evaluated. Overall, dipping broiler carcasses into four antimicrobials reduced unstressed *Enterococcus faecium* populations by 0.2 to 1.7 log CFU/mL; whereas a 1.0 to 2.0 log CFU/mL reduction on wings, compared to the untreated control sample was observed (Table 5). As for the cold-stressed cells on carcasses, they appeared to be more susceptible ($P<0.05$) to antimicrobials than unstressed cells with reductions increasing to 1.0 to 2.0 log CFU/mL. Lactic acid resulted in an additional of 0.3 log CFU/mL reduction of cold-stressed cells on wings compared to unstressed cells. *Salmonella* observations were similar for unstressed or cold-stressed-adapted *Enterococcus* cells on carcasses and wings; order of antimicrobial efficacy was: SH<LCA< LA<PAA. As an example and compared to the control, PAA reduced levels of *Enterococcus faecium* by 1.7 to 2.0 log CFU/mL on carcasses and wings for unstressed or cold-stressed adapted cells (Table 5).

Enterococcus faecium has been evaluated as a safer alternative to study *Salmonella* for thermal lethality validation studies in almonds (Jeong et al., 2011) and in a balanced carbohydrate protein meal (Bianchini et al., 2014). However, this *Salmonella* surrogate has not been studied or evaluated on poultry products, and there is limited information on appropriate *Salmonella* surrogates during poultry processing. To draw valid conclusions about antimicrobial efficacy using surrogate microorganisms, the surrogate must survive the process at least as well or better than the target pathogen (Adler et al., 2016). The current study evaluates reductions of unstressed or cold-stressed adapted *Salmonella* and *Enterococcus faecium* on broiler carcasses and wings from various antimicrobial treatments (Tables 6 and 7). Because TSA support medium permits growth of other microorganisms, TSA counts were not used for comparison in this study.

Application of PAA, LA, LCA, and SH to carcasses reduced unstressed and cold-stressed *Enterococcus faecium*, and levels were not different or less than those for *Salmonella*. PAA reduced counts by 1.7 and 2.0 log CFU/g for unstressed and cold-stressed adapted *Enterococcus faecium*, respectively; this response is similar to *Salmonella* ($P<0.05$) reductions that ranged from 0.2 to 0.5 log CFU/mL for unstressed cells and from 1.0 to 1.4 log CFU/mL for cold-stressed adapted for *Enterococcus faecium* compared to the unstressed 0.6 to 1.3 log CFU/mL on XLT04 and HardyCHROM™; Table 6). Reductions of unstressed *Enterococcus faecium* on wings treated by the antimicrobials were greater ($P<0.05$) than *Salmonella* reductions on HardyCHROM™ agar; although, results were similar ($P>0.05$) to the count for XLT-4 agar when lactic acid or the lactic/citric acid was used (Table 7). Cold-stressed *Enterococcus faecium* on wings behaved similar ($P>0.05$) to cold-stressed adapted *Salmonella* after being exposed to the antimicrobials; reductions of 1.0 to 1.9 log CFU/mL, determined with Bile Esculin agar, were observed. *Salmonella* reductions of 1.1 to 1.7 log CFU/mL and 0.9 to 1.5 log CFU/mL were observed with

XLT-4 HardyCHROM™ agar, respectively (Table 7). *Enterococcus faecium* could serve as a surrogate for *Salmonella* validating antimicrobial interventions during broiler processing.

In conclusion, results of the current study confirmed that applying a post-chilling antimicrobial dipping treatment, primarily PAA, could be used as an intervention approach to control *Salmonella* on locally processed broilers. Cold-stressed adapted *Salmonella* cells are more susceptible to antimicrobials than the unstressed cells. This is due to the cell walls being more susceptible to antimicrobials and deteriorate more easily. *Enterococcus faecium* serve as a potential nonpathogenic surrogate for *Salmonella* in in-plant validation studies for local, small poultry processors. These results suggest options for development of USDA-FSIS; 5 year strategic plan for *Salmonella* control in poultry meat products (USDA-FSIS, 2013). This data can assist local and state regulatory agencies in reducing potential risk of *Salmonella* in locally grown poultry products.

6.0 References

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Table 1. Mean, standard deviation, and range of microbial populations of aerobic plate count (APC), total coliform (TCC), *Escherichia coli*, and yeast/molds on broiler carcasses.

	Mean $\pm$ S.D.	Range
Aerobic Plate Counts (APC)	2.6 $\pm$ 0.3	2.1-3.4
Total Coliforms (TCC)	1.1 $\pm$ 0.6	0.5-2.3
<i>E. coli</i> (ECC)	0.7 $\pm$ 0.6	0.5-2.7
Yeasts and Molds	2.4 $\pm$ 0.5	1.6-4.0

Note: Each mean value represents rinsate from 42 broiler carcasses.

