COMPARISON OF THE ABILITY OF BUFFERED PEPTONE WATER AND NEUTRALIZING BUFFERED PEPTONE WATER TO OVERCOME ANTIMICROBIAL CARRYOVER IN CHICKEN CARCASSES AND PARTS

A Thesis

by

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ABSTRACT

Poultry is a known reservoir of *Salmonella enterica*, and poultry products have been repeatedly identified as transmission vehicles for this pathogen. Poultry processors have incorporated food safety antimicrobial interventions during processing, such as cetylpyridinium chloride (CPC) and peroxyacetic acid (PAA), to assist in reducing foodborne pathogen loads on raw carcasses and parts. The purpose of this study was to determine the capacity of Buffered Peptone Water (BPW) versus neutralizing Buffered Peptone Water (nBPW) to overcome antimicrobial carryover on whole chicken carcasses and chicken parts rinse collections during commercial harvest and fabrication. The null hypotheses for studies were that all rinse fluids tested (Phosphate Buffered Saline (PBS), BPW, and nBPW) would have equivalent means for presumptive-positive *Salmonella* recovery.

Detection for *S. enterica* was carried out according to biochemical testing methods designated by the U.S. Department of Agriculture for raw chicken carcasses and parts rinses. Recorded antimicrobial concentrations for PAA and CPC solutions on sampling days were 0.05%±0.007% and 0.50%±0.04%, respectively. The average presumptive-positive *Salmonella* recovery rates for PBS (control), BPW, and nBPW for chicken carcasses were 0%, 0%, and 13%, respectively, while rates for PBS, BPW, and nBPW for chicken parts were 4.8%, 12%, and 14%, respectively. Recovery rates for presumptive-positive *Salmonella* on whole carcass rinses differed as a result of rinse fluid for only nBPW ($P<0.001$, $n=20$). Statistical analysis indicated no significant

difference in presumptive-positive *Salmonella* recoveries for chicken parts as a function of rinse fluid (PBS, BPW, nBPW) (P= 0.25, *n*=14).

Given the outcomes of experiments, the null hypothesis was rejected for carcasses, but for parts, the corresponding null hypothesis was not able to be rejected. While these data show there was neutralizing ability for nBPW for carcass rinse collections, it does not provide evidence that nBPW is more effective as an antimicrobial neutralizing rinse fluid versus PBS or BPW for chicken parts. Further research must be conducted to determine if a stronger neutralizing formulation is required for parts rinses. Research should also be expanded to other chicken edible parts that are sampled per USDA-FSIS regulations to determine if results are similar to those obtained in the present study.

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NOMENCLATURE

BGS	Brilliant Green Sulfa Agar
BPW	Buffered Peptone Water
CPC	Cetylpyridinium Chloride
H_2S	Hydrogen sulfide
LIA	Lysine Iron Agar
mRV	Rappaport-Vassiliadis broth, modified
nBPW	neutralizing Buffered Peptone Water
PAA	Peroxyacetic Acid
PBS	Phosphate Buffered Saline
TSI	Triple Sugar Iron Agar
TT-H	Tetrathionate broth (Hajna)
USDA-FSIS	United States Department of Agriculture-Food Safety Inspection Service
XLT4	Xylose Lysine Tergitol-4 Agar

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1. INTRODUCTION

1.1 Foodborne illness and poultry

In the United States, it is estimated that 48 million cases of foodborne illness occur annually *(17)*. Non-typhoidal *Salmonella* is the leading cause of bacterial foodborne illness in the U.S. *(51)*. *Salmonella* spp. can be present in a variety of foods, such as beef, pork, poultry, eggs, and produce commodities *(17, 19, 41, 72)*. *Salmonella* has been a common cause of illness, but the incidence of foodborne-derived human illness from *Salmonella* does not seem to be declining; rather, the incidence rates are remaining steady *(18, 19, 38, 46)*. Salmonellosis is one of the most common foodborne diseases, with over 1.2 million illnesses and 453 deaths caused by non-typhoidal *Salmonella (20, 73)*. Although incidence rates for salmonellosis are remaining steady, these rates may be underestimated since symptoms of salmonellosis are generally perceived as gastrointestinal discomfort unless the acute disease becomes severe enough to seek medical attention *(21, 46, 69)*.

Poultry has been identified as a transmission vehicle for *Salmonella (4, 5, 19, 28, 44, 51)*. A transmission vehicle is any single animal (including humans), plant, soil, substance - or combination of the previously listed – where an infectious agent normally lives *(11)*. The transmission vehicle serves as a vehicle for the infectious substance to be transmitted to a human or other susceptible host *(11)*. The infectious agent must primarily depend on the reservoir for survival and must be able to multiply there *(11)*.

This is not to be confused with a vector which is any living creature, such as insects or animals, that transmits an infectious agent to humans, and does not necessarily have to be able to sustain the infectious agent's survival *(11)*. In 2014, poultry was associated with 14% of foodborne disease outbreaks and outbreak-associated illnesses in the U.S. – the most of any food commodity with the exception of finfish (21%) *(19)*. Also, poultry and *Salmonella* are considered the second and third food commodity/pathogen pairing for outbreak-associated illness and outbreaks, respectively *(19)*. During 2014, there were 227 illnesses in recorded outbreaks and 23 recorded hospitalizations due to *Salmonella* in chicken *(19)*. Chicken consumption per individual in the U.S. continues to increase, with the 2015 U.S. per capita consumption of boneless, trimmed chicken increasing by 8.1 pounds since 2000 *(19, 40, 52)*. For *S. enterica*, poultry, meat, and eggs serve as the main reservoir *(31)*.

A *Salmonella* performance standard was created and introduced in 1996 with the requirement for the United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) to implement a hazard analysis and critical control points (HACCP) program for pathogen reduction *(64)*. As part of this program, a *Salmonella* Verification Program was initiated, where the USDA-FSIS can assess industry performance and controls for reducing *Salmonella* contamination in raw meat and poultry products *(36, 64)*. The primary focus for foodborne pathogen control in the U.S. poultry industry is on post-harvest processing of poultry animals *(5, 55)*. In 2014, the New Poultry Inspection System was initiated by the USDA-FSIS to require all poultry companies to take measures to prevent *Salmonella* contamination, rather than addressing the contamination after the event has occurred *(66)*. Processing plants have incorporated interventions post-harvest in order to reduce microbial contamination of chicken carcasses *(5, 47, 48)*. Poultry processors can include an antimicrobial dip or spray on raw chicken carcasses and parts *(36, 55, 58)*. Antimicrobial solutions, such as peroxyacetic acid (PAA) or cetylpyridinium chloride (CPC, 1-hexadecylpyridinium chloride), are commonly used in processing *(2, 4, 5, 36, 43, 48, 71)*. However, the incidences of human *Salmonella* infection rates have remained unchanged, even though the *Salmonella* performance standards have become more stringent *(5, 9, 10, 36, 65, 66)*.

1.2 *Salmonella enterica*

Salmonella spp. are Gram-negative, facultatively anaerobic, non-sporulating regular rod-shaped bacteria that belong to the family *Enterobacteriaceae (31, 41)*. While *Salmonella* grows optimally at 37°C, it can also grow at temperatures between 5.3°C and 45°C *(41)*. Optimum pH for growth is around neutral pH (pH 6.5–7.5), but growth has been recorded in pH as low as pH 4.05 *(22, 31)*. Water activity (a_w) levels must also be at or above 0.94 for growth, with higher a_w values being required as the pH decreases *(41)*. *Salmonella* spp. can also catabolize glucose, which results in acid and gas production, but the bacterium cannot utilize lactose as a carbohydrate source *(31)*.

