EVALUATION OF SALMONELLA CONTROLS FOR OFFAL RENDERING AND FRAMES USED IN MECHANICALLY SEPARATED CHICKEN

A Dissertation

by

AMIE MARIE JONES-IBARRA

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Chair of Committee,	Christine Zocchi Alvarado
Committee Members,	Craig Daniel Coufal
	James Allen Byrd
	Thomas Matthew Taylor
Head of Department,	David James Caldwell

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ABSTRACT

This study was conducted to verify and continue to enhance the area of food safety within fresh and further processed poultry products. Objectives included: 1) determine the thermal inactivation of *Salmonella enterica* in poultry offal during rendering at differing temperatures; 2) evaluate the potential for development of systemic *Salmonella* infection in chickens as a function of challenge method and route to determine the possibility of systemic infections, and; 3) determine the effectiveness of a novel treatment of combined hydrogen peroxide (H_2O_2) and ultraviolet (UV) light with use of a Hydrogen Peroxide and UV – Advanced Oxidative Processing machine (H_2O_2/UV-AOP) to reduce aerobic bacteria and *Salmonella* in chicken frames.

Results from the rendering verification study showed mean *D*-values for the *Salmonella* cocktail at 150, 155, and 160 °F were 0.25 $\pm$ 0.05, 0.17 $\pm$ 0.01, and 0.09 $\pm$ 0.01 min, respectively, indicative of increasing susceptibility to increased application of heat during processing. Current poultry byproducts rendering procedures are effective for achieving necessary *Salmonella* control when completed under sanitary conditions.

Results from the systemic chicken infection study indicated some challenge method techniques resulted in *Salmonella* infection into bone marrow within six days of challenge, but cells were cleared by nine days post-challenge . Data show significant differences between gavage, tracheal, and transdermal scratch *Salmonella* challenge on breast muscle without feathers regarding the location of administered bacteria. Regardless of the route of administration, all challenged birds were positive for *Salmonella* by 6 days post-inoculation. Overall results indicate it is possible for a *Salmonella* infection to reach the bone marrow, signifying the needs for hygienic rearing of broilers until the point of meat

harvest in order to prevent *Salmonella* transmission to carcass components potentially used in manufacture of non-intact poultry meat products.

Results from the H_2O_2/UV-AOP application onto broiler frames for two applied concentrations of H_2O_2 indicate similar reductions in *Salmonella* of 1.1 $\log_{10}$ CFU/frame at 5.0% H_2O_2 and 1.0 $\log_{10}$ CFU/frame at 7.0% H_2O_2, compared to non-treated control. Both concentrations of H_2O_2 yielded significant reductions in APC: 0.8 and 1.5 $\log_{10}$ CFU/frame for 5.0% and 7.0% H_2O_2, respectively. These findings indicate all treatments in this study can reduce *Salmonella* on chicken frames while the 5.0% H_2O_2 and 7.0% H_2O_2 + UV treatments can reduce both *Salmonella* and APC on chicken frames.

Results from the three studies indicate the possibility of *Salmonella* reaching the bone and bone marrow via multiple pathways while giving two solutions of verifying the reduction of *Salmonella* using two processes; one currently in use for pet foods and one novel intervention for us in human consumed foods.

DEDICATION

I dedicate this dissertation to my late mother and paternal grandparents. My mother's short life was spent furthering her education to better the lives of her students falling short of her own achievement of a PhD in education. Unknowingly, she set my own ambitions into action ensuring my future. My grandfather spent his life working to ensure I had the best available options for me and to do whatever made me happy. His long life of hard work has allowed me to be on the successful path I am on today. He was the most dedicated and intelligent undereducated man I have met in my life. He created the pedestal I stand on today. My grandmother has been my number one cheerleader and influencer since I was born. She encouraged me to be a strong educated individual female that kept her goal in mind while understanding and excepting some things don't happen as planned. To these people I can only try to continue on with the examples you've left with me.

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NOMENCLATURE

ACP	African Caribbean Pacific
AIV	Avian Influenza
ANOVA	Analyzed by One-Way Analysis Of Variance
AOP	Advanced Oxidation Process
APC	Aerobic plate count
APHIS	Animal and Plant Health Inspection Service
ARS	Agricultural Research Service
AVAMA	American Veterinary Medical Association
BPW	Buffered peptone water
BS-2	Biosafety Level-2
CCP	Critical Control Point
CDC	Centers for Disease Control And Prevention
CFU	Colony forming unit
CPC	Cetylpyridinium chloride
DI	Deionized
E.U.	European Union
EHEC	Enterohemorrhagic *Escherichia coli*
EO	Essential oils
FDA	Food and Drug Administration
FMIA	Federal Meat Inspection Act of 1906
FSIS	Food Safety and Inspection Service
FSMA	Food Safety Modernization Act

FSPCs	Food Safety Preventive Controls
GFPs	Good Farming Practices
GI	Gastrointestinal
GMPs	Good Manufacturing Procedures
GRAS	Generally Recognized as Safe
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2O_2/UV-AOP	H_2O_2+UV – advanced oxidative process
HACCP	Hazard Analysis and Critical Control Points
IOBW	Inside outside bird washer
IP	Inflammatory process
ISO	International Standards Organization
LAE	Lauric arginate ester
LOD	Limit of detection
MAP	Modified atmospheric packaging
MDM	Mechanically deboned meat
MSC	Mechanically separated chicken
NA	Nalidixic acid
NASA	National Aeronautics and Space Administration
NO	Novobiocin
NPIS	New Poultry Inspection System
NRC	National Research Council
NRTE	Not-ready-to-eat

NTS	Non-typhoidal *Salmonella* spp.
PAA	Peroxyacetic acid
PBS	Phosphate buffered solution
PLNs	Peripheral lymph nodes
PPIA	Poultry Products Inspection Act Of 1957
QAC	Quaternary ammonium compound
RTE	Ready-to-eat
RV	Rappaport-Vassiliadis
SE	*Salmonella enterica* subspecies *enterica* serovar Enteritidis
SIP	*Salmonella* Initiative Program
SPARC	Southern Plains Agricultural Research Center
SS	*Salmonella enterica* subspecies *enterica* serovar Senftenberg
SSOPs	Sanitation Standard Operating Procedures
ST	*Salmonella enterica* subspecies *enterica* serovar Typhimurium
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSP	Trisodium phosphate
U.K.	United Kingdom
U.S.	United States
USDA	United States Department of Agriculture
UV	Ultraviolet
WHO	World Health Organization
WPPA	Wholesome Poultry Products Act Of 1968

XLT4	Xylose-Lysine-Tergitol 4
XLT4+NO	Xylose-Lysine-Tergitol 4 + Novobiocin

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The most current surveillance data show *Salmonella* as the leading cause of bacterial foodborne disease outbreaks and illnesses (33%), and as the cause for bacterial foodborne disease-resulting hospitalizations (62%) amongst all etiological agent categories *(14)*. Developed countries, such as the United Kingdom, the United States and Australia, have high incidence rates of *Salmonella*-derived disease, known as salmonellosis *(37, 73)*. Developing and under-developed countries presumably have high rates of salmonellosis, but because of severe under-reporting, the exact incidence is unknown *(25, 30)*. Efforts to reduce *Salmonella* around the world have been effective in decreasing its incidence in the food supply, thanks to collaborations between governmental agencies, animal producers and food processors, during pre- and post-harvesting *(7, 12, 28, 79, 85, 91, 102)*. Although their efforts have shown great success, *Salmonella* remains at the forefront of foodborne illness concerns. Salmonellosis is a special concern for one of the categories of persons who are more severely affected by foodborne illnesses, children under the age of 5 years *(45)*. Nevertheless, government agencies, food animal producers, products processors, and researchers continue to work together from around the globe to lower the overall incidences of salmonellosis to create improved public health.

Physiology and Pathogenesis

Global Foodborne Illness Incidence Rates. In the United States, 31 pathogens are estimated to cause 9.4 million human foodborne illnesses every year; of these, 39% are bacterial pathogens *(76)*. The pathogens that cause the most illnesses, in order, are

norovirus, the non-typhoidal *Salmonella* spp., *Clostridium perfringens*, and *Campylobacter* spp. *(76)*. In the U.S., over 1 million illnesses and ~400 deaths occur each year due to the NTS *(76)*. Other developed countries, such as the United Kingdom (U.K.), Australia and Japan, have high rates of salmonellosis but have a higher incidence of hospitalizations due to campylobacteriosis. Foods bearing elevated risk for pathogen transmission include poultry, red meat products, unpasteurized milk, eggs, and seafood *(1, 35, 47)*. In countries such as Jordan, salmonellosis is second compared to *Shigella* spp. *(31)*. Underdeveloped countries are far more difficult to retrieve data from, as they do not always have the means to test their cases or choose not to report. This includes countries on the continents of Africa, Asia, as well as nations within Latin America and the Caribbean *(25, 45)*. It has been theorized that because those in many underdeveloped countries are malnourished, they have an increased susceptibility to *Salmonella* infections (including Typhoid) compared to other countries with more advanced water treatment systems *(53)*.

***Salmonella* Physiology and Taxonomy.** *Salmonella* spp. are Gram-negative, facultative anaerobic, mesophilic, bacillus-shaped rods that are typically flagellated and belong to the family *Enterobacteriaceae (23, 32)*. They possess three major antigens: flagellar (H), somatic (O), and a few serovars possess the Vi antigen which partly determines their subspecies and serovars classification *(32)*. While most are motile by use of peritrichous flagella, some serovars such as *S.* Pullorum and *S.* Gallinarum are non-flagellated; others may also become non-motile due to dysfunctional flagella *(23)*. *Salmonella* ideal growth-supporting environment is 37 °C at pH of 6.5-7.5, although it has been reported to survive at pH 4.0 when put under stress *(43, 70)*. For example, when *S.* Typhimurium is briefly exposed to a mildly acidic environment (pH 5.5-6.0) followed by

exposure to a pH 4.5, it triggers a complex acid tolerance response (ATR), allowing it to persist under extreme acidic conditions (pH 3.0-4.0) *(23)*. *Salmonella* spp. utilize different sugars, minerals, and amino acids; Table 1-1 reviews the presumptive biochemical identification tests of most *Salmonella* spp. There are two species within the genus: *Salmonella bongori* and *Salmonella enterica*. *S. enterica* is divided into six different subspecies: the one of most concern for foodborne human disease being *S. enterica* subsp. *enterica*.

Salmonella Typhi and Paratyphi are causative agents of typhoid and paratyphoid fever, respectively *(5, 11)*. These bacteria are found mostly in water and cause high disease rates in countries with poor sanitation or no water and sewage treatment systems. The *Salmonella* serovars of global concern for salmonellosis are the non-typhoidal *Salmonella* (NTS). The most frequently isolated serovars for human infection are *S*. Enteritidis (65%), *S.* Typhimurium (12%), and *S*. Newport (4%) *(30)*. North America reflects the same global ranking with the fourth most frequent in serovars associated with illness in the U.S. being *S*. Heidelberg *(14, 30)*. For the experiments in this paper, the NTS serovars will be the focus, referred to as *Salmonella* and their specific subspecies identified where applicable.

***Salmonella* Pathogenesis.** Salmonellosis, a foodborne illness caused by a NTS, is contracted by food vehicle transmission. The body's reaction to the ingested bacterium includes specific and nonspecific immune responses involving but not limited to: components in saliva, gastric acidity, intestinal peristalsis, sloughing of epithelial cells that do not recognize the colonization of certain bacteria, phagocytic cells, and T and B lymphocytes. (23).

Table 1-1 Biochemical and serological characteristic reactions for *Salmonella* spp.

Test	Result		Reaction[a]
	Positive	*Negative*	
Catalase test	bubbles	no bubbles	+
Glucose (TSI)	yellow butt	red butt	+
H_2S	blackening	no blackening	+
Indole test	red color at surface	yellow color at surface	−
KCN broth	growth	no growth	−
Lysine decarboxylase (LIA)	purple butt	yellow butt	+
Lysine decarboxylase broth	purple color	yellow color	+
Malonate broth	blue color	no color change	−[c]
Methyl red test	diffuse red color	diffuse yellow color	+
Ornithine decarboxylase broth	purple color	no color change	+
Oxidase test	bubbles	no bubbles	−
Phenol red dulcitol broth	yellow color and/or gas	no gas; no color change	+[b]
Phenol red lactose broth	yellow color and/or gas	no gas; no color change	−[c]
Phenol red sucrose broth	yellow color and/or gas	no gas; no color change	−
Polyvalent flagellar test	agglutination	no agglutination	+
Polyvalent somatic test	agglutination	no agglutination	+
Simmons citrate	growth; blue color	no growth; no color change	v
Urease	purple-red color	no color change	−
Voges-Proskauer test	pink-to-red color	no color change	−

[a] +: ≥90% positive in 1-2 days; −: ≥90% negative in 1-2 days; v: variable.
[b] Majority of *S. enterica* subsp. *arizonae* cultures are negative.
[c] Majority of *S. enterica* subsp. *arizonae* cultures are positive.
Adapted from *(23, 98)*

Once the organism is ingested and makes its way through the multiple hurdles of the body's immune response, it colonizes in the lower intestine in the ileum and cecum where it begins to invade the intestinal epithelium and lymphoid follicles *(32)*. The multi-step mechanism by which *Salmonella* crosses the epithelium involves initial binding to

receptors on the epithelial cell surface and then subsequently entering the cell *(32)*. Once *Salmonella* has made its way into the intestinal cells, it begins to proliferate and once sufficient growth is reached, the body tries to purge itself by stimulating the immune release of proinflammatories, cytokines and chemokines *(32, 62)*. Chemokines are released in the early phases of an infection and result in the movement of immune cells to the source, producing chemokines and other inflammatory elements including leukocytes *(62)*. Once leukocytes have made their way to the intestinal wall, they bind to the wall and cross into the blood vessels causing inflammation, increasing in intensity as more leukocytes increase in concentration *(62)*. The release of leukocytes activates the enzyme adenylyl cyclase in the epithelial cells which results in an increase of fluid into the intestinal lumen *(23)*. This inflammatory response causes the major symptoms of gastroenteritis, inflammation of the bowels, along with mild to severe diarrhea and as the body tries to maintain homeostasis, the quick release of fluid from the intestine causes minor to cramping. Gastroenteritis is a major cause of death in under-developed countries because resources are unavailable which disallows the ability to counteract its major symptom, dehydration *(36)*. This also makes it particularly dangerous for persons at high risk for complication for infections: infants and children under 5 years, immunocompromised, pregnant women, and the elderly (65+). For those in good health and condition, the infectious dose for salmonellosis is as high as 1 million cells or more; those who are at increased risk for infection need only ingest as few as 10 cells per gram *(36, 41)*. Depending on the health of the consumer and initial dose of *Salmonella* ingested, symptoms may appear between 6 and 72 hours (incubation period) and last between 4 and 7 days *(15, 41)*. Acute sequelae result when an exposed person succumbs to symptoms and

may shed bacteria from the intestinal system. Those who do are unable to clear the bacterium from their GI tract may suffer from chronic sequelae; they become a carrier for *Salmonella* and will continue to have symptoms when the bacteria proliferate in the lower intestines, however some will remain asymptomatic *(19)*. The chronic conditions in the United States attributed to this includes aseptic reactive arthritis and Reiter's syndrome *(3)*. An outbreak report spanning from 1998 to 2012 was analyzed by Chai et al. *(16)* and found 25% of outbreaks, illnesses, and hospitalization from an implicated food or ingredient was associated with poultry, 43% of those being from *Salmonella enterica*. The most commonly reported consumption factors were food-handling errors (64%) and inadequate cooking (53%) from restaurants (37%), private homes (25%), and catering facilities (13%) *(16)*.