Table 2. Aerobic plate counts (APC) recovered from unstressed or cold-stressed *Salmonella* cells on both broiler carcasses and wings.

Treatment	Carcasses		Wings	
	Unstressed	Cold-Stressed	Unstressed	Cold-Stressed
Control	6.0 ± 0.1 ^{Aa}	6.1 ± 0.2 ^{Aa}	6.2 ± 0.1 ^{Aa}	6.3 ± 0.2 ^{Aa}
Peroxyacetic acid	4.3 ± 0.4 ^{Ca}	3.8 ± 1.0 ^{Cb}	4.8 ± 0.2 ^{Ca}	4.6 ± 0.1 ^{Db}
Lactic acid	5.1 ± 0.2 ^{Ba}	5.3 ± 0.1 ^{Ba}	5.5 ± 0.1 ^{Ba}	5.2 ± 0.2 ^{Cb}
Lactic/ citric acid	5.3 ± 0.1 ^{Ba}	5.3 ± 0.2 ^{Ba}	5.6 ± 0.1 ^{Ba}	5.5 ± 0.1 ^{Ba}
Sodium hypochlorite	5.2 ± 0.1 ^{Ba}	5.3 ± 0.3 ^{Ba}	5.5 ± 0.1 ^{Ba}	5.3 ± 0.2 ^{BCb}

Note: Each mean value represents rinsate from 6 broilers and 8 wings per treatment.

^{A,B,C,D} Mean values with different capital letters within a column different significantly (P<0.05).

^{a,b} Mean values with different lowercase letters within a row differ significantly (P<0.05).

Table 3. Unstressed or cold-stressed adapted *Salmonella* recovered on XLT-4 and HardyCHROM™ agar from inoculated broiler carcasses.

Treatment	XLT-4		HardyCHROM™ agar	
	Unstressed	Cold-Stressed	Unstressed	Cold-Stressed
Control	5.6 ± 0.2 ^{Aa}	5.4 ± 0.4 ^{Aa}	5.6 ± 0.2 ^{Aa}	5.6 ± 0.2 ^{Aa}
Peroxyacetic acid	3.9 ± 0.4 ^{Da}	3.3 ± 0.7 ^{Db}	4.1 ± 0.3 ^{Da}	3.8 ± 0.9 ^{Ca}
Lactic acid	4.3 ± 0.4 ^{Ca}	3.7 ± 0.2 ^{Cb}	4.5 ± 0.2 ^{Ca}	3.96 ± 0.3 ^{Cb}
Lactic/Citric acid	4.5 ± 0.3 ^{BCa}	4.2 ± 0.6 ^{Bb}	4.9 ± 0.2 ^{Ba}	4.5 ± 0.6 ^{Bb}
Sodium hypochlorite	4.7 ± 0.3 ^{Ba}	4.1 ± 0.3 ^{Bb}	5.0 ± 0.1 ^{Ba}	4.2 ± 0.6 ^{BCb}

Note: Each mean value represents rinsate from 6 broilers.

A,B,C,D Mean values with different capital letters within a column differ significantly ($P<0.05$).

a,b Mean values with different lowercase letters within a row differ significantly ($P<0.05$).

Table 4. Unstressed or cold-stressed adapted *Salmonella* recovered on XLT-4 and HardyCHROM™ agar from inoculated broiler wings.

Treatment	XLT-4		HardyCHROM™	
	Unstressed	Cold-Stressed	Unstressed	Cold-Stressed
Control	6.0 ± 0.1 ^{Aa}	5.9 ± 0.1 ^{Aa}	5.8 ± 0.4 ^{Aa}	5.9 ± 0.1 ^{Aa}
Peroxyacetic acid	4.4 ± 0.2 ^{Da}	4.2 ± 0.3 ^{Cb}	4.6 ± 0.3 ^{Da}	4.4 ± 0.9 ^{Da}
Lactic acid	4.7 ± 0.1 ^{Da}	4.2 ± 0.3 ^{Cb}	5.0 ± 0.2 ^{Ca}	4.5 ± 0.3 ^{Cb}
Lactic/Citric acid	5.0 ± 0.1 ^{Ca}	4.6 ± 0.4 ^{Bb}	5.3 ± 0.1 ^{Ba}	5.0 ± 0.2 ^{Bb}
Sodium Hypochlorite	5.2 ± 0.1 ^{Ba}	4.8 ± 0.1 ^{Bb}	5.3 ± 0.1 ^{Ba}	5.0 ± 0.2 ^{Bb}

Note: Each mean value represents rinsate from 8 wings per treatment.

^{A,B,C,D} Mean values with different capital letters within a column differ significantly ($P < 0.05$).