In the genus *Salmonella*, there exists two species: *S. bongori* and *S. enterica*. *S. enterica* can be further classified into six subspecies, including *enterica*, *arizonae*, *diarizonae*, *salamae*, *houtenae*, and *indica (41)*. While most *Salmonella* spp. are motile with peritrichous flagella, *S. enterica* subsp. *enterica* serovar Pullorum and *S. enterica* subsp. *enterica* serovar Gallinarum are non-motile strains due to the lack of functional flagella *(31, 50)*. Table 1 summarizes the biochemical characteristics of most *S. enterica* strains.

Table 1. Biochemical characteristics for most *S. enterica* strains

Biochemical Test	Reaction[a]
Glucose (TSI)	+
Lysine decarboxylases (LIA)	+
Hydrogen sulfide (TSI and LIA)	+
Urease	-
Lysine decarboxylase broth	+
Phenol red dulcitol broth	+(b)
Potassium cyanide broth	-
Malonate broth	-(c)
Indole test	-
Polvalent flagellar test	+
Polyvalent somatic test	+
Lactose fermentation	-(c)
Sucrose fermentation	-
Voges-Proskauer test	-
Methyl red test	+
Simmons citrate	v

[a] + 90% or more positive in 1 or 2 days; -: 90% or more negative in 1 or 2 days; v: variable
[b] Majority of *S. enterica* subsp. *arizonae* strains are negative
[c] Majority of *S. enterica* subsp. *arizonae* strains are positive
Adapted from *(33)*

The primary environment for *Salmonella* spp. is in the intestinal tract of animals, such as birds, reptiles, humans, and farm animals *(41)*. However, humans and animals are the primary reservoirs *(41)*. *Salmonella* can be excreted in feces, and this can result in transmission to humans by insects and other living creatures, which serve as vectors. For insects that serve as vectors, *Salmonella* can be transmitted whenever the insect bites and injects the infectious agent or when contaminated appendages touch a person *(11)*. Animals can serve as a vector when *Salmonella* is present in the gastrointestinal tract and are excreted in the animal's feces, resulting in an unsanitary environment. Animals can also have *Salmonella* present on the exterior surface of the animal, and *Salmonella* contamination can occur when contact is made with the fecal matter. As humans and other animals consume contaminated foods and water, and the organisms are shed through fecal matter, the cycle will continue. Through these dissemination vehicles, *Salmonella* spp. can eventually be found in water, soils, and farms, resulting in their presence on meat commodities through cross-contamination and natural occurrence *(41, 72)*.

1.3 Salmonellosis

Salmonellosis is a gastrointestinal disease which typically lasts 4–7 days, although chronic salmonellosis can occur *(32, 35)*. *Salmonella* infections can occur with as few as 10 cells per gram *(41)*. Rates of salmonellosis usually peak during the summer months, with rates being the highest from May through October *(13)*. This could be due to the increased occurrence of temperature abuse of foods and/or cross-contamination of foods at cookouts during the summer months *(72)*. However, the more likely cause is the correlation between higher ambient temperatures providing the ability for more rapid replication *(1)*. Children under the age of 5, older adults, and immunocompromised individuals are at the highest risk for infection, requiring the consumption of fewer cells in order to develop symptoms *(20, 41, 72)*.

Symptoms for salmonellosis can appear in as few as 4 hours, but a 12-14 hour incubation period until onset of clinical illness is average *(32, 41)*. Salmonellosis can cause lower gastrointestinal tract symptoms within an infected person *(32, 41)*. These symptoms can include abdominal cramps, diarrhea, vomiting, fever, chills, nausea, and possible headache. Most individuals recover without needing antibiotic treatment, but in severe cases, *Salmonella* can infect the bloodstream or other parts of the body *(35)*. Severe cases result in an individual needing medical attention, and death can occur if the disease is not treated *(20)*.

Although salmonellosis has been causing illness for over 125 years, it has only been a notifiable disease in the U.S. since 1942 *(20, 72)*. While it is mandatory that reportable disease cases are reported to the state and territorial jurisdictions, it is voluntary that notifiable diseases are reported to the CDC by state and territorial jurisdictions *(15)*. Since 1942, the rate of reported cases for salmonellosis has increased over time, but this could be attributed to more awareness, surveillance, and sampling *(20, 72)*. Recently, rates of *Salmonella* cases have remained relatively constant, with 1 million confirmed *Salmonella*-derived foodborne illnesses occurring per year in the U.S *(17)*. With non-typhoidal *Salmonella* resulting in an annual estimate of 378 deaths a year in the U.S., decreasing the rates of *Salmonella* spp. in the food supply is a priority

(51). This is a priority not only due to the hospitalizations and/or fatalities that may occur, but also due to the economic impact that salmonellosis can have annually *(73)*. In the U.S., the economic impact of salmonellosis can be as high as $2.3 billion per year, and the cost of illness is estimated at over $3.3 million per year *(3, 35)*.

1.4 Poultry as a reservoir for *Salmonella*

Post-rigor meats have pH values suitable for growth for a variety of organisms. Bacteria prefer to grow at a pH 6.5 to 7.5, but they can also tolerate growth with pH ranges from pH 4 to 9 *(29)*. With an approximate pH between 6.2 and 6.4, chicken meat has a pH environment suitable for harboring bacteria *(46)*. Meat also provides a sufficient nutrient source and has enough moisture content to sustain microbial growth *(46)*. Table 2, which has been reprinted with permission from Springer, displays a list of bacteria most frequently found on fresh meats and poultry *(41)*. The primary source of salmonellae accumulation in poultry is within the gastrointestinal tract *(8, 41, 72)*. Contamination of the exterior and cavity of the bird carcass can occur during the slaughter operation *(41)*. Cross-contamination can also occur after evisceration if contaminated knives, blades, and other processing equipment come into contact with edible carcasses or parts.

Table 2. Genera of bacteria most frequently found on meats and poultry. Reprinted with permission of Springer, Modern Food Microbiology, Fresh Meats and Poultry, 7th ed., 2005, pg. 65, James M. Jay, Martin J. Loessner, David A. Golden, 29 November 2016.

Genus	Gram Reaction	Fresh Meats	Poultry
Acinetobacter	-	✓✓[a]	✓✓
Aeromonas	-	✓✓	✓[b]
Alcaligenes	-	✓	✓
Arcobacter	-	✓	
Bacillus	+	✓	✓
Brochothrix	+	✓	✓
Campylobacter	-		✓✓
Carnobacterium	+	✓	
Caseobacter	+	✓	
Citrobacter	-	✓	✓
Clostridium	+	✓	✓
Corynebacterium	+	✓	✓✓
Enterobacter	-	✓	✓
Enterococcus	+	✓✓	✓
Erysipelothrix	+	✓	✓
Escherichia	-	✓	
Flavobacterium	-	✓	✓
Hafnia	-	✓	
Kocuria	+	✓	✓
Kurthia	+	✓	
Lactobacillus	+	✓	
Lactococcus	+	✓	
Leuconostoc	+	✓	
Listeria	+	✓	✓✓
Microbacterium	+	✓	✓
Micrococcus	+	✓	✓✓
Moraxella	-	✓✓	✓
Paenibacillus	+	✓	✓
Pantoea	-	✓	✓
Pediococcus	+	✓	
Proteus	-	✓	✓
Pseudomonas	-	✓✓	✓✓
Psychrobacter	-	✓✓	✓
Salmonella	-	✓	✓
Serratia	-	✓	✓
Shewanella	-	✓	
Staphylococcus	+	✓	✓
Vagococcus	+		✓✓
Weissella	+	✓	✓
Yersinia	-	✓	✓