Poultry Industry

History of U.S. Poultry Industry Regulation. For some time before 1906, lobbyist and advocates for social justice sought to urge the government to develop regulations for the food and drug industry *(26, 29)*. The Association of Official Agricultural Chemists (now AOAC International), formed by the USDA, had been raising awareness of the health hazards associated with colorings and preservatives found in canned meats. This included an incident with Armour & Co. that was accused of sending rotten canned beef to U.S. Army soldiers in Cuba that contained a visible layer of boric acid, then used as a preservative, which was used to mask the stench of the rotted meat *(26)*. While many working parts were trying to get legislation passed, the input for new regulations continued to stall in Congress until the publishing of "The Jungle" by author Upton Sinclair *(26)*. The books described the working conditions of meat packing houses

including how the meat packing practices that produced and packaged the meats themselves were done. There was an enormous public outcry for control of the contamination and sanitation issues described in the novel *(26)*. President Theodore Roosevelt was given an advanced copy of the manuscript and subsequently threatened to release details of unsanitary and unsafe working environment in order to speed along two congressional acts then in standstill, which then became law that very same day *(26)*. These laws are now known as the Pure Food and Drug Act and the Federal Meat Inspection Act of 1906 (FMIA). The Pure Food and Drug Act was enacted to ensure the prevention of the manufacture, sale, or transportation of adulterated or misbranded foods, drugs, medicine, and liquors, and for regulating the trafficking of these items *(99)*. At the same time the FMIA was passed to ensure meat and meat food products distributed were wholesome, not adulterated, and properly marked, labeled, and packaged *(81)*. During this time, chickens and turkeys were mainly produced on small farms and sold as live birds or slaughtered for local customers or transported to markets in nearby cities *(66)*. The FMIA did not cover poultry as it was seen as a minor meat product, typically purchased as a live bird or New York Dressed, with only blood and feathers removed *(64, 66)*. The need for these inspections became afterwards apparent as poultry began to rise in popularity, which led to the USDA creating the Federal Poultry Inspection Service (FPIS) in 1926 to provide inspection services for those processors who voluntarily participated in the USDA's inspection program *(64, 66)*. In 1942, an Illinois plant was the first to roll out a government-approved on-line evisceration system which led to the eventual norm of evisceration and packaging of ready-to-cook whole carcasses in iced wooden crates *(64)*. By 1952, broilers (meat chickens) surpassed the number of farm chickens as the number

one source of chicken meat in the U.S., leading to the creation of the National Broiler Council in 1954. They later changed their name to the National Chicken Council to better reflect all poultry products they would come to represent *(64)*. With a new poultry advocate in Washington, D.C., the push and eventual creation of the Poultry Products Inspection Act of 1957 (PPIA) came to fruition. The PPIA was created in response to changes in consumer perceptions and marketing which increased during World War II after the increase of sales of poultry products bearing the FPIS certification marking *(66)*. The PPIA required inspection of poultry prior to and after slaughter, plant facilities, all operating procedures, product labeling, and imported poultry products *(66)*. A restriction to the PPIA was an exemption that stated slaughterers and processors of poultry were exempt from federal inspection if the product did not cross states lines for commerce *(82)*. To address this, the Wholesome Poultry Products Act of 1968 (WPPA) was created to bring inspection to poultry products whether they were state or federally operated and crossed or did not cross state lines; it required inspection of essentially all poultry sold to consumers *(66)*. By the 1970s the poultry industry had evolved into a modern state with enhanced animal nutrition, disease prevention and/or treatment programs, genetic improvements through breeding, and automation technologies *(64)*. During the 1980s, consumers switched to more convenient foods, which for poultry meant cut-up and further processed chickens rather than whole birds *(64)*. During the 1990s, international poultry exports began to increase, reaching about 20% of the U.S. poultry products going to foreign markets by 2001*(64)*.

The USDA introduced a system called Hazard Analysis Critical Control Point (HACCP) in 1994, adopted from a program developed by NASA and the Pillsbury Co. for

the astronaut program in the 1960s. This was in response to an outbreak of the pathogen *Escherichia col*i O157:H7 which resulted in the death of 4 children the previous year at a fast food chain from undercooked hamburger meat. With HACCP implementation mandated by 1998, there was a reduction of pathogenic microbes' prevalence *(86)*. *Salmonella,* however, even with significant decline in red meats, has shown persistence in other foods *(101)*. In 2008, there was a boom in severe salmonellosis cases including a multi-state outbreak of peanut butter and multi-national outbreak of produce, simultaneously with ongoing poultry and meat incidences. With increasing frequency of outbreaks of *Salmonella* spp. since the implementation of HACCP, the USDA Food Safety and Inspection Service (USDA-FSIS) needed to create a way to reduce the incidence of *Salmonella* disease. Between 1998 and 2000, USDA-FSIS phased in implementation of collecting *Salmonella* data and after full completion, they found an upward trend in *Salmonella* positives between 2002 and 2005*(24, 88, 101)*. In 2008, USDA-FSIS announced a program called the *Salmonella* Initiative Program (SIP) that sought to give better performance standards using national baseline studies that would be conducted before the program's eventual implementation in 2011 *(88, 90, 92)*. In 2013, the *Salmonella* Action Plan was positioned with six high-priority goals with parameters including public education, collaborations, inspection, sampling, import/export restrictions, and the enforcement of all of parameters to ensure meeting the food safety goal of Healthy People 2020 by improving food safety-related behaviors and practices *(67, 91, 93)*. The inspection part of this plan, known as Modernization of Poultry Slaughter Inspection, was put into final rule in 2014 and is referred to as the New Poultry Inspection System (NPIS) *(93)*. NPIS gives mandate that poultry facilities take scientifically based measures to

prevent contamination rather than addressing it after it occurs in addition to revised *Salmonella* and *Campylobacter* performance standards in 2015 and USDA-FSIS published the results in 2016 *(93)*. The sampling aspect of the action plan is called the Microbial Sampling Plan and requires almost all poultry slaughter facilities to develop, implement, and maintain written procedures to prevent carcass contamination by enteric pathogens and fecal material throughout the entire slaughter and dressing operation (9 CFR §381.65) *(92)*. These procedures include but are not limited to description of sample collection procedure and locations, laboratory and analytical assays performed, and scientific and technical documentation to support sampling program *(92)*. U.S. Department of Agriculture-FSIS also recommend the testing for enteric pathogens be compared to results on the presence or absence of other non-pathogenic organisms as some of these are indicators for known pathogens *(92)*.

While HACCP is applied to both USDA-inspected and some FDA-inspected foods, the Food Safety Modernization Act (FSMA) was signed into law in 2011 by President Barack Obama. It increases the ability of the FDA to provide means of preventing contamination of the food supply in contrary to reactionary methods. This act provides legal authority to regulate human and animal food products. There are five key areas of focus, including: food safety preventative controls, inspection and compliance, imported food safety, response, and enhanced partnerships with global partners. The final rule for animal foods was published in 2015 and compliance began the following year (21 CFR Parts 11, 16, 117, 500, 507, and 579). Full compliance of the entire industry will be completed by the end of 2019. Facilities that manufacture products for animals may choose to follow CGMPs of animal food or those for human consumed foods. With the passage of

the FMSA and subsequent regulatory procedures by the U.S. Food and Drug Administration (FDA), the capacity of the U.S. further processing industry to effectively process raw materials for microbiological safety is being re-evaluated in preparation for preventive controls development and implementation. Multiple severe U.S. outbreaks of human following cross-contamination of pet foods with enteric pathogens such as *Salmonella enterica* spp., cases of exposure of consumers to *Salmonella*-contaminated pet food, and contamination of processed and further processed poultry items have resulted in increased concern regarding their microbiological safety *(96)*.

The PPIA included any poultry product that contained added water due to absorption or retention to be calculated and internally recorded but was not required to be labeled (9 CFR 317.2 and 381.117) *(64, 84)*. An amendment (9 CFR 381.169) to the PPIA in 1968 added the requirement that the amount of retained water for an RTC poultry bone-in carcasses and parts to be limited to approximately 3% weight increase after chilling and washing. It also required that any solution added to these products that are more than 1% be labeled on the packaging. But as the poultry industry began to improve with disease programs, nutritional advances, genetic improvements thought breeding, and processing automation techniques, the amount of added solutions were increasing and becoming harder to adhere *(64)*. In 1997, the court case Kenny et al v Glickman brought the attention to the industry that consumers felt there was not an easy to understand explanation of water retention in the products and a justification for the added water/solution. This later resulted in USDA creating amendments beginning in 2001 over labeling regulations (9 CFR 441.10) which were finalized in 2014 and began implementation in 2016. These require any amount of retained water to be indicated on the product's package when no ingredients

have been added and no longer limited to the 3% rule, as the industry uses a standard calculation based on a weight -in, weight-out method *(65)*. This regulation is to help distinguish between water being an incidental addition to the raw product due to processing steps necessary for food safety purposes (such as washing or chilling) and allows for better consumer awareness *(6, 87)*. The methods being researched by the current authors would not require labeling of an added solution or added water as the system uses H_2O_2+UV as a food safety measure to reduce *Salmonella* and overall APC.

Pre-Harvest Transmission Prevention. The industry has been lowering the prevalence of *Salmonella* found on final poultry products as a result of the impacts of pre-harvest interventions and practices. The CDC has identified poultry as a major source of *Salmonella (94)*. Pre-harvest interventions, as previously identified by World Health Organization *(102)*, include non-therapeutic use of antimicrobials, biosecurity, feed and feed additives managements, manipulation of microbiota, vaccination, and GFPs. These precautions are applied in an effort to eliminate the numbers of *Salmonella* shed in feces by the bird, rodent and wild bird populations, and prevent cross-contamination during transport. Birds are kept in an environment where fecal material may be easily transmitted onto their bodies or orally ingested through horizontal transmission. Horizontal transmission is accomplished by a direct route such as food-borne or air-borne *(18)*. Hence, one bird that is initially *Salmonella*-negative encounters a *Salmonella*-shedding bird; this may result in the initial bird becoming a carrier and eventually the majority or entire flock become *Salmonella* positive. Rodents can be a *Salmonella* vector for poultry producers as they are attracted to covered, warm, and safe environments where there is easy access to food and water. A study in the Netherlands observed when rodents were found to have

high counts of *Salmonella*, the flocks they came into contact had similar high counts of the same bacterium. Flocks with minimal infection rates only had rodents found with similar low to negative counts of *Salmonella (56)*. This study also pointed out that having organic and free-range chickens may result in a higher incidence of *Salmonella* because of the likelihood of the birds encountering rodents more readily due to the types of rodent deterrent methods used. The birds may have also received initial *Salmonella* via vertical transmission which involves the microorganism being passed from mother via offspring on the surface of or within the egg *(18)*.

Vaccinations are another pre-harvest disease intervention used to reduce the numbers of *Salmonella* carried by poultry animals. Vaccinations for *Salmonella* were once only considered for layers, hens used to produce eggs. This was due to time requirements needed to acquire immunity following vaccination using dead cells. While layers are not immediately used in meat production, they are later processed once their egg production no longer performs at the desired levels *(83)*. A meta-analysis of four studies done in 2012 on the effectiveness of live and dead vaccinations reducing *Salmonella* spp. in broilers found that a live vaccine for *Salmonella* Typhimurium showed promising results in being able to effectively reduce the prevalence by 36% of *Salmonella* cecal content in broiler chickens *(78)*. Currently, there are *Salmonella* live culture vaccines available for commercial use however some broilers are not currently being vaccinated *(61)*. Broiler chickens populated from vaccinated breeders tend to have 15% lower *Salmonella* prevalence, approximately half the environmental samples containing *Salmonella* at the farm, and about 10% lower *Salmonella* prevalence in the broilers when they enter the processing plant compared to those from unvaccinated breeders *(22)*.

Lastly, the use of antimicrobial drugs has been reported to prevent *Salmonella* from colonizing the GI tract of animals. However, the World Health Organization (WHO) *(102)* does not recommend the use of these antimicrobial drugs as a food safety intervention due to inconsistent evidence for the efficacy and the serious potential for the development of antimicrobial resistant strains of human pathogens. Instead, they recommend overall biosecurity protection and the hygienic maintenance of transport vehicles to help reduce the incidence of pre-harvest *Salmonella*.