^{a,b} Mean values with different lowercase letters within a row differ significantly ($P < 0.05$).

Table 5. Unstressed or cold-stressed adapted *Enterococcus faecium* recovered from inoculated both broiler carcasses and wings.

Treatment	Carcasses		Wings	
	Unstressed	Cold-Stressed	Unstressed	Cold-Stressed
Control	5.4 ± 0.2 ^{Aa}	6.1 ± 0.1 ^{Ab}	6.3 ± 0.1 ^{Aa}	6.1 ± 0.2 ^{Aa}
Peroxyacetic acid	3.7 ± 0.4 ^{Db}	4.0 ± 0.4 ^{Da}	4.3 ± 0.3 ^{Da}	4.2 ± 0.2 ^{Da}
Lactic acid	4.8 ± 0.2 ^{Ca}	4.6 ± 0.3 ^{Ca}	5.3 ± 0.1 ^{Ca}	5.0 ± 0.3 ^{Cb}
Lactic/Citric acid	5.2 ± 0.2 ^{ABa}	5.0 ± 0.3 ^{Ba}	5.2 ± 0.1 ^{Ca}	5.2 ± 0.2 ^{Ba}
Sodium hypochlorite	5.0 ± 0.1 ^{BCa}	5.1 ± 0.2 ^{Ba}	5.0 ± 0.2 ^{Ba}	5.0 ± 0.2 ^{Ca}

Note: Each mean value represents rinsate from 6 broilers and 8 wings per treatment.

^{A,B,C,D} Mean values with different capital letters within a column differ significantly ($P < 0.05$).

^{a,b} Mean values with different lowercase letters within a row differ significantly ($P < 0.05$).

Table 6. Comparing reduction of unstressed or cold-stress adapted *Salmonella* and *Enterococcus faecium* recovered from inoculated broiler carcasses.

Treatment	Unstressed			Cold-Stressed		
	XLT-4	HardyCHROM	Bile Esculin	XLT-4	HardyCHROM	Bile Esculin
Peroxyacetic acid	1.7 ± 0.4 ^A	1.5 ± 0.3 ^A	1.7 ± 0.4 ^A	2.1 ± 0.7 ^A	1.8 ± 0.9 ^A	2.0 ± 0.4 ^A
Lactic acid	1.3 ± 0.4 ^A	1.1 ± 0.2 ^A	0.5 ± 0.2 ^B	1.7 ± 0.2 ^A	1.7 ± 0.3 ^A	1.4 ± 0.3 ^A
Lactic/Citric acid	1.1 ± 0.3 ^A	0.7 ± 0.2 ^B	0.2 ± 0.2 ^C	1.2 ± 0.5 ^A	1.4 ± 0.5 ^A	1.1 ± 0.3 ^A
Sodium hypochlorite	0.9 ± 0.3 ^A	0.6 ± 0.1 ^B	0.4 ± 0.1 ^B	1.3 ± 0.3 ^B	1.7 ± 0.6 ^A	1.0 ± 0.2 ^B

Note: Each mean value represents rinsate from 6 broilers carcasses per treatment.

A,B,C,D Mean values with different capital letters within a within a row under unstressed or cold-stressed column different significantly ($P < 0.05$).

Table 7. Comparing reduction of unstressed or cold-stress adapted *Salmonella* and *Enterococcus faecium* recovered from inoculated broiler wings

Treatment	Unstressed			Cold-Stressed		
	XLT-4	HardyCHROM	Bile Esculin	XLT-4	HardyCHROM	Bile Esculin
Peroxyacetic acid	1.3 ± 0.2 ^B	1.1 ± 0.2 ^B	2.0 ± 0.3 ^A	1.7 ± 0.3 ^A	1.3 ± 0.3 ^B	1.9 ± 0.2 ^A
Lactic acid	1.3 ± 0.1 ^A	0.8 ± 0.1 ^B	1.0 ± 0.1 ^A	1.7 ± 0.3 ^A	1.5 ± 0.5 ^B	1.2 ± 0.3 ^B
Lactic/Citric acid	1.0 ± 0.1 ^A	0.4 ± 0.1 ^B	1.0 ± 0.1 ^A	1.2 ± 0.4 ^A	0.9 ± 0.2 ^A	1.0 ± 0.2 ^A
Sodium hypochlorite	0.9 ± 0.1 ^B	0.5 ± 0.1 ^C	1.3 ± 0.2 ^A	1.1 ± 0.1 ^A	0.9 ± 0.2 ^A	1.1 ± 0.2 ^A

Note: Each mean value represents rinsate from 8 wings per treatment.

^{A,B,C,D} Mean values with different capital letters within a row under unstressed or cold-stressed column different significantly ($P < 0.05$).