[a] ✓✓ represents the genera being most frequently reported

[b] ✓ represents the genera being known to occur

Adapted from *(41)*

Poultry has been repeatedly implicated or identified in the occurrence of foodborne disease outbreaks in the United States, especially with salmonellosis *(17, 19, 31)*. Salmonellosis cases vary with poultry animals serving as the vector or poultry meat serving as the transmission vehicle. Most recently, several *S. enterica* subsp. *enterica* serovars were associated with causing human infections originating from the handling of domestic poultry chicks *(16)*. This is unique considering the outbreak occurred due to *Salmonella* being transmitted with poultry chicks servings as a vector. Since July of 2016, *Salmonella* serovars *S.* Enteritidis, *S.* Muenster, *S.* Hadar, *S.* Indiana, *S.* Mbandaka, *S.* Infantis, *S.* Braenderup, and *S.* Infantis have caused 895 cases of infection, 1 death, and 209 hospitalizations in 48 states due to the handling of poultry chicks *(16)*. In 2013 and 2014, *S.* Heidelberg was identified as the culprit of a multistate outbreak *(12, 14)*. This outbreak of *S.* Heidelberg persisted from March 2013 until July 2014, and was traced back to Foster Farms branded chicken, chicken parts, and marinated products. It resulted in 634 cases of salmonellosis occurring in 29 states and Puerto Rico *(14)*. Both of these outbreaks demonstrate that poultry can serve as both a vector and transmission vehicle for *Salmonella.*

1.5 Antimicrobial use as a food safety intervention in the poultry industry

In the poultry industry, organic and inorganic acids are incorporated as an antimicrobial intervention in some instances *(41, 46, 49)*. The antimicrobial effect that organic acids have comes from their ability to lower pH and the toxicity to microorganisms from the undissociated form of the acid *(29, 46)*. All microorganisms have a maximum, minimum, and optimum pH level for growth, and if hydrogen ion concentrations are changed, it can influence the inhibition or growth of the organism *(29)*. Undissociated acid molecules can easily cross cell membranes of microbial cells and enter the cytoplasm, where the molecules will dissociate due to the cytoplasm pH being more than 6.0 *(46)*. As a result, the cytoplasm pH will be lowered, causing the cell to use energy to force excess hydrogen ions out of the cytoplasm to regain metabolic pH *(46)*. Eventually, the cytoplasm pH falls below the level of homeostasis, and the cell dies *(46)*.

Addition of using organic and inorganic acids for use in the production of meat, poultry, and egg products must be approved by the USDA-FSIS *(63)*. The ingredients that are safe and suitable are updated quarterly and are provided in the FSIS Directive 7120.1 *(60, 63)*. Currently, both CPC and PAA are approved as antimicrobial interventions to treat the surfaces of raw poultry carcasses or parts (skin-on or skinless) *(60)*. When applied as a dip or spray, PAA and CPC should not exceed 2,000 parts per million (ppm) and 8,000 ppm, respectively *(60)*.

CPC is a quaternary ammonium compound that is used as a cationic surfactant *(39)*. When alkaline CPC ions interact with acid groups in bacterial cells, the ions form weakly ionized compounds that subsequently inhibit bacterial metabolism *(39, 45)*. CPC can be applied as either a fine mist spray or as a liquid solution directly to raw poultry carcasses prior to immersion in a chiller *(23)*. Chronic exposure to CPC can cause microorganisms to become less sensitive to the compounds *(68)*.

PAA is a quaternary equilibrium mix of acetic acid and hydrogen peroxide, ultimately breaking down into acetic acid, water ,and oxygen *(37)*. PAA's antimicrobial mechanism is due to the release of reactive oxygenated species. The active oxygen can oxidize sulfhydryl and sulfur bonds in proteins. Oxidizing these bonds can result in the disruption of proton transfer at the cell membrane, which ruptures cell walls *(6, 37)*. PAA can be applied as an antimicrobial for poultry carcasses, parts, and organs during processing as a dip or a spray and can function under low temperature conditions, such as at 4°C *(6, 24)*.

1.6 *Salmonella* testing for poultry products

With the exception of very low volume establishments (ones that annually slaughter no more than 440,000 chickens, 60,000 turkeys, ducks, geese, guineas, or squabs), chicken processing establishments must take a minimum of one carcass and part sample weekly for *Salmonella* testing *(25, 62)*. While establishments must take a minimum one carcass and part sample weekly, they must also sample at a frequency that is adequate to monitor their ability to maintain process control for enteric pathogens *(25)*. Samples are collected on a moving window basis, meaning that when a new sample is taken, the oldest sample is removed from the window *(62)*. Samples must be taken based according to USDA-FSIS Microbiology Laboratory Guidebook, chapter 4.08, which took effect June 29, 2014 *(57)*.

According to current USDA-FSIS recommendations, samples collected for *Salmonella* prevalence testing should be allowed to drip for 1 min prior to rinse fluid addition to reduce the possibility of antimicrobial carryover from the antimicrobial dip application *(57, 59)*. However, it is not possible to completely remove all antimicrobial solution from the sample unless the sample undergoes an extensive wash. It is possible that antimicrobial carryover can occur in sampled carcass or parts rinse fluids that are submitted for *Salmonella* testing. This was supported by Gamble et al. (2016) when looking at the efficacy of BPW being able to neutralize a variety of antimicrobial solutions that were applied to *Salmonella*-inoculated male broilers *(36)*. Results indicated that BPW as a pre-enrichment solution was not able to fully overcome antimicrobial carryover that collected in the rinse fluid *(36)*. In response to the issue of antimicrobial carryover and its possible impacts on pathogen recovery for performance standard adherence determination, USDA-FSIS issued a notice for poultry rinsate collections to occur with neutralizing Buffered Peptone Water (nBPW) instead of Buffered Peptone Water (BPW), which took effect July 1, 2016 *(61)*.

In order to determine the rate of *Salmonella* recovery of BPW rinse fluid compared to nBPW, *Salmonella* testing was performed using the biochemical testing methods described in the USDA-FSIS guideline testing method for both chicken carcass and chicken parts rinses *(57)*. A preliminary study was conducted with *Salmonella* spp.-inoculated young chicken carcasses undergoing an antimicrobial application of either CPC or PAA prior to rinse fluids being collected. Once it was apparent that nBPW did possess the ability to recover *Salmonella* spp. from the preliminary study, chicken carcasses and parts were sampled at a USDA-FSIS inspected poultry slaughter and fabrication facility, and *Salmonella* testing was performed outside of the facility. The null hypothesis was that all rinse fluids would have equivalent means for presumptive-positive *Salmonella* recovery, and the alternative hypothesis was that at least one rinse fluid would have presumptive-positive *Salmonella* recovery means be different.

2. MATERIALS AND METHODS

2.1 *Salmonella* inoculated preliminary neutralizer efficacy trial

As indicated previously, there are several antimicrobial processes that a broiler slaughter and fabrication facility can use in order to reduce levels of enteric bacteria. In order to determine if the antimicrobial-neutralizing ability of nBPW would be effective in a commercial poultry abattoir setting that could have very low cell prevalence rates of *Salmonella* spp., the rinse fluid needed to be able to demonstrate that it had the ability to overcome any antimicrobial carryover on a chicken carcass that was a carrier for *Salmonella* spp. In order to accomplish this, a preliminary experiment was conducted to determine the neutralizing abilities of BPW and nBPW on young chicken carcasses that were inoculated with a *Salmonella* spp. cocktail.