Post-Harvest Contamination Prevention. While the E.U. and U.S. both focus on pre-harvest prevention of *Salmonella* occurrence in poultry production, the U.S. poultry industries also puts significant thought and effort into post-harvest pathogen prevention and reduction strategies development and implementation. Post-harvest includes the harvesting step, after receiving, to packaging of product(s). Careful attention is put upon the steps of poultry meat harvest known to cause a high frequency of carcass cross-contamination, including picking (feather removal) and chilling (reducing internal carcass temperature to 4 °C (39.2 °F), specifically immersion chilling *(69)*. Other steps that contribute to higher levels of carcass cross-contamination are scalding (opening of feather follicles) and evisceration (removal of the viscera) *(12)*. *Salmonella* food safety interventions for post-harvest carcass handling include reduction of fecal cross-contamination to meet the zero-tolerance requirement before reaching the chill tank, visual post-mortem in-line inspection by USDA-FSIS and use of sanitizers and antimicrobials. These are managed under an establishment's HACCP system. Each point of contamination is identified and a step to reduce or eliminate the identified hazard(s) is put into place, termed a critical control point (CCP) which when used for the reduction of *Salmonella* on

carcasses and other edible parts can be antimicrobials. There are two kinds of antimicrobial interventions, chemical and physical (i.e. heat). One method of physical intervention are to lower the carcass and parts to 4 °C to prevent further proliferation of possible present pathogens to reduce and/or eliminate them completely *(69)*.

Various antimicrobial chemicals/sanitizers are approved for use to reduce numbers of contaminating pathogens and are commonly applied during poultry processing on raw carcasses and parts such as chlorine-based compounds, quaternary ammonium compounds, organic acids, peroxides, peracetic acid, and other chemical treatments *(12, 21, 38, 95)*. Traditionally used antimicrobials, chlorine and chlorine-based compounds, have been profusely researched and widely applied as the antimicrobial treatments of choice during the poultry process *(40, 80, 103)*. Currently in the poultry industry, the three most frequently used antimicrobials are PAA, CPC, and chlorine followed by organic acids *(54)*.

Chlorine is a non-selective bactericidal agent that was first used in the food industry to clean dairy equipment followed by major industry facilities for use on processing equipment *(49, 57, 74)*. Currently, chlorine is added as a disinfectant to water systems in different forms, most commonly as chlorine gas, sodium hypochlorite (liquid), and calcium hypochlorite (solid tablets) *(75)*. The pH of the water is kept between 5.0-6.5; the higher pH in the range is to which the hypochlorite ion can be maintained in the hypochlorous acid form which is more effective at killing bacteria than the hypochlorite ion alone, while the low range of the pH insures no corrosion of the equipment *(75)*. In 1951, Goresline et al. *(33)* reported on the use of 10 and 20 ppm chlorine solution in a poultry evisceration facility and reported a 78% reduction of bacterial contamination on the final wash of a carcass and a 90% reduction on equipment. Over the next decade,

researchers learned antimicrobial effectiveness of chlorinated water will decrease when the quality of processing water and amount of organic load in the chill water has changed *(9, 17, 71)*. Though chlorine proved to be a viable solution for reducing bacterial loads on poultry carcasses and equipment, the abundance of organic material caused a lack of free chlorine available in lower concentrations over time *(80)*. To address this issue, an increase in strength of chlorinated water was implemented but led to a growing concern about chemical derivatives being formed by the higher concentrated chlorination of water *(80)*.

Other chemicals have since been researched and found to be more effective than chlorine including PAA, a chemical oxidant with equal parts hydrogen peroxide, acetic acid, and water used to lower microbial counts in post-chill dips or sprays *(2, 9, 10, 17, 103)*. PAA rapidly decomposes to acetic acid, oxygen, and water without the formation of toxic side effects which makes it ideal for food use. When surveyed in 2012, PAA was found to be the most commonly used post-chill antimicrobial when immersion chilling systems were in use and is currently one of the more inexpensive options *(2, 9, 54, 63, 100)*. When researched, PAA (0.0025%, 0.01%, 0.02%, 0.07 and 0.1%) outperformed chlorine controls (0.003%, 0.005%) and unlike chlorine, PAA was observed to have the ability to overcome the high organic load found in chill water and on carcass and parts *(9, 17, 58)*.

Cetylpyridinium chloride(1-hexadecylpyridinium chloride, CPC) is a quaternary ammonium-based neutral pH compound (QAC), a commonly found ingredient in household mouthwash *(54, 69, 97, 100)*). It is stable, water-soluble, non-volatile, and colorless and frequently used as a post-chill antimicrobial when drenched or sprayed with an inside-outside (IO) cabinet but is also used in the main chill tank after carcasses have

reached the appropriate internal temperature *(13, 39, 54, 58, 100)*. QACs are antimicrobials that are able to absorb into the cell surface of a bacterium and destroy the cell's wall and membrane which can have a direct or indirect lethal effect on the cell with little effect on morphological structure *(44, 51)*. CPC is able to kill bacteria by forming weakly ionized compounds which subsequently inhibit bacterial metabolism; it has been shown to reduce *S.* Typhimurium by 6.0 $\log_{10}$ in a liquid suspension within 1.0 min *(20, 70)*. Yang et al. *(103)* and Loretz et al. *(50)* found a 0.5% CPC spray used on carcasses and skin reduced *S.* Typhimurium by 2.0 and 0.9-2.5 $\log_{10}$-cycles, respectively, as well as reduction of aerobic counts by 2.2 and 1.6 $\log_{10}$-cycles, respectively. CPC has also been shown to not cause adverse effects, such as a chemical odor or discoloration of the chicken skin, which allows for broiler carcasses to be sold for retail *(44)*.

Once the carcasses have gone through the chill process, they will either be packaged whole or continue to the debone processes *(34)*. Once each part is cut, the product is sprayed with an antimicrobial before packaging, preventing pathogens from adequately attaching to the surface of the meat or skin over time *(55)*. An organic acid is commonly used for this step in a combination of lactic and acetic acid due to their synergistic effect *(12)*. These organic acids are inexpensive to produce, GRAS, and environmentally friendly but limited in resources because they are naturally occurring *(69)*.

Alternative Interventions for *Salmonella* Reduction. While the need exists for better consumer education to improve knowledge of how to keep food safe, the industry continues to innovate and examine alternative methodologies of reducing the overall counts of *Salmonella* on raw and further processed products. An increasingly frequent method for post-process intervention is high pressure processing (HPP), allowing for a

non-thermal reduction in *Salmonella* spp *(4, 77)*. HPP inactivates microorganisms while retaining food quality attributes and allows for food processing at ambient temperatures *(72)*. Salmonella spp. has been shown to decrease in range between 1.3 to over 5.0 log CFU/g on chicken breast fillets following HPP treatments at 400 MPa at 12 °C for a 0 min and 20 min cycle, respectively *(59)*.

Other technologies, such as plant essential oils, are relatively new with respect to their application in pre- and post- harvest interventions and have been shown to minimize the prevalence of *Salmonella* on raw poultry a well as shelled eggs *(8)*. The use of plant essential oils (EO), which have been widely studied to reduce the presence of pathogens on meats and poultry, most commonly carcacol and thymol. A review by Bajpai et al. *(8)* reported the use of various EOs (thyme, oregano, sage, rosemary, cinnamon bark) minimized found *Salmonella* in various meat products, while, clove oil was found to reduce pathogens on shelled eggs when supplemented in feed formulations for live birds *(68)*. These studies also found using EO in post-harvest packaging materials resulted in a reduction of pathogens over time while maintaining meat quality *(8, 68)*.

Another commonly used practice is electron beam ionizing radiation, food irradiation, which reduces *Salmonella* and other pathogens by creating mutations through the generation of free radicals during processing, causing loss of normal cellular functions *(48)*. However, irradiation can result in the degradation of sensory attributes and loss of overall consumer acceptability, preventing producers from implementation within the industry as widely as other methods *(46, 48, 52)*. Other alternative interventions and some of their benefits include pulsed electric field processing (preserved sensory qualities and uses less energy, ozone processing (highly reactive and penetrable), ultra-violet light (non-

thermal, short time, and allows for continuous process), ultra-sound (preserves food quality and integrity), electrolyzed oxidized water (only uses water and salt with no residual disinfectant contamination), high pressure CO_2 processing (non-thermal, non-toxic, nonflammable, bath and continuous processes), and bacteriophage treatment (targets only pathogenic bacteria, and widespread availability) *(60)*.

Poultry Frames for Further Processing. Poultry frames consist of parts such as ribs, keel, sternum, necks, skin, and sometimes remnants of whole muscles and other gizzard parts. While poultry whole muscle is generally recognized as a lower fat protein option (3 g or less of fat in 100 g total), MSC on average is 14.4% fat according to research in 1979 *(27, 89)*. Juneja and Eblen found that *Salmonella* was more resistant to heat treatment when in higher fat levels of beef compared to beef with lower fat contents *(42)*. They observed an increase in fat created a decrease in water activity (a_w) that may have led to poor heating quality allowing for pathogens to survive longer in the medium *(1)*. Explanation of how frames and MSC is used, is discussed in a subsequent chapter. The importance of being able to reduce the initial pathogen load on poultry frames before they are stored in a more ideal environment for pathogen reduction, causes a need for higher cook temperatures or times for a further processor. The authors intend to validate the ability *Salmonella* can adhere to bones of poultry carcasses and recommend a treatment to reduce presence at *Salmonella* and overall APC.

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CHAPTER II

VALIDATION OF THERMAL LETHALITY AGAINST *SALMONELLA ENTERICA* IN POULTRY OFFAL DURING RENDERING*

Introduction

The rendering of byproducts following the harvest of various food-yielding animals, including poultry and livestock species, supplies the U.S. agricultural and process industries with raw materials for the manufacture of various products. These products include components for manufacture of foods for livestock and companion animals, as well as cooking oils and other industrial products (e.g., tallows, soaps). Rendering allows for a total use of the animal and prevents excessive dumping of animal carcass tissues in landfills. Nevertheless, rendered products may present risks of microbiological hazard being present, despite the intensity of processing. Thus there is a consequent need for further research into the development and validation of rendering procedures to inactivate and microbial pathogens *(9)*. Hofacre et al. *(7)* reported the presence of *Salmonella* serovars in bovine meat and bone meal and a 5% *Salmonella* prevalence in blended meals. Serovars recovered included Arkansas, Livingstone, Brandenburg, Tennessee, and Mbandaka, all bearing resistance to at least one therapeutic antimicrobial *(7)*. Similarly, Kinley et al. *(10)* reported the prevalence of *Enterococcus* spp. in various rendered meals at approximately 81%, while *Salmonella* was recovered from 8.7% of total sampled rendered material. *Salmonella* was recovered from 13.7% of poultry meal samples,

indicating the potential for a higher rate of cross-contamination of poultry-sourced rendered materials.

The presence of microbial pathogens in rendered animal products may result from the survival of pathogens during processing or from post-process cross-contamination *(4, 5, 9, 10)*. The rendering industry commonly employs a high-heat continuous-type process in which an indirect heat exchanger heats high moisture raw materials to temperatures between 250 and 280 °F for periods of between 20 and 90 min *(12)*. Following thermal processing, excess fat is separated by a mechanical press, producing both non-fat solids and extracted fat fractions that may be further processed into various products for animal consumption or use. The occurrence of multiple recalls of raw, dry and processed pet foods since August 2013 *(16)*, and the passage of the Food and Drug Administration Food Safety Modernization Act (FDA-FSMA), has resulted in renewed attention being given to the need for validated process control and food safety preventive controls (FSPCs) to prevent cross-contamination of final products *(15)*. Between 2013 and 2015, 30 recalls of differing pet foods due to concerns over pathogen cross-contamination occurred; while the source(s) of the contaminating pathogen was not identified in all of these recalls, product cross-contamination has been reported to contribute to the onset of human disease outbreaks following consumer exposure to pet foods *(3, 16)*.

The development of thermal process lethality parameters for the determination of minimum required cooking procedures is critical for the safe completion of animal rendering. The decimal reduction time (*D*-value) is the time at constant temperature required to inactivate 90% of a microbe's population (1.0 $\log_{10}$), whereas the Z-value relates the process equivalency – the change in temperature required to achieve a 10-fold

change in the microbe's *D*-value. Kinley et al. *(10)* reported *D*-values of various *Salmonella* serovars in buffered saline at 55, 60, and 65°C; for all serovars, *D*-values decreased as process temperature increased, while Z-values for salmonellae approximated 7°C. However, reported *D*-values in saline were of limited value in terms of predicting the resistance to heating of *Salmonella* when applied to rendering processes. Hays *(6)* reported the *D*-values of multiple *Salmonella* serovars in a blend of poultry crax (leftover protein, mineral, and residual fat content following cooking and fat separation) *(11)* and fat (50% fat final concentration) ranged between 0.67 and 0.70 min at 115.6 °C (240 °F). These findings indicate proper achievement of minimum rendering temperatures effectively inactivate even large populations of *Salmonella* when it enters a rendering process on surfaces of raw poultry offal.

Previous research has ignored the effects of process come-up time on the cumulative lethality of rendering processes to microbial pathogens such as *Salmonella*. Thus, research is warranted in order to properly define minimal processing conditions to achieve a cumulative process lethality of 7.0 $\log_{10}$-cycles in *Salmonella* during rendering. A lethality criterion of 7.0 $\log_{10}$-cycles reduction of *Salmonella* is targeted based on U.S. Department of Agriculture (USDA) performance standards for fully-cooked poultry products *(14)*. The primary objective of this research was to conduct thermal lethality experiments in poultry offal in order to determine the *D*-values of *Salmonella enterica* serovars linked to human or animal disease at differing processing temperatures. Consequently, gathered data were utilized to determine a process z-value useful for the prediction of necessary lethality to achieve the performance objective of a 7.0 $\log_{10}$-cycle *Salmonella* inactivation.