2.1.1 Inocula preparation

Isolates (one strain each) of *Salmonella* Typhimurium, *S.* Heidelberg, and *S.* Enteritidis were obtained from the culture collection of Dr. Christine Alvarado (Department of Poultry Science, Texas A&M University, College Station, TX) and revived in 10 ml of Tryptic Soy Broth (TSB; Becton, Dickinson and Co., Franklin Lakes, NJ) by incubating aerobically, without shaking at 35±2°C for 21±3 h. One loopful of each culture then was transferred into a sterile tube containing 10 ml TSB and incubated aerobically without shaking at 35±2°C for 21±3 h. Cultures were transferred to labeled, pre-sterilized, conical tubes, which then were wrapped in Parafilm M® (Bemis, Oshkosh, WI) to prevent contamination and/or leakage during transport to the Poultry Science Center at Texas A&M University (College Station, TX). Immediately prior to use, incubated cultures were serially diluted in Phosphate-Buffered Saline solution (PBS; EMD Millipore, Temecula, CA) to a final target concentration of approximately 6.0 $\log_{10}$ colony forming units per milliliter (CFU/ml). Tubes containing 6.0 $\log_{10}$ CFU/ml cells were centrifuged (2191 x g in a Jouan B4i centrifuge, 25±2°C, 15 min), supernatants aseptically removed, and pellets suspended in 10 ml sterile PBS. Suspended pellets then underwent a second centrifugation in identical fashion to the conditions described for the initial centrifugation, and the process was repeated a third time in identical fashion to the first two centrifugations and washings of culture pellets. After the third resuspension in PBS, *Salmonella* serovars in PBS were combined to create a mixture of strains for subsequent inoculation ("cocktail"). To verify the concentration of the inoculum cocktail, a 5 ml aliquot was serially diluted in 0.1% peptone water (PW, Becton, Dickinson and Co.) and dilutions were aseptically spread on surfaces of Xylose Lysine Tergitol-4 (XLT4; Becton, Dickinson and Co.) agar supplemented with Niaproof-4 (Sigma-Aldrich Co., St. Louis, MO)-containing Petri plates. XLT4 Petri plates were inverted and incubated aerobically at 35±2°C for 21±3 h. After incubation, only black or red colonies with or without black centers were selected for colony counting. Colony counts then were transformed and expressed as $\log_{10}$ CFU/ml. The remaining cocktail solution went into a sterile spray bottle.

2.1.2 Young chicken carcass collection

Five boxes, each containing 20 young chicken carcasses (*Gallus domesticus*), were purchased from a local purveyor one day prior to the date that carcasses were to be inoculated (i.e. the project initiation date). Packages were transported to the Texas A&M Poultry Science Center and placed in a walk-in cooler (4±2°C).

2.1.3 Poultry carcass inoculation of *Salmonella* cocktail

In order to have the maximum amount of external carcass surface exposed to air for drying after inoculation, metal racks were used for carcass placement after inoculation. Racks were placed in plastic pans, sprayed with 70% ethanol, and allowed to air dry. Each carcass was aseptically transferred into a polyethylene bag (15” by 20” polyethylene, poultry rinse bag, 12 liter capacity, VWR Int., Radnor, PA) and placed on a sterilized rack.

For the *Salmonella* cocktail-containing bottle, the volume of a full spray was measured as being 1±0.1 ml. To prime the pump, an individual wearing a shoulder-length glove placed the inoculum-containing spray bottle into a separate, empty, polyethylene bag, and 3 pumps were sprayed into the bag to prime the spray pump to ensure a full spray onto carcass surfaces. After priming, the individual used a gloved hand to lift the plastic bag away from the anterior surface of the carcass. The bag containing the carcass was opened, and one spray (1±0.1 ml) of inoculum was applied at approximately 8–10 in. away from the carcass. Once sprayed, the bottle was removed, and the bag ends were closed to prevent aerosols from being released. This process was repeated for all bird carcasses being inoculated in the experiment.

After approximately 2 min post-inoculation, carcasses were removed from bags, and each carcass was placed on an individual metal rack. The posterior surface of the carcass was in contact with the metal rack, with anterior sides facing upwards. After placement, the carcasses were dried for 30 min to allow for bacterial attachment.

2.1.4 CPC antimicrobial solution preparation and application methods

CPC was one of two antimicrobial solutions used after inoculation and the 30 min bacterial attachment occurred. There were two CPC application methods; a drench (CPC_a) and a drench followed immediately by an 80 min chill in ice-water (CPC_b). CPC_a method was conducted in order to determine *Salmonella* spp. recovery in carcasses that were immediately sampled after antimicrobial treatment and water rinse. CPC_b method was conducted in an attempt to imitate an ice-chill that would take place in a slaughter and fabrication facility. A CPC (Cecure™ , SafeFoods Corp., Rogers, AR) and water (H_2O) solution was created immediately prior to application at an ingoing concentration of 8,000 ppm CPC (0.8%) by combining 14.7 liters H_2O and 300 ml commercial CPC-containing solution (40± 2% concentration according to manufacturer guidance) and stirring. Ingoing CPC concentration was verified using a cetylpyridinium chlorine titration kit (SafeFoods Cecure Titration Kit, SafeFood Corp., Rogers, AK) prior to CPC application.

Approximately 2 liters of CPC solution then was drenched onto a carcass for each treatment. This drench was accomplished by an individual, wearing shoulder-length gloves, holding the carcass by the drumsticks while another individual, with shoulder-length gloves, poured approximately 1 liter of the CPC solution over the carcass and into the interior cavity. After approximately 1 liter was poured, the carcass-holding individual would remove one hand, and the solution-holding individual would pour approximately 500 ml over the drumstick area that was previously covered by the glove, with a small amount of solution being poured over the carcass-holding individual's gloved hand. The carcass-holding individual would replace the gloved hand back onto the drumstick, remove the other hand, and the process was repeated for the opposite side. After the drench was applied, CPC_a carcasses were allowed to drip for 1 min in order for excess CPC solution to be removed. The CPC_a carcasses then were sprayed with 50±5 ml of water in order to simulate process methods in the collaborating poultry abattoir facility. After the water spray, carcasses were allowed to drip for an additional 1 min before being placed into a polyethylene bag. Conversely, CPC_b birds were allowed to drip for 1 min after the CPC drench occurred in order for excess CPC solution to be removed, and then, CPC_b carcasses were placed into an ice water bath for 80 min. The CPC_b birds were removed from the bath and allowed to drip for 1 min before being placed into a polyethylene bag for carcass rinsing.

2.1.5 PAA antimicrobial solution and application method

PAA was the second of two antimicrobial solutions used after inoculation and 30 min bacterial attachment occurred. An aqueous PAA (Paragonn XP, SafeFoods Corp.) solution was prepared immediately prior to application at an ingoing concentration of 2,000 ppm PAA (0.2%) by combining 14.8 liters H_2O and 200 ml of PAA (14–17% concentration per manufacturer-provided product description) and stirring. Ingoing PAA concentration was tested using a hydrogen peroxide and peracetic acid test kit (LaMotte, Chestertown, MD) prior to PAA application. Approximately 2 liters of PAA antimicrobial solution was drenched onto a carcass for each treatment conducted using the same method previously mentioned for CPC_a carcasses. After the drench was applied, PAA carcasses were allowed to drip for 1 min in order for excess PAA solution to be removed. Post 1-min PAA drip, the PAA-treated birds were immediately placed into polyethylene bags for carcass rinsing.

2.1.6 Preparation of rinse fluid

Rinse fluid solutions of PBS (control), BPW, and nBPW were prepared in 100±10 ml and 30±2 ml volumes, with 100±10 ml volumes serving as rinse fluids for carcass rinses, and 30±2 ml volumes serving as sterile enrichment fluids. It has been shown that a reduced, 100 ml rinse fluid volume has no affect on the recovery of *Salmonella,* so these volumes were used in an effort to reduce media preparation and waste *(26)*. Rinse fluids included 100±10 ml and 30±2 ml volumes each of PBS, BPW, and nBPW. PBS was chosen as a control fluid based on its buffering abilities.

PBS and BPW (Hardy Diagnostics, Santa Monica, CA) were prepared according to manufacturer instructions. One PBS tablet was added per 1 liter distilled H_2O and dissolved via agitation. After tablets were completely dissolved, PBS solution was aliquoted into autoclavable, screw-cap polypropylene bottles (Nalge Nunc International, Rochester, NY) and autoclaved-sterilized for 15 min at 121ºC. Once cooled, bottles were stored at 4±2°C until sample rinse fluid collection was to occur. For BPW preparation, 20 g of Buffered Peptone (Hardy Diagnostics) free-flowing medium powder was added per 1 liter of distilled H_2O and dissolved via agitation with a stir rod. After the powdered media was completely dissolved, the BPW solution was aliquoted into autoclavable, screw-cap polypropylene bottles, and autoclaved-sterilized for 15 min at

121ºC. Once tempered, bottles were stored at 4±2°C until sample rinse fluid collection was to occur.

For nBPW, preparation was prepared based on the USDA-FSIS preparation method *(67)*. Twenty (20) g Buffered Peptone, 7 g Refined Soy Lecithin powder (Alfa Aesar, Haverhill, MA) and 1 g sodium thiosulfate (EMD Millipore) was added per 833 ml distilled H_2O and stirred via agitation for 5 min. After stirring, the solution was autoclave-sterilized for 15 min at 121ºC. In 167 ml sterile distilled H_2O, 12.5 g sodium bicarbonate (EMD Millipore) was dissolved via agitation and heating. After the sodium bicarbonate solution was dissolved, the solution was vacuum-sterilized via a filtration system (0.45 µm pore size, VWR Int.) and added to the autoclaved broth after it had tempered to at least 55ºC. The nBPW was stirred for 1 min to homogenize after the addition of the sodium bicarbonate solution, and it remained stirring while being aliquoted due to precipitation occurring in the broth. After the nBPW solution was aliquoted into sterilized, screw-cap polypropylene bottles, it was stored at 4±2°C until sample rinse fluid collection was to occur.

2.1.7 Carcass rinse and sample rinse fluid collection

For laboratory experiments, carcasses that did not undergo inoculation were used as negative control carcasses. Negative control carcasses were immediately placed into polyethylene bags for carcass rinsing with PBS. Positive control carcasses underwent spray inoculation in identical fashion to the conditions described for inoculation in Section 2.1.3. After the bacterial attachment period, carcasses were immediately placed into polyethylene bags for rinsing with PBS. The negative control was used for determining the presumptive-positive *Salmonella* prevalence on carcasses prior to inoculation. The positive control was used for determining the amount of presumptive-positive *Salmonella* bacterial attachment after the 30 min inoculation period. After the antimicrobial-treated carcasses were placed in sterile, polypropylene bags, either BPW or nBPW (100±10 ml) was poured into the bag. The top of the bag was twisted several times, and the bird was rinsed by moving the bag back and forth in an arc motion (21±3 in.) for 1 min. This was accomplished by holding the twisted top of the bag with one hand, placing the other hand on the bottom of the bag to stabilize the carcass, and then moving the bag back and forth in an arc motion. This ensured that both the interior and exterior surface of the carcass was rinsed. After 1 min was completed, the corner of the bag was cut with flame-sterilized scissors and sample rinse fluid was collected back in the respective container it was poured from.

2.1.8 *Salmonella* microbiological testing method

In order to simulate USDA-FSIS *Salmonella* testing procedures, the biochemical testing methods described in the USDA-FSIS Microbiological Laboratory Guidebook (MLG) 4.08 were used to test for recovered inoculated *Salmonella* spp. *(57)*. While samples collected in plants are sealed, refrigerated, and shipped to an FSIS laboratory for *Salmonella* detection, all samples taken during this study were transferred on day 0 of the experiment in order to allow for *Salmonella* spp. to have the greatest opportunity for recovery.

After sample rinse fluid was collected, 30 ml of sample rinse fluid was added to a bottle containing 30 ml of sterile, matching rinse fluid (BPW, nBPW) for pre-enrichment purposes to allow injured organisms the opportunity for recovery *(30)*. This resulted in a pre-enrichment container volume of 60 ml. The 60 ml pre-enrichment container was incubated at 35±2°C for 22±2 h. Following incubation, 0.5±0.05 ml and 0.1±0.05 ml pre-enrichment solution was added to 10 ml Tetrathionate Hajna (TT-H; Becton, Dickinson and Co.) broth and 10 ml modified Rappaport-Vassiliadis broth (mRV; Sigma-Aldrich Co.), respectively, for selective enrichment. Inoculated selective enrichment broths were incubated at 42±2°C for 22±2 h. One loopful each of post-incubated TT-H and mRV was streaked onto Brilliant Green Sulfa (BGS; Becton, Dickinson and Co.) agar-containing Petri plates and XLT4 agar supplemented with Niaproof-4-containing Petri plates. BGS and XLT4 Petri plates were inverted and aerobically incubated at 35±2°C for 22±2 h. Three *Salmonella*-typical colonies from each sample set were selected with a pre-sterilized plastic needle. Triple Sugar Iron agar (TSI; Hardy Diagnostics) and Lysine Iron Agar (LIA; Becton, Dickinson and Co.) slants were inoculated with the picked colony by stabbing the inoculated needle into the butt of the slant, removing the needle, and then streaking the needle across the lawn of the slant. Slants were aerobically incubated at 35±2°C for 22±2 h before being assessed for typical *Salmonella* biochemical characteristics.

TSI slants were recorded as presumptive-positive *Salmonella* if a yellow butt and red slant was produced after incubation, with or without gas production from glucose fermentation or blackening of medium from hydrogen sulfide (H_2S) production, which occurs due to the interaction between sodium thiosulfate and ferric ammonium citrate in the media *(34, 57)*. LIA slants were recorded as positive if there was a purple butt, indicating the decarboxylation of lysine, with or without blackening of medium, a result of H_2S production between sodium thiosulfate and ferric ammonium citrate *(30, 34, 57)*. In the event that an atypical *Salmonella* result occurred during TSI and LIA result recording, the Rule-Out Reactions guidelines were followed to classify questionable colony slants being classified as positive or negative *(34)*. The Rule-Out Reactions guidelines states that if any of the following occur, the isolate tested is considered negative: (a) isolates producing "no change" or an alkaline slant and butt in both TSI (red) and LIA (purple), (b) isolates having three atypical reaction results (TSI having an acidic or yellow butt and slant , LIA having an acidic or yellow butt), or (c) isolates producing a burgundy or brick red slant in LIA *(34)*. A listing of typical and atypical biochemical test results are provided in Table 3. If both the TSI and LIA sample from the colony set were considered presumptive-positive *Salmonella*, then the colony sample was considered "positive" for *Salmonella* spp. through biochemical media testing *(34)*. Characteristics were recorded and presumptive-positive *Salmonella* colonies were determined as "present" if *Salmonella* positive morphologies were present in either XLT4 or BGS Petri plates and on matching TSI and LIA slants and met Rule-Out Reactions guidelines.

Table 3. *Salmonella enterica* subspecies *enterica* biochemical test result possibilities

Media	**Typical result[a] (T)**	**Atypical result[a] (A)**
XLT4	Colonies: Black (H_2S +[b]) or red colonies with (H_2S +) or without (H_2S -[c]) black centers Colony Rim: May be yellow in 24 hr but should later turn red	Colonies: Pink (Xylose -[d]); pink-yellow color (lactose + e and/or sucrose +[f]) Colony rim: May be yellow in 24 hr but should later turn red
BGS	Colonies: Pink and opaque with a smooth appearance Colony rim: entire colony edge should have red color in medium	Colonies: Green Colony rim: no reddening of agar surrounding colony
TSI	Butt: Yellow with (H_2S +) or without (H_2S -) blackening of the media Slant: Red to pink	Butt: Yellow without (H_2S -) blackening of the media; Yellow with (H_2S +) or without (H_2S -) blackening of the media Slant: Red; Yellow
LIA	Butt: Purple with (H_2S +) or without (H_2S -) blackening of the media Slant: Purple, unless blackening has extended onto the slant	Butt: Yellow with (H_2S +) without (H_2S -) blackening of the media Slant: Purple with (H_2S +) or without (H_2S -) blackening of the media

[a] Results are characteristic for *Salmonella enterica* subsp. *enterica*
[b] hydrogen sulfide (H_2S) producing
[c] non-hydrogen sulfide producing
[d] xylose fermentation negative
[e] lactose fermentation positive
[f] sucrose fermentation positive
Adapted from *(34, 57)*

2.2 Chicken carcass or parts *Salmonella* detection in a commercial abattoir

As indicated previously in Section 1.5, there are several antimicrobial processes that poultry slaughter and fabrication facilities can use in order to reduce levels of enteric bacteria. After the preliminary experiment, PBS, BPW, and nBPW rinse fluids were utilized in chicken carcass and chicken parts rinses in a commercial abattoir to determine recovery rates for presumptive-positive *Salmonella* spp.