Materials and Methods

Acquisition of *Salmonella* serovars for use in experimental procedures. Isolates of *Salmonella enterica* serovars Enteritidis (recovered from a federally inspected commercial poultry slaughter facility) and Senftenberg 775W were obtained from the Texas A&M University Food Microbiology Laboratory (Department of Animal Science, Texas A&M University, College Station, TX) for use in *D*-value study completion. Wild-type (SG60; Fowl typhoid outbreak isolate) and virulence-attenuated isolates (SG70, SG 98) of *Salmonella* Gallinarum (causative of Fowl Typhoid) were donated by Kenneth Roland (The Biodesign Institute, Arizona State University, Tempe, AZ). *S.* Gallinarum mutant isolates were described to bear attenuated virulence/reduced ability to synthesize lipopolysaccharide components through deletion mutations. Virulence-attenuated *S.* Gallinarum isolates were obtained and compared to the wild type isolate in order to verify no differences in heat sensitivity, with the intent of using attenuated isolates during experimental trials. This was done for purposes of preventing unintentional exposure of research poultry flocks (Texas A&M University Department of Poultry Science) to the wild-type pathogen.

All *Salmonella* cultures were cryo-preserved at -80 °C in Cryo-Care vials (Key Scientific Products, Inc., Round Rock, TX) following overnight growth in tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD) at 35 °C. In addition, each culture was prepared on slants of tryptic soy agar (TSA; Becton, Dickinson and Co.) from overnight cultures of isolates in TSB. Inoculated TSA slants were incubated 24 h at 35 °C, removed

from incubation and aseptically layered with sterile mineral oil (to prevent oxidative damage), and placed under refrigeration (5 °C) until required for use. New slants were utilized for each experimental replication, and slants were not retained for greater two months. Cultures from slants were activated by aseptically collecting culture using sterile inoculating loops into TSB, followed by overnight (18 h) incubation at 35 °C.

In vitro thermal death time trials for *Salmonella* isolates. Thermal inactivation trials to determine the decimal reduction times (*D*-values) for each of the experimental *Salmonella* isolates (*S.* Gallinarum 60 [Wild type], 70, and 98, *S.* Enteritidis and *S.* Senftenberg) were completed in liquid phosphate buffered saline (PBS; Becton, Dickinson and Co) in micro-capillary vials (glass: 9 cm x 1 mm internal diameter), with three replicates being completed (N=3). Isolates were prepared by reviving in tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD) for 18 h at 35 °C, followed by a subsequent passage in TSB with 18 h incubation at 35 °C, placing isolates into stationary phase at point of use for inoculation. Isolates were then aseptically pipetted (50 µl) into sterile glass micro-capillary tubes that had been previously flame-sealed at one end. Following loading of cultures, tubes were carefully flame-sealed and cooled prior to submerging in a flowing water bath tempered to 60 °C. A submerged thermometer and a type-K thermocouple were used to monitor water temperature in the bath and an opened capillary, respectively. *Salmonella*-loaded micro-capillary tubes were removed at increasing time intervals from the water bath, immediately cooled in ice-cold water, sanitized with 70% ethyl alcohol, then rinsed twice in ice-cold sterile water to remove sanitizer, and placed in sterile plastic 50 ml conical vials. A flame-sterilized glass rod was used to pulverize micro-capillary vials to allow enumeration of surviving *Salmonella*.

Surviving *Salmonella* cells were spread on surfaces of TSA-containing Petri plates after being serially diluted in 0.1% peptone diluent (Becton, Dickinson and Co.); inoculated plates were incubated aerobically for 36-48 h at 35 °C. Following incubation, *Salmonella* colonies were counted and recorded; plate counts were calculated, and CFU/ml values were subjected to $\log_{10}$-transformation prior to data analysis.

Replicate-specific *D*-values were determined for each isolate by plotting the $\log_{10}$-transformed counts (y-axis) of surviving *Salmonella* isolates against the heating interval (x-axis) and utilizing the linear regression tool in Microsoft Excel™ (Redmond, WA). The *D*-value was taken as the negative inverse of the slope of the linear regression best-fit line from at least three connected points on the survivor curve demonstrating good linearity (R^2 >0.92). *D*-values were determined in this manner for each *Salmonella* isolate and averaged together to gain a mean *Salmonella* serovar-specific *D*-value. Additionally, the thermal death time required to achieve a 5.0 $\log_{10}$-cycle reduction ($F^5_{140°F}$) was determined from the survivor curve of each isolate in a manner similar to that of Jackson et al. *(8)*. This was done through visual inspection of survivor curves and use of drop lines to identify the time period required for the 5.0 $\log_{10}$ CFU/ml reduction. This was completed for all *Salmonella* isolates except for *S.* Senftenberg, due to lack of completion of a 5.0 $\log_{10}$-cycle reduction during the experimental time course. In the case of *S.* Senftenberg, for the purposes of statistical analysis and comparison of *Salmonella* F-values, the time to achieve a 5.0 $\log_{10}$-cycle reduction was determined by the equation $F_{X°F}=D_{X°F}(5.0)$.

Poultry byproduct source and preparation. Chicken offal, containing viscera, necks, feathers, paws, and heads, not previously subjected to moisture removal was obtained from a commercial poultry byproducts rendering facility in TX. Obtained product

was immediately returned within 2 hours under ambient temperature to the Department of Poultry Science Microbiology Laboratory on the Texas A&M University campus and stored under refrigeration (4-5 °C) until ready for use.

Procedures for *Salmonella* inoculation onto poultry offal. Following the completion of *in vitro Salmonella D*-value experiments, it was determined that *S.* Gallinarum SG60, SG70, and SG98 isolates demonstrated thermal resistances not differing from *S.* Enteritidis. In consultation with Department of Poultry Science and Texas A&M University Biosafey leadership, it was decided to discontinue the use of all *S.* Gallinarum isolates based upon: i) the lack of statistically significant differences in thermal resistance from human foodborne pathogenic salmonellae; ii) their numerically smaller *D*-values versus *S.* Enteritidis and Senftenberg isolates, and; iii) concerns over accidental cross-contamination of research and teaching poultry flocks despite the implementation of procedures to prevent such occurrences. Subsequent experiments utilized a *Salmonella* cocktail to simulate natural contamination of poultry crax via *Salmonella* serovars, for completion of poultry offal-inoculated experiments. *Salmonella* cocktails (10^8 CFU/ml) were prepared by first reviving *S.* Enteritidis (SE) and *S.* Senftenberg (SS) separately at 37 °C in 10 ml of TSB filled in 15 ml sterile conical tubes for 18-24 h. Following revival, the conical tubes were centrifuged to pelletize the bacterial cells (3,500 x *g*, 15 min, 23 °C). Following centrifugation, supernatants were gently poured off and pelleted bacterial cells were suspended in 10.0 ml sterile peptone buffer and centrifuged again in identical fashion as before. Upon completion of the second centrifugation step, supernatants were again poured off and pellets suspended in 5.0 ml phosphate buffered saline (PBS; Becton, Dickinson and Co.) to increase the number of cells inoculated and improve detection

opportunities during heating trials. From the 5.0 ml volumes, 1.0 ml aliquots of each *Salmonella* serovar/isolate were combined into a new sterile reaction tube and vortexed to form the working 2 serovar cocktail.

To confirm researchers' ability to consistently inoculate poultry offal samples to a desired *Salmonella* count, aliquots (~10.0 g each) of non-inoculated sample tissue were placed inside a bottom sealed steel piping canister (base width: 2.5 cm, top width: 4.5 cm, height: 13.8 cm, depth: 10 cm), base end welded closed, inoculated with 100 µl concentrated overnight culture (10 x 10 µl), mixed with a sterile metal stir-rod to homogenize the inoculum throughout the sample, and covered with aluminum foil. Samples were then aseptically extracted from canisters, serially diluted in buffered peptone water (BPW; Becton, Dickinson and Co.), and transferred to brilliant green sulfa agar (BGS; Becton, Dickinson and Co.). Following transfer, plates were aerobically incubated for 24-48 h at 36 °C prior to colony enumeration. Colonies counted were red to pink-white with red zones surrounded each colony. The recovery and enumeration of sublethally injured cells was attempted during initial trials by the use of a tryptic soy agar (TSA; Becton, Dickinson and Co.) thin layer agar overlay. Nonetheless, due to frequent, excessive over-growth of naturally occurring heat-resistant microbes on Petri dishes, researchers' ability to accurately enumerate surviving *Salmonella* (both non-injured and injured) was significantly hindered. It was thus decided to disallow background microbiota growth through elimination of overlaid TSA. Counts of *Salmonella* from these samples were used to establish the pre-heating *Salmonella* loads on inoculated poultry offal samples prior to initiation of thermal death time experiments.

Inactivation of *Salmonella* during sample heating come-up periods. An experiment was initiated to determine the lethality of poultry offal heating on inoculated *Salmonella*. Following inoculation, the same steel offal-containing canisters were loaded into an oil bath set to 160, 170, or 180 °F (71.1, 76.7, or 82.2 °C). Additionally, a non-inoculated sample canister was loaded into the heated oil bath with a type-J thermocouple inserted through the punctured aluminum foil, to monitor internal sample temperature. A timer was started immediately upon loading and stopped upon achieving internal temperatures of 160, 170, or 180 °F. Canisters were removed, submerged in ice-cold water for at least 1 min, and surviving *Salmonella* were enumerated by spread-plating on surfaces of BGS agar Petri plates following extraction of sample with a sterile spatula and preparation of decimal dilutions. Inoculated plates were incubated up to 48 h at 36 °C prior to colony counting.

Sample heating and temperature monitoring during processing. Inactivation of *Salmonella* serovars was determined in inoculated chicken offal samples; samples (~10.0 g) of non-inoculated chicken offal were placed in a sealed metal canister, top-covered with aluminum foil, and placed into an oil bath set to 150, 155, or 160 °F (65.5, 68.3, 71.1 °C), selected to allow for enumeration of surviving *Salmonella* colonies. A hole was poked through the aluminum to allow for escaping steam, mimicking the continuous cooking of the commercial process. During preliminary experiments, temperatures closer to the current industry critical control point (CCP) procedures (180 °F), minimum cook temperature for effective fat extraction during rendering (235 °F), and minimum cooking temperature for poultry rendering (265 °F) were shown to be extremely effective at

eliminating *Salmonella*, but disallowed survivor enumeration (Data not shown). As a result, the experimental temperatures were lowered.

Non-inoculated samples were allowed to sit in the oil bath until the coldest spot reached the desired temperature (typically the approximate geometric center, about 2.5 cm up from the base of the sample tube). At that time, each tube was inoculated with 100 µl of concentrated culture and then stirred with a sterile metal implement. For cooking to 150 °F (~65.5 °C), 7 min was required to achieve uniform temperature throughout samples, whereas for 160 °F (71.1 °C), an average of 10.17 min was required to achieve target temperature through the samples according to thermocouple data (Data not shown). A timer was started immediately upon sample inoculation and mixing completion and samples were removed at 0, 0.25, 0.5, 0.75, 1.0, 5.0, 10.0, and 15.0 min of heating for each of the three experimental temperatures. At each time point, samples were removed and placed immediately in an ice-cold water bath to chill samples and halt heating-induced *Salmonella* inactivation. Samples were held on ice for at least 1 min, and no longer than 5.0 min, prior to being removed for enumeration of surviving *Salmonella*. As described above, surviving *Salmonella* were enumerated on BGS. Inoculated Petri plates were incubated aerobically at 36 °C for up to 48 h prior to colony enumeration. Plate counts were then $\log_{10}$-tranformed for statistical analyses and calculation of *D* and z-values.

***Salmonella D*-value and process z-value determination.** *D*-values for *Salmonella* cocktails were determined for each process temperature (150, 155, 160 °F) for each of three distinct replicates in like fashion. Survivor curves were analyzed by linear regression and *D*-values at each temperature were determined from the negative inverse of the slope of the best-fit line. The process z-value was determined by first plotting the $\log_{10}$-

transformed *D*-values for the *Salmonella* cocktail calculated for each individual experimental replication (y-axis) against the processing temperature for which each *D*-value was determined (x-axis). The replicate-specific z-value was determined as the negative inverse of the slope of the best-fit line; this process was completed for each of three independently completed replications. Finally, values were averaged together to give the process Z-value.

Experimental design and statistical analysis. Thermal inactivation trials were designed and analyzed as factorials; all trials were completed in identical fashion three times (N=3). *D*-values were then averaged together and subjected to statistical analysis to determine significant differences in *Salmonella* heat resistance as a function of heating temperature. Data were analyzed by one-way analysis of variance (ANOVA) to determine significant differences between treatments (main effects: heating temperature, period, and their interaction) at p=0.05, and significantly differing means were separated by use of Tukey's Multiple Comparisons Test at $p<0.05$. Statistical analysis of data was completed using Prism v.6.0 (GraphPad Software, Inc., La Jolla, CA).

Results

D-values and thermal resistance of *Salmonella* serovars in vitro. Figure 2-1 depicts the survival of individual *Salmonella* serovars in liquid buffer heated at 140 °F (60 °C). All *S.* Gallinarum survivor curves demonstrated a rapid, initially steep death phase, followed by a shallow 'tailing' phase characterized by little additional inactivation. Similarly, *S.* Enteritidis numbers declined while heated, though the rate of reduction accelerated after 1.5 min exposure. Unlike the *S.* Gallinarum and *S.* Senftenberg, *S.*

Enteritidis demonstrated a shouldering-type inactivation curve, initially shallow, followed by a steeper rate of pathogen decline (Figure 2-1).