2.2.1 Sample size determination for Salmonella detection in a commercial abattoir

Monthly *Salmonella* testing data from a USDA-FSIS inspected poultry slaughter and fabrication facility was reviewed in order to determine sample sizes that would be able to capture the normal rates of occurrence in the facility. To determine the sample size necessary to achieve a minimum power of 80%, the following equation was utilized, where P is the chosen power (1-β) and *p'* is the proportion of carcasses or parts that are not contaminated *(27)*.

$$n = \log(P) / \log(p')$$

Based on the above equation and nationwide USDA-FSIS-collected microbiological baseline data for *Salmonella* prevalence in raw young chicken carcasses and raw chicken parts, sample sizes (*n*) for chicken carcasses and chicken edible parts were determined *(54, 56)*. If the equation resulted in an odd number sample size, the sample size was increased to an even number for matching purposes. Per rinse fluid treatment for each replication of chicken parts, 3 samples of 16 wings, 3 samples of 9 thighs, 4 samples of 4 split breasts and 4 samples of 11 drumsticks were sampled to ensure a variety between light and dark meat pieces (*n*=14). The numbers of pieces per sample correlates to the poultry facility's collection standard for how many pieces yield an average of 4 pounds, equivalent to USDA-FSIS weight requirement for all chicken part samples for *Salmonella* detection sampling *(57)*. Per rinse fluid treatment for each replication for chicken carcasses, 20 chicken carcasses were selected to be sampled (*n*=20). After three replications, this resulted in parts having an N=42 and carcasses having an N=60.

2.2.2 Comparison of chicken carcass or parts sample rinse fluids for *Salmonella* detection in a commercial abattoir

Chicken carcasses and parts (*Gallus domesticus*) were collected from a USDA-FSIS inspected poultry slaughter and fabrication facility located in Texas with establishment permission. All sample collections occurred during the first shift, and all replications occurred on separate days. After evisceration, carcasses underwent a CPC spray (0.525±0.025% CPC) application in a spray cabinet that applied CPC solution to the exterior surface of the carcass and the interior cavity. Ingoing CPC concentration was verified using a cetylpyridinium chlorine titration kit (SafeFoods Cecure Titration Kit, SafeFood Corp.), and antimicrobial solution was collected from the CPC antimicrobial solution container in the commercial abattoir. Carcasses then were chilled in an ice-water chiller for approximately 2 h. All carcasses were selected at random, collected post-chill, and were allowed to drip for 1 min before being placed into a polyethylene bag for rinsing.

All parts samples collected from the facility were taken from carcasses that underwent CPC-treatment prior to fabrication. For cut chicken edible parts, chicken carcasses were placed onto fabrication lines that continued onto cut chicken edible parts processing. Parts were cut in the sequence of wings, split breasts, thighs, and drumsticks. After parts were cut, they were dropped into a chute and sent to the respective parts conveyer lines. The parts were subjected to a PAA dip tank (0.05%±0.007%) before being sprayed with water as they left the dip tank. Ingoing PAA concentration was tested using a hydrogen peroxide and peracetic acid test kit (LaMotte), and antimicrobial solution was collected from the PAA dip tanks that parts were to be collected from in the commercial abattoir. After the water spray, edible parts were collected from the conveyer line, allowed to drip for 1 min, and then placed into a polyethylene bag for rinsing.

2.2.3 Sample rinse fluid collection of antimicrobial-treated chicken carcasses or parts

Rinse fluid solutions were prepared in 400±10 ml and 30±2 ml volumes to simulate USDA-FSIS *Salmonella* testing procedures. Rinse fluid volumes of 400 ml of PBS, BPW, or nBPW were poured into a bag containing either a whole chicken carcass or parts that underwent the respective antimicrobial treatment (CPC application to the carcass and PAA dip tank application post-cut). The top of the bag was twisted several times, and the bird was rinsed by moving the bag back and forth in an arc motion (21±3 in. range) for 1 min *(57)*. After 1 min, the corner of the bag was cut with flame-sterilized scissors and sample rinse fluid was collected back in the respective container it was poured from *(57)*.

2.2.4 *Salmonella* detection on antimicrobial-treated chicken carcasses or parts following rinsing

Salmonella survival on commercially harvested chicken carcasses and cut parts were completed in identical fashion to methods described above for the detection and identification of *Salmonella* from the first study using *Salmonella*-inoculated chicken carcasses in Section 2.1.8. Data were coded in like fashion as to that described for presumptive-positive *Salmonella*, -negative status on tested carcasses or cut parts for subsequent statistical analysis in Section 2.1.8.

2.3 Statistical analysis

From the *Salmonella*-inoculated carcass study, carcasses bearing detectable, presumptive-positive *Salmonella* spp. were converted into binomial data, with presumptive-positive *Salmonella* samples coded as 1 and *Salmonella*-negative samples coded as 0. All analyses were performed using JMP Pro v12 (SAS Inc., Cary, NC). Analysis of variance (ANOVA) was used to determine differences among rinse fluids' main effects and/or interactions of these main effects. Main effects for the preliminary experiment were identified as antimicrobial intervention and rinse fluid whereas main effects for commercial chicken carcasses and parts were rinse fluids. Statistically significant differences among main effects ($p<0.05$) were analyzed further and compared using either Student's t test or Tukey's Honest Significant Differences (HSD) test. The determination of differences in frequencies of presumptive-positive *Salmonella* survival was analyzed as a function of sample rinse fluid (PBS, BPW, or nBPW) and antimicrobial application (CPC, PAA). At the conclusion of the second study testing

Salmonella survival on commercially processed/harvested chicken carcasses and parts, resulting data was analyzed in identical fashion as that described for determination of differences in *Salmonella* survival as a function of sample rinse fluid (PBS, BPW, or nBPW).

3. RESULTS

3.1 *Salmonella* inoculated chicken carcasses

Following the completion of XLT4 and BGS recording, it was observed that only the PAA-treated, BPW-rinsed samples (PAA-BPW) yielded negative *Salmonella*-typical results (Table 4). Overall, 53.3% of the PAA-BPW-treated samples were presumptive-positive for the presence of *Salmonella*. All other treatments had presumptive-positive *Salmonella*-typical results recovered at a rate of 100% across collected samples. For all colonies that were stab/streaked onto TSI and LIA slants, 100% of samples were recorded as presumptive-positive *Salmonella* after the designated incubation period mentioned previously in Section 2.1.8

When comparing the interaction between rinse fluid formula and antimicrobial intervention, both fixed main effects (rinse fluid formulations, antimicrobial intervention) and the rinse fluid formulation x antimicrobial intervention were statistically significant at an $\alpha = 0.05$ (Table 5). A student's t test was done in order to explain pair-wise comparisons among rinse fluid and the antimicrobial intervention (Table 6, Table 7, respectively). When comparing individual antimicrobial intervention/rinse fluid combinations, it was found that only the BPW-PAA combination was statistically different from over all rinse fluid formulation x antimicrobial interaction combinations (Table 8).

Table 4. Presumptive-positive *Salmonella* recovery from inoculated young chicken carcasses[a]

Antimicrobial Intervention[b,c]	Rinse Fluid[d]	% Presumptive-Positive *Salmonella*[e, f]
CPC	BPW	100.0
	nBPW	100.0
CPC w/ 80 minute chill	BPW	100.0
	nBPW	100.0
PAA	BPW	53.3
	nBPW	100.0

[a] The *Salmonella* spp. inocula was 1.1 X 10^6 CFU/ml.

[b] CPC, cetylpyridinium chloride, 0.8% CPC, PAA, peroxyacetic acid, 0.2% PAA.

[c] CPC ingoing level was 0.8%, PAA ingoing level was 0.2%.

[d] BPW, buffered peptone water, nBPW, neutralizing buffered peptone water.

[e] Positive was defined as a sample that tested positive on either XLT4 or BGS Petri plates and on matching TSI and LIA slants. Negative results were determined as any sample that either did not have *Salmonella*-typical colony growth on XLT4 or BGS or did not have a presumptive-positive *Salmonella* interpretation from matching TSI and LIA slants*(34, 57)*.

[f] Calculated by taking the number of presumptive-positive results, dividing it by the number of samples overall for the respective Antimicrobial Intervention*Rinse Fluid combination and multiplying by 100.

Table 5. Fixed Effects Test for presumptive-positive *Salmonella* recovery from inoculated young chicken carcasses[a]

Source[b]	**Nparm**	**DF**	**DFDen**	**F Ratio**	**Prob > F**
Rinse Fluid	1	1	82	12.0299	0.0008*
Antimicrobial intervention	2	2	82	12.0299	<0.0001*
Rinse Fluid X Antimicrobial intervention	2	2	82	12.0299	<0.0001*

[a] The *Salmonella* spp. inocula was 1.1 X 10^6 CFU/ml.
[b] Rinse fluids used were BPW, Buffered Peptone Water, nBPW, neutralizing Buffered Peptone Water. Antimicrobial interventions used were CPC, cetylpyridinium chloride, PAA, peroxyacetic acid.
[c] *Prob >F significant, $\alpha = 0.05$

Table 6. Least Squares Means Differences Student's t for presumptive-positive *Salmonella* recovery from inoculated young chicken carcasses as a function of rinse fluid

Level[a]	Least Squares Means[b]	Standard Error
nBPW	1.00 A	0.025
BPW	0.84 B	

[a] nBPW, neutralizing Buffered Peptone Water, BPW, Buffered Peptone Water.
[b] Levels not connected by the same letter(A, B) differ at $p<0.05$.

Table 7. Least Squares Means Differences Student's t for presumptive-positive *Salmonella* recovery from inoculated young chicken carcasses as a function of antimicrobial intervention

Level[a]	**Least Squares Means**[b]	**Standard Error**
CPC_a	1.0 A	0.033
CPC_b	1.0 A	
PAA	0.77 B	

[a] CPC_a, CPC-treated carcasses without a chill step, CPC_b, CPC-treated carcasses with an 80 min chill, PAA, PAA-treated carcasses.
[b] Levels not connected by the same letter(A, B) differ at $p<0.05$.

Table 8. Least Squares Means Differences Student's t for presumptive-positive *Salmonella* recovery from inoculated young chicken carcasses as a function of rinse fluid x antimicrobial intervention

Level[a]	**Least Squares Means[b]**	**Standard Error**
BPW X CPC_a	2.03 A	0.051
BPW X CPC_b	1.74 A	
nBPW X CPC_a	1.61 A	
nBPW X CPC_b	1.31 A	
nBPW X PAA	1.22 A	
BPW X PAA	0.99 B	

[a] BPW, Buffered Peptone Water, nBPW, neutralizing Buffered Peptone Water, CPC_a, cetylpyridinium chloride-treated carcasses without a chill step, CPC_b, cetylpyridinium chloride-treated carcasses with an 80 min chill, PAA, PAA-treated carcasses

[b] Levels not connected by the same letter(A, B) differ at $p<0.05$.

3.2 Commercial chicken carcasses

Unlike the inoculated *Salmonella* carcass study, not every sample in the commercial plant carcass study was able to be labeled as presumptive-positive *Salmonella* or negative without going through TSI and LIA biochemical testing. While in the inoculated *Salmonella* carcass study, XLT4 and BGS agars displayed typical *S. enterica* subsp. e*nterica* morphologies, in the commercial carcass samples, atypical colony morphologies were seen across all rinse fluid collections. The majority of BGS Petri plates showed bright, yellow-green colonies with a yellow-green or green halo, while only select few showed red colonies with a red agar halo surrounding the colony. These various characteristics were seen in all experimental replications, regardless of day of collection. In replicate 3, 0% of XLT4 and BGS agars displayed recovery after 24 hour incubation for CPC-treated, PBS-rinsed samples (CPC-PBS) and CPC-treated, BPW-rinsed samples (CPC-BPW) only (data not shown).

Plates displaying no growth were incubated at 35±2°C for an additional 21±3 h *(57)*. After the additional incubation time, plates displaying no colony growth were discarded and recorded as negative *(57)*. Plates with colony growth were recorded based on the procedure previously stated in Section 2.18. After completion of TSI and LIA incubation, Rule-Out Guidelines were used in order to classify questionable, atypical colony slants being classified as presumptive-positive or negative *(34)*.

The percentage of presumptive-positive *Salmonella* rinse fluid results can be seen in Table 9. The only rinse fluid that had presumptive-positive *Salmonella* recovery was nBPW at a 13.3% recovery rate. After running an ANOVA for presumptive-positive *Salmonella* results, there was a statistically significant difference between rinse fluids (α=0.05) (Table 10). Due to this, a Least Squares Means Differences Tukey's HSD test was conducted to determine differences between rinse fluids (Table 11). There was a statistical difference between the nBPW rinse fluid from both the BPW and PBS rinse fluids, but there was no difference seen between BPW and PBS.

Table 9. Percent of presumptive-positive *Salmonella* results for chicken carcasses

Rinse Fluid[a]	% Presumptive-Positive *Salmonella*[b]
PBS	0.0%
BPW	0.0%
nBPW	13.3%

[a] PBS, Phosphate Buffered Saline, BPW, Buffered Peptone Water, nBPW, neutralizing Buffered Peptone Water.

[b] % presumptive-positive *Salmonella* was calculated by taking the number of presumptive-positive results, dividing it by the number of samples overall for the respective rinse fluid (N=60) and multiplying by 100.

Table 10. Analysis of variance for chicken carcass presumptive-positive *Salmonella* results

Source	Degrees of Freedom	Sums of Squares	Means of Squares	F Ratio	Prob > F
Model	4	1.80	0.450	11.6667	<0.0001*
Error	175	6.75	0.039		
C. Total	179	8.75			

* Prob > F significant at $\alpha=0.05$

Table 11. Least Squares Means Differences for chicken carcass presumptive-positive *Salmonella*

Level[a]	**Least Squares Means**[b]	**Standard Error**
nBPW	0.15 A	0.025
BPW	1.9E-16 B	
PBS	1.9E-16 B	

[a] PBS, Phosphate Buffered Saline, BPW, Buffered Peptone Water, nBPW, neutralizing Buffered Peptone Water.
[b] Levels not connected by the same letter are significantly different at $p<0.05$.

3.3 Commercial chicken edible parts

Similar to the commercial abattoir chicken carcass study, not every sample in the parts study was able to be labeled as presumptive-positive *Salmonella* or negative without going through TSI and LIA biochemical testing. BGS and XLT4 Petri plates showed similar morphologies as what was seen in the commercial carcass section. The majority of BGS Petri plates showed bright, yellow-green colonies with a yellow-green or green halo, while only select few showed red colonies with a red agar halo surrounding the colony. These various characteristics were seen in all sample replications, regardless of day of collection, or part sample piece that was collected. After completion of TSI and LIA incubation, Rule-Out Guidelines were used in order to classify questionable, atypical colony slants being classified as presumptive-positive or negative *(34)*.

The percentage of presumptive-positive *Salmonella* rinse fluid results can be seen in Table 12. There was a 0% recovery of presumptive-positive *Salmonella* in reps 2 and 3 for the PBS rinse fluid (data not shown). After running an ANOVA for presumptive-positive *Salmonella* results, there was no statistically significant difference between rinse fluids (α =0.05) (Table 13).