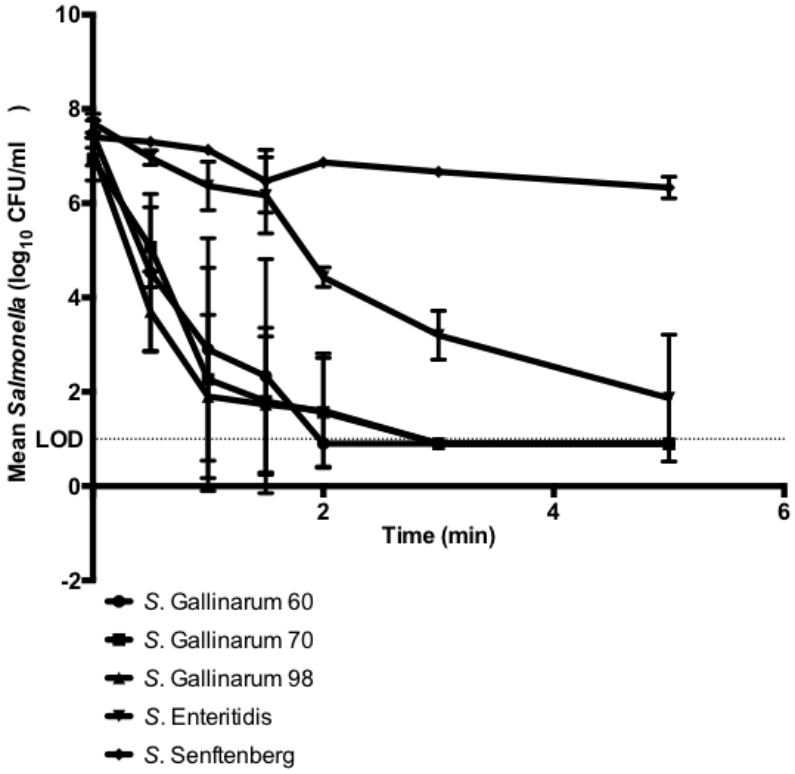

Figure 2-1. Reprinted: Survival of *Salmonella* serovars during heating in buffer at 140 °F (60 °C). Symbols depict mean Salmonella survivors as determined from enumeration on tryptic soy agar-containing Petri plates from triplicate identical replicates (N=3). Error bars indicate one sample standard deviation from mean. LOD: limit of detection (1.0 $\log_{10}$ CFU/ml).

D-values and $F^5_{140°F}$ values are depicted in Figures 2-2A and 2-2B, respectively. *Salmonella* Senftenberg exhibited the greatest resistance to heating, with a mean *D*-value of 3.48 $\pm$ 0.63 min. By comparison, *S.* Enteritidis and *S.* Gallinarum Wild-type (SG60) had *D*-values at 140 °F of 0.89 $\pm$ 0.48 and 0.41 $\pm$ 0.37 min, respectively. Statistical analysis indicated that while the mean *S.* Enteritidis *D*-value did not significantly differ from those of *S.* Gallinarum isolates, *S.* Senftenberg was statistically more heat resistant than other *Salmonella* organisms (Figure 2-2A). Similarly, analysis of $F^5_{140°F}$ values for *Salmonella*

isolates revealed no significant differences in the times required to achieve a 5.0 $\log_{10}$-cycle reduction at 140 °F between *S.* Gallinarum and *S.* Enteritidis. The *S.* Senftenberg $F^5_{140°F}$= 17.40 $\pm$ 3.14 min was statistically significantly higher than all other experimental salmonellae $F^5_{140°F}$ values (Figure 2-2B).

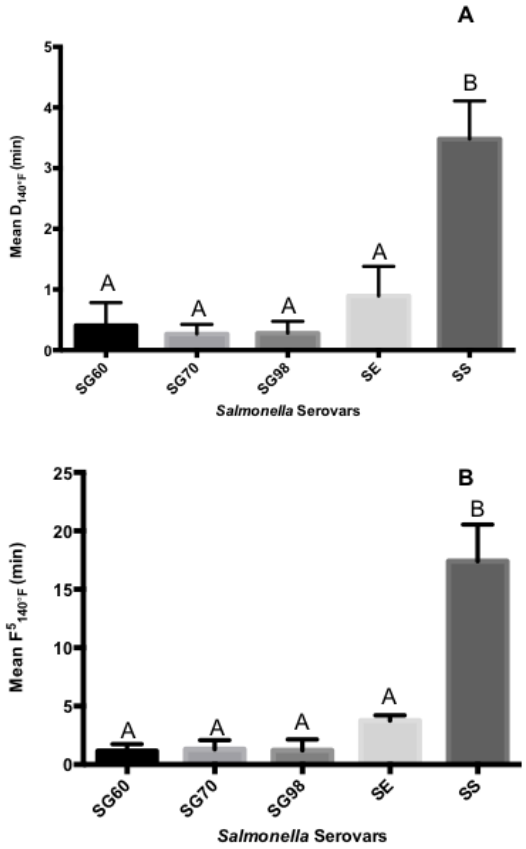

Figure 2-2. Reprinted: Mean D (A) and $F^5_{140°F}$ (B) values of Salmonella serovars determined from heating experiments at 140 °F. D-values were determined by regression of linear components of survivor curves as the negative inverse of the slope of the best fit line. $F^5_{140°F}$ values were determined as the total time to achieve a 5.0 $\log_{10}$ cycle inactivation. Bars depict means from triplicate identical replicates (N=3); error bars depict one sample standard deviation from the mean. SG60: S. Gallinarum 60; SG70: S. Gallinarum 70; SG98: S. Gallinarum 98; SE: S. Enteritidis; SS: S. Senftenberg. Bars not sharing letters (A, B) differ at $p<0.05$.

Inactivation of *Salmonella* during heating come-up periods. During experiments to determine the inactivation of *Salmonella* following inoculation and come-up to

processing temperatures (initially set at 160, 170, and 180 °F), we observed *Salmonella* inocula were inactivated to below the limit of detection for plating assays (LOD: 1.0 $\log_{10}$ CFU/g). This precluded the completion of an integrated analysis of thermal lethality for offal rendering. Across replications, a minimum reduction of at least 6.5 $\log_{10}$-cycles was achieved for the *Salmonella* inoculum during come-up to the targeted rendering temperatures (Data not shown). This indicated that at the outset of rendering, poultry offal-contaminating *Salmonella* were unable to adapt to increasing exposure to heat. Observing these results, we revised the inoculation protocol as described above to allow for *D*-value determination by adding inoculum only after the cooking temperature was achieved, not prior to heating.

Lethality of heating to *Salmonella* in offal when inoculated post-come-up. The survival of *Salmonella* inoculated into poultry offal following the heating come-up period is depicted in Table 2-1. For all three rendering temperatures, 0.25 min exposure to heat was able to produce statistically significant reductions in *Salmonella* numbers, while further numerical reductions were obtained at 0.5 min or longer. At 150 °F a slow decline in survivor counts was observed, with survivors being detected from up to 5 min of heating. Conversely, inactivation of *Salmonella* was much more severe and rapid at 155 and 160 °F. At 160 °F *Salmonella* survivors were unable to be enumerated after only 0.25 min of heat exposure, achieving 5.8 $\log_{10}$-cycles' reduction (a statistically significant reduction). A similar reduction in *Salmonella* numbers to below the detection limit was observed for 155 °F-treated samples at 1.0 min of heating, also a 5.8 $\log_{10}$ reduction. A 5.1 $\log_{10}$-cycle reduction to non-detectable levels was observed for 150 °F-heated *Salmonella* after 10 min of heating (Table 2-1).

Table 2-1. Reprinted: Least square means of *Salmonella* survivors (log_{10} CFU/g) in poultry crax as a function of the interaction of rendering temperature x period (*p*=0.006).

Rendering Period (min)	Rendering Temperature (°C)[a]		
	150	155	160
0.0	6.13^{AY}	6.78^{AY}	6.76^{AY}
0.25	3.49^{BY}	1.58^{BZ}	ND
0.50	3.20^{BY}	1.71^{BZ}	ND
0.75	2.16^{BY}	1.29^{BY}	ND
1.0	2.13^{BY}	ND[b]	ND
5.0	$1.92B^{Y}$	ND	ND
10.0	ND	ND	ND
15.0	ND	ND	ND

[a] Values depict least square means from three identically completed replicates (*N*=3). Values not sharing a letter (A,B,C) within a column (rendering temperature), or within a row (rendering period) not sharing a common letter (Y,Z), differ at *p*=0.05. Data were analyzed by two-way analysis of variance and means separated by Tukey's Honestly Significant Differences (HSD) test.

[b] Limit of detection of plating assay: 1.0 log_{10} CFU/g. ND: non-detectable.

D-values and z-values for *Salmonella* inactivation in poultry offal. Inactivation of *Salmonella* cocktails in poultry offal following a heating come-up period is presented as mean *D*-values at 150, 155, and 160 °F in Table 2-2. *D*-values for the *Salmonella* cocktail differed as a function of heating temperature ($p<0.05$), and ranged from 0.086 to 0.25 min. Following completion of *D*-value calculations from across replications, a z-value was calculated as the negative inverse of the slope of the best fit line connecting the *D*-values from the three process temperatures for each replicate. The mean z-value for the process was 21.948 $\pm$ 3.87 °F, indicating the temperature change in the process required to achieve

a 10-fold increase or decrease in the *D*-value for *Salmonella* destruction during poultry offal rendering.

Table 2-2. Reprinted: Mean *Salmonella* Senftenberg and Enteritidis cocktail *D*-values in poultry offal heated at 150, 155, or 160 °F.

	Heating Temperature (°F)		
	150	**155**	**160**
Mean ± SD[a]	0.254 ± 0.045[A]	0.172 ± 0.012[B]	0.086 ± 0.002[C]
Lower 95% CL[b]	0.141	0.143	0.076
Upper 95% CL[b]	0.367	0.201	0.096
SEM[c]	0.022		

[a] *D*-values were calculated as the negative inverse of the slope from the best-fit line equation from each replicate/temperature-specific survivor curve (*N*=3). Values presented are means from triplicate replications ± one sample standard deviation from the mean. Values not sharing capital letters (A, B, C) differ at $p<0.05$.
[b] Lower 95% CL: lower 95% confidence limit; Upper 95% CL; upper 95% confidence limit.
[c] SEM: standard error about mean.

Discussion

D-values of *Salmonella* isolates in the current study at 60 °C in buffer (140 °F) ranged from 0.27 to 3.5 min (*S.* Senftenberg) (Figure 2-2A). Blankenship *(2)* reported the $D_{138.2°F}$ of *S.* Typhimurium in buffer was 0.63 min, placing its heat resistance between that observed for *S.* Gallinarum and *S.* Enteritidis isolates used in the current study. As described, *S.* Senftenberg exhibited significantly higher heat resistance versus other *Salmonella* isolates, a phenomenon reported by other researchers *(1)*. Stopforth et al. *(13)*reported differences in *D*-values at 60 °C for multiple *Salmonella* serovars, ranging from 0.16 min to 0.39 min, heated in buffer in experiments similar to the current study.

Jackson et al. *(8)* utilized the $F^{5}_{55°C}$ to relate the inactivation of *E. coli* O157:H7 cells in hamburgers when thermal inactivation curves did not adhere strictly to a linear kinetic, displaying shouldering or tailing elements. In their study, stationary phase cells revived at 37 °C, inoculated into ground beef, and then cooked at 55 °C required 106.4 min to achieve a 5.0 $\log_{10}$-cycle reduction. In the current study, the $F^{5}_{140°F}$ values for *Salmonella* serovars ranged from 1.2 to 17.4 min in buffer (Figure 2-2B). These results are not necessarily unexpected, given the higher heating temperature used in the current study, inherent differences in heat resistance between *E. coli* O157:H7 and *Salmonella* serovars used, and the use of an aqueous buffer lacking protein and lipid which may have produced insulatory effects to heat transfer in beef hamburger.

The USDA Food Safety Inspection Service (USDA-FSIS) previously developed performance standards for the control of *Salmonella* in fully cooked poultry products, calling for a 7.0 $\log_{10}$-cycle reduction through processing *(14)*. In the current study, *D*-values of *Salmonella* cocktails in poultry offal at 150, 155, and 160 °F were 0.254, 0.172, and 0.086 min, respectively (Table 2-2). Application of *D*-values to achieve a predicted 7.0 $\log_{10}$-cycle inactivation at process temperatures utilized would produce minimum required holding times of 1.78, 1.21, and 0.602 min at 150, 155, and 160 °F, respectively, provided the death rate was constant and heat was evenly distributed throughout the entire processing system (i.e. no cold spots). From *in vitro* trials and survivor curves, it is likely that cold spots did exist in our sampling chamber. We sought to control for this by use of thermocouples in poultry offal cooking experiments inserted at multiple points in a sample canister and requiring all three thermocouples to read the minimum desired cook temperature before inoculating *Salmonella*. Such practices are recommended for

processors who have mapped the distribution of heat throughout a system and have knowledge of potential cold spot locations in order to achieve minimum necessary lethality for pathogen control.

In the current study, researchers attempted to quantify *Salmonella* resistance to heating of poultry offal in a manner that would account for the cumulative rendering process-achieved lethality. Nevertheless, researchers were unable to do so due to inactivation of *Salmonella* during come-up at 160, 170, and 180 °F to numbers below the limit of detection. For this reason, testing at these process temperatures was discontinued. Subsequent experiments followed the method of poultry offal (crax, poultry fat) sample inoculation utilized by Hays *(6)*. This method allowed for the thermal come-up of samples prior to inoculation, allowing for improved enumeration of surviving *Salmonella* for purposes of *D*-value and z-value calculation. Taken together, lethality achieved during the product come-up period in combination with the *D*-value obtained at 160 °F in poultry offal, and inactivation of *Salmonella* to non-detectable numbers within 0.25 min, indicates rendering of poultry offal to 160 °F should satisfy renderers' needs to produce safe poultry byproduct meal for further processing. This, in combination with application of other types of food safety preventive controls, is expected to assist the U.S. companion and agricultural animal food manufacturing industries with providing safe raw materials for animal food manufacture.