Table 12. Percent of presumptive-positive *Salmonella* results from chicken edible parts study

Rinse Fluid[a]	**% Presumptive-Positive *Salmonella***[b]
PBS	4.8%
BPW	11.9%
nBPW	28.6%

[a] PBS, Phosphate Buffered Saline, BPW, Buffered Peptone Water, nBPW, neutralizing Buffered Peptone Water.
[b] % presumptive-positive *Salmonella* was calculated by taking the number of presumptive-positive results, dividing it by the number of samples overall for the respective rinse fluid (N=42) and multiplying by 100.

Table 13. Analysis of Variance for chicken edible parts presumptive-positive *Salmonella* results

Source	**DF**	**Sums of Squares**	**Means of Squares**	**F Ratio**	**Prob > F**
Model	4	0.51	0.13	1.4	0.25
Error	121	11	0.09		
C. Total	125	12			

* Prob > F significant

4. CONCLUSION

There is debate on whether the current testing methods provide the greatest opportunity for *Salmonella* recovery from raw poultry. Bourassa et al. (2015) determined that after performing neck skin, whole carcass rinse fluid and whole carcass enrichment on raw broilers treated with either air or immersion chilling, the only way to definitively declare that a raw poultry item that was tested for *Salmonella* can be declared "*Salmonella*-free" would be to sample every carcass by whole carcass enrichment, leaving no carcasses for consumption *(7)*. Gamble et al.(2016) discovered that at 0- and 1-min drip time intervals for carcasses treated with PAA, CPC and acidified sodium chlorite, the collected drip fluid displayed statistically significant ($P<0.0001$) antimicrobial carryover activity with 0% of the samples detecting *Salmonella*, meaning that false-negatives could occur if the antimicrobial carryover is stronger than the neutralizing ability of the rinse fluid *(36)*. Also, *Salmonella* cells could easily not be taken up when transferring occurs in the testing method. Of 400ml rinse fluid, 100% of rinse fluid capture and retention from the sample is unlikely with rinse fluid inevitably remaining on the carcasses, parts and/or bag. Of the rinse fluid that is collected, only 30 ml is taken to be diluted in a 1:1 ratio with sterile rinse fluid and pre-enriched. Of the 60 ml pre-enriched fluid, only 0.6±0.1 ml is transferred into selective enrichment broths. This leaves room for a failure to detect to occur in the event that *Salmonella* counts were not high enough after selective enrichment occurred.

Unlike the inoculated *Salmonella* carcass study, not every sample in the commercial whole carcass and edible parts study was able to be labeled as presumptive-positive *Salmonella* or negative without going through TSI and LIA biochemical testing. This could be due to the inoculated carcass study being inoculated with a high population of *Salmonella* (10^6 CFU/ml), resulting in the *Salmonella* serovars used in the cocktail to be recovered at a high rate through pre- and selective enrichments that followed after rinse fluid collection. Since low numbers of *Salmonella* are present in foods, pre-enrichment enables injured organisms the opportunity for recovery *(30)*. However, this may not always mean the successful recovery of *Salmonella* spp. The ability to detect any *Salmonella* present can be dependent on the amount of *Salmonella* that is on the food initially *(7, 53)*. Processed raw poultry carcasses usually have low *Salmonella* counts, with previous studies showing that chicken carcasses that are recorded as *Salmonella* positive can typically have no more than 100 cells of *Salmonella* *(7, 42, 70)*. Berghaus et al. (2013) was able to show that as broiler chickens moved from farms and are processed in plants, the *Salmonella* prevalence decreases from the time the birds arrive at the plant to the time they exited a chlorinated-immersion chill tank *(5)*. This demonstrates that antimicrobial interventions reduce the amount of *Salmonella* on carcasses, but also explains the low numbers of *Salmonella* from commercially collected samples. While Ukuku et al. (2004) applied a *Salmonella* cocktail to cantaloupes, they still found that as the $\log_{10}$ CFU/ml inoculum level was increased, the recovery rates for *Salmonella* survival also increased for cantaloupes that underwent a hot water treatment or a 5% hydrogen peroxide treatment *(53)*. This correlates to the data collected for the 10^6 CFU/ml inoculated carcass preliminary study where recovery was observed from all antimicrobial intervention/rinse fluid treatment combinations. This could also explain the low recovery rates seen in both the commercial carcass and edible parts studies also. When comparing varying levels of PAA and 0.003% chlorine solutions on inoculated chicken carcasses, Bauermeister et al. (2008) found that PAA levels as low as 0.0025% were more effective in decreasing *Salmonella* spp. compared to the chlorine solution *(4)*. This could explain the low recovery rate seen in the PAA-treated, BPW-rinsed samples in the inoculated chicken carcass study.

Since raw poultry can also harbor bacteria other than *Salmonella*, the variation in morphologies on XLT4 and BGS Petri plates could be explained by the potential that other bacteria were able to replicate in the enrichment broths *(41)*. There were atypical S. *enterica* subsp. *enterica* morphologies on XLT4 and BGS Petri plates in all reps for commercial carcasses and parts. Plates exhibiting *Salmonella* atypical and typical colonies had typical colonies sampled for TSI and LIA stab-streaking. For commercial chicken carcasses and parts, atypical morphologies were tested in the event that an atypical *Salmonella* spp. was present. After TSI and LIA incubation, there was a 0% recovery of presumptive-positive *Salmonellae* for all atypical morphology colonies across all rinse fluids for both commercial carcasses and parts. This supports the suggestion that there were other bacteria that were able to thrive in the enrichment broths and were able to grow XLT4 and/or BGS.

Following the completion of the *Salmonella*-inoculated carcass study, findings suggest that the use of nBPW provided for a higher rate of *Salmonella* recovery post-antimicrobial application versus conventional BPW rinse fluid when PAA was the applied antimicrobial. Conversely, for CPC-treated inoculated carcasses, presumptive-positive *Salmonella* survival rates did not differ, indicating that there was no effective impact of the rinse fluid formula on *Salmonella* recovery. For the remaining antimicrobial intervention/rinse fluid interactions, there was no significant difference between the treatments, other than the previously mentioned PAA-BPW interaction. PAA application at maximum applied concentrations may be effectively neutralized with the use of nBPW post-dripping based on the preliminary experiment results.

After running a one-way ANOVA for presumptive-positive *Salmonella*, there was no significant difference (α =0.05) between rinse fluids for parts, but there was a significant difference between rinse fluids for commercial carcasses rinsed with nBPW only. These data indicate that in samples with low *Salmonella* counts, nBPW does have a neutralizing affect against any antimicrobial solution that could have remained post-1 min drip, and it was greater than both the control (PBS) and the previous rinse fluid (BPW).

This is the first study comparing the nBPW rinse fluid formulation to BPW rinse fluid in both an inoculated carcass setting and a commercial poultry abattoir *(67)*. While these data show there was neutralizing ability for nBPW for carcass rinse collections, it does not provide the security that nBPW is more capable as a neutralizing rinse fluid versus PBS or BPW for chicken parts rinse collections. In order to obtain more concrete results, further studies should be done to determine if there is a statistical difference between BPW and nBPW rinse fluids when sampling chicken edible parts or if a stronger neutralizing formulation is required for parts rinses due to chicken parts in this setting having undergone two antimicrobial intervention applications versus the one application for carcasses. Different approved antimicrobial solutions at varying levels should also be tested to ensure that nBPW has a greater neutralizing ability across all antimicrobial solutions available for use by poultry processors. Also, carcass studies should be extended to determine if BPW versus nBPW rinse fluid collections could be replicated with similar results. Research should also be expanded to other chicken edible parts that are sampled per USDA-FSIS regulations to determine if results are similar to those obtained in the present study.

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