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CHAPTER III

IMPACT OF ROUTE OF INFECTION IN THE ONSET OF SYSTEMIC *SALMONELLA* INFECTION IN EXPERIMENTALLY CHALLENGED CHICKENS†

Introduction

The non-typhoidal salmonellae are known to maintain a reservoir in poultry, can persist in food production systems, and have been recovered from poultry carcasses and derived food products in the past. Hsi et al. *(15)* reported higher risk of human salmonellosis following poultry consumption for U.S. consumers versus other meat types based on serving size and frequency of consumption, following the evaluation of USDA Economic Research Service data. Painter et al. *(20)* ascribed a higher number of fatalities resulting from foodborne disease occurring between 1998 and 2008 following consumption of pathogen-contaminated poultry products versus other food types. A number of food products are produced with the inclusion of mechanically separated chicken (MSC), defined as any product resulting from mechanical separation of bone from chicken skeletal muscle and/or other tissue *(1)*. Food products may include MSC as a component in their formulation, and may undergo either partial or full cooking prior to consumption *(14, 21)*. *Salmonella*-contaminated MSC consumption has been twice linked to human foodborne disease outbreaks in the U.S., likely the result of under-cooking of foods incorporating MSC or cross-contamination of fully cooked products with products not fully cooked *(16, 22)*.

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Not unlike the red meat animal, chickens may contract symptomatic or asymptomatic infections through differing routes of exposure to an infectious agent, including but not limited to: pecking on skin, consumption of contaminated feed, water, or litter, scratching, biting insects, toe scratches by other chickens or through inhalation *(12, 13, 18)*. Respiration in chickens is accomplished via a network of air sacs in addition to lungs, and in some cases air sacs are located within the marrow of the bird's pneumatic bones *(2, 10, 26)*. Consequently, endo-tracheal challenge with *Salmonella* has been reported to produce a greater likelihood of systemic *Salmonella* infection versus other challenge methods such as oral gavage *(18)*, and may result in *Salmonella* accessing the marrow of chicken bones. *Salmonella* contact with chicken bones, and thus marrow, via inhalation or other exposure routes may facilitate its eventual transmission to MSC or fresh cut parts obtained during further processing. This presents a challenge to the U.S. poultry industry's ability to produce *Salmonella*-free cut poultry and MSC for human consumption, potentially reducing adherence to federally mandated *Salmonella* performance standards for MSC. Currently, the U.S. Department of Agriculture Food Safety and Inspection Service *(29)* has set a performance standard of no more than 13 *Salmonella*-positive samples out of 52 samples (325 g each) collected within a year's time (using a rolling window method for performance standard adherence determination).

Reports of *Salmonella* carriage in chicken bones and/or marrow is sparing. Wu et al. *(31)* reported a prevalence of only 0.7% in chicken bone marrow from naturally infected commercially raised chickens, though a prevalence of 9.3% in turkey bone marrow from naturally infected birds was later reported *(8)*. The primary objective of the current study was to ascertain whether chicken bone marrow was susceptible to *Salmonella* infection as

a function of challenge site and method, and the number of days lapsed between challenge and *Salmonella* status assessment. A secondary objective of this study was to determine whether differing pathogen challenge methods resulted in detectable, countable *Salmonella* in the cecum as a model internal gastric organ serving as a source of *Salmonella* potentially contaminating carcasses during harvest and further processing. Should *Salmonella* access bone marrow or adjacent air sacs, this presents an obstacle to production of *Salmonella*-free cut poultry or MSC in compliance with USDA-FSIS performance standards for *Salmonella*.

Materials and Methods

Bacterial culture preparation for experimentation. Commercial chicken production facility-recovered isolates of *Salmonella enterica* belonging to serovars Typhimurium and Heidelberg (one isolate per serovar), naturally resistant to novobiocin (25.0 µg/mL) and nalidixic acid (20.0 µg/mL), were revived from -80°C storage from the culture collection of the Avian Microbial Physiology Laboratory at the USDA Agricultural Research Service (ARS) Southern Plains Agricultural Research Center (SPARC) (College Station, TX). Before culture thawing could complete, sterile loops were used to scrape frozen culture into 10.0 mL volumes of tryptic soy broth (TSB; Difco, Detroit, MI) supplemented with 10.0 µL (25.0 µg/mL) novobiocin (Sigma-Aldrich Co., St. Louis, MO) and 10.0 µL (20.0 µg/mL) nalidixic acid (Sigma-Aldrich Co.). Inoculated TSB tubes were then incubated for 8.0 h at 37 °C without shaking. Following incubation, 100 µL of each culture was aseptically passed into 10.0 mL sterile TSB containing antibiotics and cultures again incubated for 8.0 h at 37 °C without shaking.

Following completion of strain activation, *Salmonella* isolates were transferred to 100.0 mL volumes of sterilized TSB (not containing antibiotics) and incubated under conditions identical to those used for culture revival. After incubation, 25.0 mL volumes of each culture fluid were prepared in conical vials (four vials per isolate) and centrifuged at 5,000 x *g* at ambient temperature for 10.0 min. Resulting supernatants were poured off and culture pellets were suspended in 10.0 mL phosphate buffered saline (PBS; Thermo-Fisher Scientific, Waltham, MA) to wash cultures of excess microbial fermentate. The process of centrifugation, supernatant removal, and bacterial pellet suspension was completed twice identically. The final resulting bacterial pellet in each culture vial was suspended in 4.0 mL PBS to increase the cell count per mL of fluid; suspended pellets were mixed vigorously by vortexing. A cocktail of the two isolates was then prepared by mixing equivalent volumes of each suspended pellet in a new, sterile screw-cap conical vial and vortexing. Prepared cocktails of *Salmonella* cultures were expected to produce an approximate count of 1 x 10^9 cfu/mL *Salmonella* isolate; counts of *Salmonella* in the cocktail preparation were determined by preparation of decimal dilutions in PBS and spreading on tryptic soy agar (TSA; Difco), followed by incubation of Petri plates at 37 °C for 24 h prior to colony enumeration. Isolate-containing vials were then decimally diluted in PBS to a target of 10^7-10^8 cfu/mL for use in poultry bird challenge experiments.

Experimental design for poultry animal challenge with S*almonella* inoculum. During experiment planning, the number of chickens (*Gallus domesticus*) required to achieve at least 90% statistical power was calculated at $\alpha = 0.01$ with a SD = 0.5, and df = 5, by JMP v9.0 (SAS Institute, Inc., Cary, NC) *(19)*. A minimum of 24 birds per treatment group (4 birds each per treatment sampled at 6, 9, or 12 days post-challenge) were required

to fulfill desired power; an additional animal was then added into each treatment group to account for the possibility of animal mortality occurring during the experimental trial (n=25). A resulting N=75 animals per trial were tested over duplicate identically completed trials (total N=150). All animal production, handling, and euthanasia procedures were conducted according to the USDA-ARS SPARC Institutional Animal Care and Use Committee (IACUC) protocol 2016022 and were completed at the USDA-ARS SPARC. Animal feed/diets met or exceeded chicken nutritional requirements; feed and drinking water was made available *ad libitum (7)*.

Straight-run chicks were collected (equal numbers male and female) from a commercial hatchery located within Texas on day of hatch and transported to the USDA-ARS SPARC. During transit, animals were kept at 75-78 °F (23.9-25.6 °C) with ventilation to minimize animal stress; transportation conditions conformed to all institutional policies and federal regulations. Before departing the hatchery, all chicks were vaccinated against coccidiosis but not *Salmonella*. Paper towels used in chick trays at the hatchery were also collected and were tested for the presence of naturally occurring *Salmonella* bearing similar antibiotic resistance capacities, in order to exclude chicks from further use as necessary *(25)*. Upon arrival at the USDA-ARS SPARC, chicks were randomly assigned to into one of six pens, each designating a differing *Salmonella* challenge method; pens were separated from one another to prevent cross-over of *Salmonella*.

***Salmonella* challenge of birds.** At 30 days of age, all birds were administered a cocktail of *S.* Heidelberg and Typhimurium isolates, prepared to a concentration of 10^8 cfu/mL using the following methods of challenge: oral gavage, endo-tracheal gavage, or

one of four variations of transdermal scratch method modified from the method of Edrington et al. *(9)*. In order to minimize stress of birds during challenge, the environment was kept quiet and overhead lights turned down. After dose administration, birds remained hand-restrained for 5-10 s to confirm full dose delivery and assess for stress incurred. Observed labored breathing, or other visible signs of stress, were recorded; birds showing visible signs of stress were held for an additional 5 min to allow birds to calm. No birds continued showing signs of distress after this time period resulting in no removal of any animals from the experiment.

Oral gavage has been used previously in research investigating the utility of various antimicrobial interventions in poultry production, as it mimics the ingestion of a bacterial pathogen from feed, litter, water, or oral contact with a pathogen by pecking of chickens *(11, 23)*. A 10 mL syringe with a 20 gauge needle was used to deliver 1.0 mL inoculum to administer *Salmonella* to the esophagus. Endo-tracheal gavage was completed according to the method of Alworth and Kelly *(2)*, using a sterilized 0.5 mL repeater pipet used to dose 0.25 mL *Salmonella* cocktail. No birds subjected to endo-tracheal gavage were observed to have respiratory distress following challenge. Transdermal challenge of chickens was completed using a patented disposable 10 lancet (1.2 mm stainless steel) allergy skin test kit (HolliStier Allergy, Spokane, WA). Birds were held with wings to the side to ensure their comfort and safety. The lancet device was dipped into the broth containing the *Salmonella* cocktail and applied with light pressure onto the animal's skin, administering 20.0 µL total inoculum. Chickens were monitored following transdermal challenge for onset of external infections; none were observed to occur throughout experimental trials.

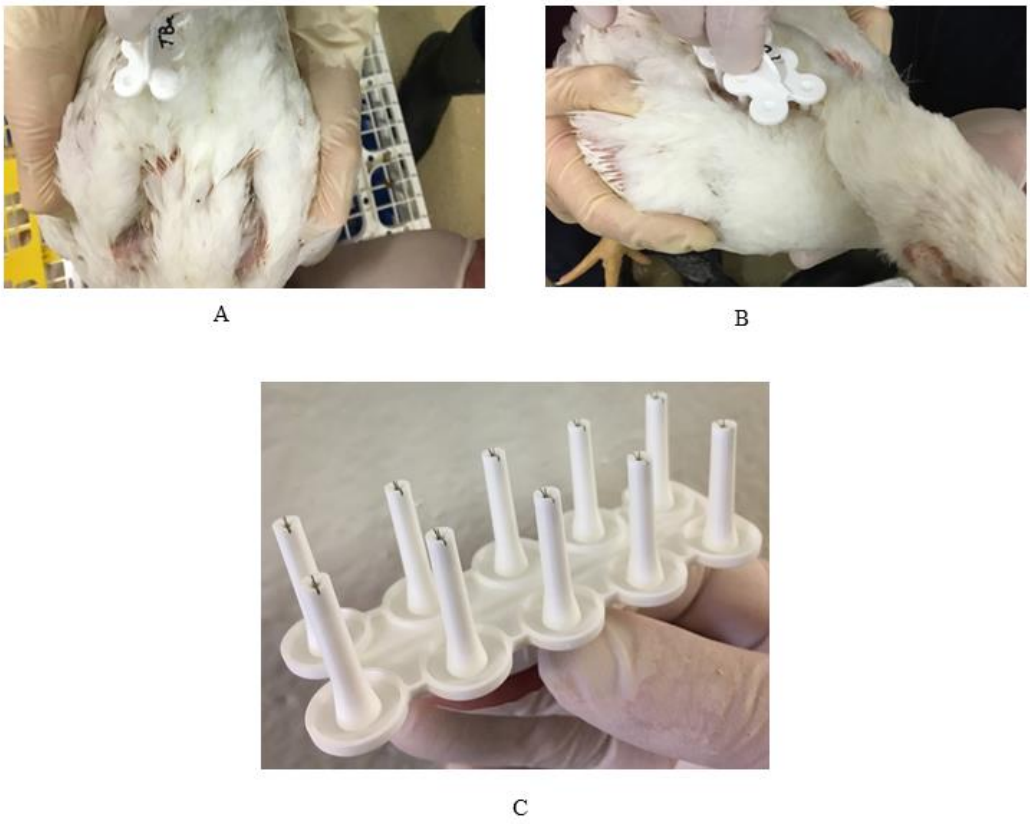

Figure 3-1. Back of experimental chicken positioned for transdermal *Salmonella* scratch challenge with feathers (A), without feathers (B), and 10-lancet device used for transdermal *Salmonella* challenge (C).

Two anatomical transdermal locations selected for *Salmonella* challenge were selected: the pectoralis major and latissimus dorsi muscles. Breast muscles were scratched to imitate an open wound arising from direct contact via pecking. Latissimus dorsi was selected to mimic similar potential for *Salmonella* infection occurring via scratching by birds, though back muscle was presumed to be less susceptible to infection after inoculation as the back does not directly contact litter, feed, and since chickens cannot reach their backs with their beaks. Two physiological conditions were also selected in combination with selected anatomical locations: animal back with or without feathers (apteric regions between feathers/plumage-bearing regions) (Figure 3-1).

Animal euthanasia, necropsy, and sample recovery. On each day of testing for all treatments, five randomly chosen birds were gathered and euthanized with gaseous CO_2 followed by cervical dislocation in accordance with practices recommended by the USDA Animal and Plant Health Inspection Service (APHIS) and the American Veterinary Medical Association (AVMA), and were then transported into the USDA-ARS SPARC Biosafety level (BSL) 2 laboratory for tissue recovery and immediate initiation of microbiological analysis of sampled tissues *(4, 27)*. Bird carcasses were dipped into 2.0% chlorhexidine (Henry Schein Animal Health, Memphis, TN; prepared for use according to manufacturer's instructions) to reduce dander and lower cross-contamination during necropsy. Tissues were harvested using sanitized implements dipped in 2.0% chlorhexidine followed by flame sterilization and were inspected for abnormalities following harvest. Ceca were removed to verify intake of *Salmonella* inoculum; liver/spleen samples were collected to verify systemic infection post-challenge. Both humerus bones were taken; each end was aseptically removed by flame-sterilized poultry shears. The left humerus was then aseptically broken to open and expose marrow for further testing, while the right humerus was kept to test for *Salmonella* from whole bone.

Microbiological analysis of sampled tissues. Gastrointestinal tract counts of *Salmonella* were determined by cecal counts. Cecum samples (0.25 g each) were diluted in 2.25 mL PBS (pH 7.0; MP Biomedicals, LLC, Santa Ana, CA), homogenized by stomaching (230 rpm) for 1.0 min, and then decimal dilutions were prepared in PBS. Dilutions were spread on surfaces of xylose lysine tergitol 4 (XLT4) agar (Becton, Dickinson and Co., Franklin Lakes, NJ) supplemented with 25.0 and 20.0 µg/mL novobiocin and nalidixic acid, respectively. Following preparation of Petri plates, 0.25 mL

of diluted ceca sample was inoculated into Rappaport Vassiliadis (RV) broth (Becton, Dickinson and Co.) and incubated for 24 h at 42 °C to selectively enrich for *Salmonella*. Bone and bone marrow were individually placed into filter-containing stomacher pouches and diluted with 10.0 mL buffered peptone water (BPW; Becton, Dickinson and Co.), manually crushed with a hammer, and then placed into a stomacher for 60 s to further pulverize tissue or bone. Bone and marrow samples were decimally diluted in PBS and dilutions spread on surfaces of XLT4 agar supplemented with novobiocin and nalidixic acid (at identical concentrations as that listed above). All XLT4 plates were incubated for 24-48 h at 37 °C before inspecting for *Salmonella*-typical colonies. Following preparation of plates, remaining bone or marrow sample fluid was incubated for 24 h at 37 °C to enrich remaining sample fluid for *Salmonella*. Following non-selective enrichment in BPW, 0.25 mL of BPW fluid was aseptically transferred into 10 ml RV broth for *Salmonella* selective enrichment (24 h at 42 °C). Samples of liver and spleen were combined together into 50 mL conical vials containing 10 mL RV broth and subjected to selective enrichment (24 h at 42 °C), in order to detect onset of systemic infection following *Salmonella* challenge. Following selective enrichment, all samples were streaked onto surfaces of XLT4 agar supplemented with antibiotics and plates incubated for 24-48 h at 37 °C before inspecting for *Salmonella*-typical colonies. Samples producing *Salmonella*-typical colonies were recorded. For every sample producing *Salmonella* colonies, all *Salmonella*-typical colonies from the XLT4 plate was streaked onto new XLT4 plates containing antibiotics (25.0 and 20.0 µg/mL novobiocin and nalidixic acid, respectively) and thereafter incubated for 24-48 h at 37 °C. Isolated colonies from Petri plates were then lifted and subjected to PCR to confirm identity as *Salmonella.*

Confirmation of *Salmonella* by PCR. Confirmation of presumptive positive *Salmonella* isolates was carried out by PCR, using the DiversiLab *Salmonella* kit and LabChip® (bioMérieux N.A., Durham, NC). DNA extraction was completed using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to manufacturer-provided instructions. For each DNA sample, 23.0 µL of the master mix was created using 18 µL re-PCR MM1, 2.5 µL GeneAmp 10x PCR buffer, 2.0 µL Primer mix, and 0.50 AmpliTaq DNA polymerase, all supplied by the kit. The master mix was aliquoted into reaction tubes containing 2.5 µL of DNA. Thermal cycling totaled 35 cycles and included an initial denaturation at 94 °C for 120 s followed by another denaturation period at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 70 °C for 90 s, and a final extension at 70 °C for 180 s. Strain typing was done using microfluidics chips in the Agilent Bioanalyzer (Agilent technologies, Inc., Santa Clara, CA). DNA fingerprints were then compared using the DiversiLab Software. Analysis was performed with the generated DNA fingerprints from *Salmonella* samples and compared to the DiversiLab Software database. The results were given in form of a dendrogram and Top Match, a DiversiLab calculation matching serotypes with a typical gel-electrophoresis computer produced image. Isolates were considered matches when identified to be >79.9% similar to the library serotype.

Statistical analysis of data. Frequency of *Salmonella* recovery by necropsied tissue sites/locations were analyzed by ANOVA, testing the effect of days post-challenge, method of challenge, and the interaction of these effects. Data were blocked by trial; means differing at P=0.05 were separated by Student's t-test. All data were analyzed by JMP Pro v.13.0 (SAS Institute, Inc., Cary, NC) at α=0.05. Frequencies (%) of *Salmonella* recovery

by challenge method were also similarly analyzed by ANOVA, testing the main effects of days post-challenge and carcass sampling site, and their interaction on pathogen recovery; data were blocked by trial.

Counts of *Salmonella* on XLT4 plates supplemented with antibiotics were $\log_{10}$-transformed prior to data analysis. For samples not yielding detectable *Salmonella* colonies by plating, below the limit of detection (LOD) of 100 cfu/g, the value 2.0 $\log_{10}$ cfu/g was assigned solely to facilitate statistical analysis of data, testing for *Salmonella* recovery by challenge method. Counts of *Salmonella* ($\log_{10}$ cfu/g) from chicken ceca following challenge and sampling of euthanized birds were tested for differences in cecal *Salmonella* counts by ANOVA, testing the effect of days post-challenge, challenge method, and the interaction of these effects (α=0.05). Means differing at P=0.05 were separated by Student's t-test via JMP Pro v.13.0.

Results

Frequency of *Salmonella* recovery differed between sampled chicken tissues/organs by challenge method and days post-challenge. Figure 3-2 depicts the differing frequencies of *Salmonella* recovered from challenged chickens following microbiological analysis of bone, marrow, ceca, and liver/spleen samples as a function of challenge method and number of days post-challenge. Analysis of data indicated no differences in mean frequencies of *Salmonella* by the interaction of main effects (days post-challenge x challenge method) and no differences amongst means for the interaction of main effects for all of the sampled chicken carcass sites ($P \geq 0.05$) except for the bone marrow. Oral and endo-tracheal gavage methods tended to produce the highest frequencies of *Salmonella* uptake into birds as compared to transdermal methods, particularly as

observed in *Salmonella* recovered from organs (ceca, liver/spleen) (Figure 3-2). Transdermal challenge produced *Salmonella* uptake into chickens at rates not differing statistically by anatomical (back, breast) or physiological conditions (with, without feathers), although varying frequencies of *Salmonella* recovery were observed to occur both by days post-challenge and by transdermal method for all carcass sampling sites except for marrow (Figure 3-2).

In contrast to the recoveries of *Salmonella* from ceca, whole bone, and liver/spleen samples collected from *Salmonella*-challenged poultry birds, the frequency of *Salmonella* detection from bone marrow differed by the interaction of number of days post-challenge when sampling was completed and the method of challenge (P=0.005) (Table 1). Whereas oral and endo-tracheal gavage yielded recoveries of *Salmonella* at 6 days post-challenge (10 and 20%, respectively), subsequent samplings at 9 and 12 days post-challenge yielded no detection of *Salmonella* in marrow samples, suggesting any infections had been cleared by the bird's immune system. Given the location of air sacs surrounding some of the animal's bones, translocation into bone marrow from respiratory challenge (endo-tracheal gavage) was expected to occur. By comparison, liver/spleen samples demonstrated longer-lasting systemic infection following *Salmonella* challenge for all methods of challenge except for transdermal breast without feathers (Figure 3-2).

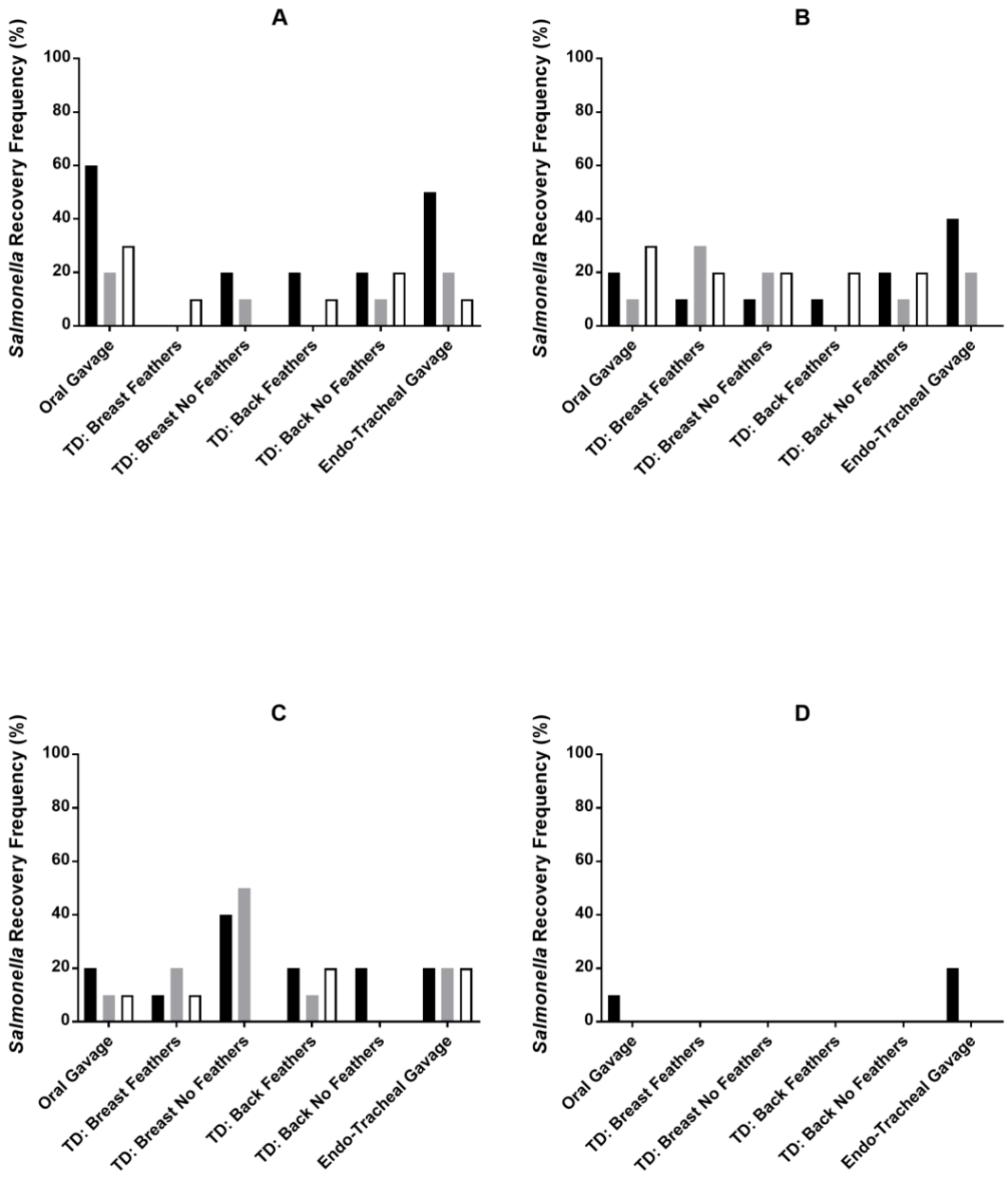

Figure 3-2. Least squares means (%) of *Salmonella* recovery frequencies for chickens (N=150) sampled at the ceca (A), liver/spleen (B), whole bone (humerus) (C), and bone marrow for 6 (black-filled bars), 9 (grey-filled bars) and 12 days post-challenge (open bars). *Salmonella*-typical colonies were subjected to PCR confirmation following selective enrichment and plating.

Table 3-1. Least squares means of *Salmonella* recovery frequencies (%) from chicken humerus bone marrow by the interaction of challenge method by number of days post-challenge (*P*=0.005).

	Days Post-*Salmonella* Challenge		
Challenge Method[1]	**6**	**9**	**12**
Oral Gavage	10.0[a2]	0.0[c]	0.0[c]
Endo-Tracheal Gavage	20.0[b]	0.0[c]	0.0[c]
TD: Back with Feathers	0.0[c]	0.0[c]	0.0[c]
TD: Breast with Feathers	0.0[c]	0.0[c]	0.0[c]
TD: Back no Feathers	0.0[c]	0.0[c]	0.0[c]
TD: Breast no Feathers	0.0[c]	0.0[c]	0.0[c]
Pooled SE = 2.36			

[1] Birds were challenged with cocktail of *Salmonella* Heidelberg and Typhimurium. TD: trans-dermal challenge.
[2] Numbers depict means collected from birds per challenge method, with duplicate identical trials completed (*N*=150). Means not sharing a letter ([a, b, c]) differ (P<0.05) by ANOVA and Student's t-test.

***Salmonella* populations from ceca samples differed by challenge method and the number of days post-challenge.** Mean counts of *Salmonella* recovered from chicken cecal samples post-challenge differed from one another by challenge method (P=0.032) but not by number of days post-challenge before euthanasia and sampling, nor by the interaction of these effects (Table 2). Ingestion of *Salmonella* by oral gavage resulted in the highest mean number of recovered *Salmonella* from ceca (3.0 $\log_{10}$ cfu/g cecal sample) as compared to endo-tracheal gavage and transdermal methods. Surprisingly, trans-dermal

challenge on both back and breast without feathers yielded higher numbers of *Salmonella* recovered from cecal samples versus both trans-dermal challenges at the same locations with feathers and the endo-tracheal gavage challenge. While the presence of feathers would be expected to reduce the efficiency of *Salmonella* trans-dermal delivery, the lower mean count of *Salmonella* recovered from endo-tracheal challenge versus some trans-dermal methods was surprising given the differences in volumes used in these challenge methods (20.0 µL versus 250.0 µL). Nevertheless, statistical differences were not determined to exist except for oral gavage-challenged versus trans-dermal challenge on back and breast with feathers.

Table 3-2. Least squares means (log_{10} CFU/g) of *Salmonella* counts from chicken ceca samples by challenge method (*P*=0.032).

Challenge Method[1]	***Salmonella* Cecal Count**
Oral Gavage	3.04 ± 1.21^{a}[2]
Endo-Tracheal Gavage	2.62 ± 0.71^{ab}
TD: Back with Feathers	2.42 ± 0.68^{b}
TD: Breast with Feathers	2.35 ± 0.68^{b}
TD: Back no Feathers	2.78 ± 1.06^{ab}
TD: Breast no Feathers	2.78 ± 0.97^{ab}
Pooled SE = 0.16	

[1] Birds were challenged with cocktail of *Salmonella* Heidelberg and Typhimurium. TD: trans-dermal challenge. Mean *Salmonella* in inoculum = 8.4±0.1 log_{10} cfu/ml.
[2] Numbers depict means from *n*=24 birds ± one sample SD. Values not sharing a letter (^{a,b}) differ by ANOVA and Student's t-test at P=0.05.

Discussion

The current study investigated the potential for a cocktail of two *Salmonella enterica* isolates to gain entry into differing organs, bone, and bone marrow of chickens following challenge with *Salmonella* by differing challenge methods. Data obtained from experimental trials indicate that *Salmonella* may gain access to multiple organs (ceca, liver, and spleen) following ingestion or inhalation, but even more concerning, that transdermal challenge facilitated internal translocation of *Salmonella* inoculum to organs as well as to bone. While frequencies of *Salmonella* positive samples were not determined to differ statistically for liver/spleen, ceca, and bone-derived samples collected 6, 9, and 12 days post-challenge as a function of challenge method, *Salmonella* detection frequencies varied, ranging from 10% *Salmonella* for some tissue samples, to 50% *Salmonella* detection from liver/spleen samples challenged by endo-tracheal gavage 6 days post-challenge (Figure 3-2). These data demonstrate that normal activities engaged by chickens during their life cycle present a risk of *Salmonella* acquisition into internal organs and tissues (including bone marrow), including consumption of pathogen-contaminated feed or water, pecking and fighting with other birds.

Interestingly, only oral or endo-tracheal gavage produced detectable *Salmonella* in bone marrow of challenged birds, with endo-tracheal gavage resulting in twice the *Salmonella* detection frequency as that of oral gavage (Table 3-1). Detection of *Salmonella* in bone marrow was potentially the result of translocation through adjacent air sacs surrounding pneumatic bones in the chicken (Figure 3-2), and occurred within a week post-challenge, not unlike previous research indicating detection of orally dosed *S.*

Pullorum from chickens 7 days post-challenge *(30)*. In the current study, *Salmonella* was nonetheless cleared rapidly from marrow at 9- and 12-days post-challenge.

In contrast to the current study, however, other researchers have demonstrated longer-lasting infections following *Salmonella* challenge, in some cases requiring multiple weeks for infections to clear. Harbaugh et al. *(12)*infected turkeys with *S.* Typhimurium by 2 or 4 h exposure to airborne *Salmonella*-inoculated feces (10^5 and 10^9 CFU/g feces). Following 2 h exposure to 10^5 CFU/g *S.* Typhimurium-containing feces dust, at least one experimental bird tested positive for *S.* Typhimurium in nasal passages, ceca, liver, and the small intestine *(6)*. Likewise, others reported successful infection of young chicken ceca and livers following tracheal gavage with a cocktail of *Salmonella* Typhimurium and Enteritidis, noting *Salmonella*-positive ceca samples occurred more frequently following endo-tracheal challenged as compared with oral gavage challenge *(17)*. These authors also reported in another set of studies that intra-tracheal gavage was the only experimental challenge method that facilitated cecal colonization of *S.* Senftenberg versus oral gavage or even intravenous challenge *(18)*.

As compared to gavage methods, transdermal challenge applied to skin with or without feathers on the breast and back muscles were capable of producing systemic infections in organs within experimental birds. Previous research has indicated reliable outcomes in using trans-dermal type challenge methods for producing infections or testing the utility of vaccines in poultry animals *(1, 24)*. The lower dose application (20.0 µL) versus larger inoculum volumes may also improve researcher safety by lowering the volume of infectious agent inoculum that must be prepared or handled during challenge, as

well as by lowering risk of challenged birds expectorating challenged agent following oral or endo-tracheal application.

Numbers of *Salmonella* resulting in ceca samples differed by challenge method, but not the numbers of days lapsing from challenge to sampling (P=0.032) (Table 2). Mean numbers of *Salmonella* enumerated from cecal samples did not differ by the volume of inoculum applied during a specific challenge procedure, except that oral gavage yielded the highest count of *Salmonella* from cecal samples and also used the highest inoculum volume (1.0 mL). Nevertheless, differences in numbers of *Salmonella* counted from trans-dermal challenged birds did not differ from those from orally gavaged birds except for birds challenged on back and breast bearing feathers. Trans-dermal challenge resulted in systemic infections in various organs and tissues (with the exception of bone marrow).

The presence of pneumatic bones, with air sacs surrounding these bones to assist in respiration, the presence of marrow in bones, the potential for ground bone and marrow to be incorporated into MSC, and the potential for bone-contaminating *Salmonella* to be contacted during fresh cut poultry fabrication, demonstrate MSC bears a risk of *Salmonella* transmission to consumers of foods containing MSC or fabricated fresh poultry *(1, 16)*. Others have reported that in beef and dairy cattle, the inclusion of peripheral lymph nodes (**PLNs**) in ground beef produces a risk of *Salmonella* transmission to consumers *(3, 5)*. In the current study, *Salmonella* was not detected from chicken bone marrow after selective enrichment from chickens challenged via trans-dermal scratch on breast or back muscles (Figure 3-2). Surprisingly, in intact humeral bone samples, trans-dermal scratch challenge did yield detectable *Salmonella* at 6, 9, and 12 days post-challenge, indicating *Salmonella* is able to access bones following challenge but may not penetrate intact bone to access

marrow, or its survival in the marrow is short-lived (Figure 3-2). That endo-tracheal gavage was able to yield detectable *Salmonella* in marrow indicates *Salmonella* may access marrow via networked air sacs following aerosol inhalation.

The consumption of *Salmonella*-contaminated MSC-containing foods, or fresh intact cut poultry, particularly in instances where the food product is mishandled or undercooked, represents a food safety hazard for poultry products consumers *(16, 21)*. During the normal life cycle of broiler chickens, multiple routes of exposure to non-typhoidal salmonellae, including contaminated feed, water, litter, inhalation of pathogen-contaminated aerosols, as well as epidermal wounding by fighting or toe scratches, are known to exist and present risk of pathogen acquisition. Production and handling of fabricated intact and non-intact poultry products, including MSC, must be carried out in sanitary fashion *(28)*, using thermal or non-thermal food processing systems validated to effectively reduce *Salmonella* and protect consumer safety.

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CHAPTER IV

CONCLUSION: CHICKEN FRAME SURFACE SANITIZATION BY APPLICATION OF ADVANCED OXIDATIVE PROCESSING COMBINING HYDROGEN PEROXIDE AND ULTRAVIOLET LIGHT

Introduction

Since 2011 there have been ten *Salmonella* outbreaks announced by the USDA involving raw poultry products *(16, 24)*. In these cases, *Salmonella* was transmitted through food products including retail chicken, whole birds, ground poultry, and mechanically separated chicken (MSC) *(24)*. In 2013, after the development of a risk assessment motivated by multiple *Salmonella* outbreaks related to use of MSC, the United State Department of Agriculture Food Safety Inspection Services (USDA-FSIS) conducted a 6-month sampling period targeting *Salmonella* prevalence in Not-Ready-to-Eat (NRTE) comminuted poultry products, which included ground chicken and MSC *(20)*. The USDA-FSIS reported 82.9% of 697 samples of MSC were positive for *Salmonella*, double that of *Salmonella*-positive ground chicken samples (42.3% of 691) *(21)*. In February 2016, new performance standards for *Salmonella* in NRTE comminuted chicken were released and included comminuted chicken with an allowable 25.0% positive (13 of 52 samples), comminuted turkey with 13.5% positives (7 of 52 samples), and chicken parts with 15.4% positive (8 of 52 samples) *(22)*. USDA-FSIS has not included MSC in its mandatory sampling of comminuted chicken, but MSC is included in on-going exploratory testing *(22)*.

In 1995, the USDA allowed the use of MSC in hot dogs, luncheon meat, and other further processed products, without restrictions other than the labeling requirements of "mechanically separated chicken or poultry" *(19)*. MSC is produced by separating edible tissue from bones, cartilage and connective tissue from chicken frames, or backs, using a mechanical deboner. The equipment uses small holes, such as a sieve or similar device, and pressure to separate bone from the edible tissue, thus creating a paste or batter-like poultry meat product *(19)*. Chicken frames are the result of a fully deboned or cut-up carcass from processing. These frames may not receive antimicrobial treatment before being processed, yielding the potential to cause cross-contamination if the bird had a systemic bacterial infection.

In order to meet performance standards, carcass and parts are either dipped or sprayed with sanitizer such as peracetic acid (PAA) containing hydrogen peroxide (H_2O_2). Hydrogen peroxide was previously researched as a bactericide in poultry chill water and was found to reduce aerobic microorganisms by 94% at a concentration at 11,000 ppm *(13)*. However, Lillard and Thompson *(13)* reported catalase from blood in the water interacted with H_2O_2 (9,200-12,00 ppm), resulting in bloated and bleached carcasses, leading to less desirable carcasses and parts for commercial sales at this high concentration. Regardless, H_2O_2 continues to be studied for applications in conjunction with other known sanitizing methods such as ultraviolet light (UV). Currently, H_2O_2/UV, an advanced oxidation process (AOP), has been researched on shell eggs to reduce *Salmonella* and other bacteria which might otherwise contribute to spoilage *(26)*.

The term "Advanced Oxidation Process" was first introduced by Glaze et al. *(8)* in 1987 when studying the observed effects of combining ozone (O_2) and/or H_2O_2 with UV

light to describe the processes in which the hydroxyl radical, an oxidizing agent, is generated. Hydrogen peroxide produces a hemolytic cleavage while the subsequent exposure to the UV-C light produces two hydroxyl radicals per photon absorbed *(7)*. This photolytic reaction produces a two-electron reduction state which gives H_2O_2 the ability to diffuse across microbial cell membranes *(9)*. This reaction occurs more quickly with the supplement of UV light, at wavelengths 200-300 nm, which decomposes the structure of H_2O_2 into hydroxyl radicals, initiating a photochemical reaction with the microorganism's nucleic acids *(3, 4, 10, 14)*. This diffusion of H_2O_2 causes irreparable damage to nucleic acids, proteins, and lipids, effectively disabling the ability of a microorganism to survive and/or reproduce *(12)*. The UV radiation damages the DNA of the microorganism which inhibits the microorganism's ability to replicate *(5)*.

In 2010, Wells et al. *(26)* found applying H_2O_2 and UV independent of each other on eggs resulted in a reduced aerobic bacterial count up to 2.0 $\log_{10}$ CFU/egg. However, when the two applications were combined with a lower concentration of 1.5% H_2O_2 followed by a UV light (254 nm) treatment of 8 min, APC were reduced by more than 3.0 $\log_{10}$ CFU/egg. Al-Ajeeli et al. *(2)* and Gottselig et al. *(9)* showed this procedure was capable of significantly reducing *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (ST) on eggs with the use of 3.0 and 3.5% H_2O_2 in conjunction with a shortened UV light exposure time. Gottselig et al. *(9)* determined there was not a difference in the reduction of ST achieved by UV light exposure of 60 sec with the use of 3.0% H_2O_2 as compared to 5.0 sec UV application 3.0% H_2O_2. In addition, the authors observed a reduction of 5.3 $\log_{10}$ CFU/egg with the twice repeated application of H_2O_2 plus 5 s UV-C. This process was subsequently studied and observed using 3.5% H_2O_2 by Al-

Ajeeli et al. *(2)*. The authors reduced SE and APC counts on shell eggs to below the limit of detection (1.3 $\log_{10}$ CFU/egg) *(2)*.

The use of a typical household concentration of H_2O_2 (3.0%) and a 5 s UV-C exposure period was effective at reducing APC and *Salmonella* spp. loads on eggshells. Examining the use of this sanitation technique on chicken frames before entering the MSC process may help reduce the contamination of *Salmonella* present in the end raw product. The overall objectives of this study were to: 1) determine the effectiveness of H_2O_2 + UV light application to reduce APC and *Salmonella* spp. on chicken frames, and 2) determine the minimum concentration of H_2O_2 needed to maximize the reduction of *Salmonella* and APC on chicken frames.

Materials and Methods

Bacterial culture preparation and maintenance of cell suspensions. Isolates of *Salmonella enterica* serovar Enteritidis (SE, recovered from a commercial poultry slaughter facility) and *Salmonella enterica* serovar Typhimurium American Type Culture Collection 13311 (ST, ATCC, Manassas, Va.) were obtained from refrigerated storage (4 °C) in the Food Microbiology Laboratory culture collection (Department of Animal Science, Texas A&M University, College Station, TX). Tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD)-containing test tubes were inoculated separately with each *Salmonella* isolate and incubated aerobically without agitation at 35 °C for 24 h to revive cultures. Overnight cultures were loop-inoculated into sterilized TSB in 15 mL sealable conical centrifuge tubes (VWR International, West Chester, PA), and then incubated aerobically without agitation at 35 °C for 24 h to complete revival. Conical tubes were centrifuged at 2,500 x *g* in a biosafety level (BL)-2 compliant centrifuge for 10 min at

ambient temperature. The supernatant was gently poured off and the pellet was resuspended into 10 ml phosphate buffered saline (PBS; Milipore-Sigma Corp., St. Louis, MO), sealed and then vortexed to suspend the pellet. Centrifugation and wash procedures were repeated twice in the same manner to wash cultures of excess microbial fermentate. Final pellets were resuspended in 9 mL of PBS and vortexed to ensure pellet was completely suspended in diluent. Both strains were aseptically combined into a 50 mL conical vial and vortexed to homogeneously mix to make an inoculum cocktail.

Chicken frame sample collection. Chicken frames containing sternum, meat tissue, ribs, and neck were obtained from a commercial chicken processing facility and refrigerated (4-5 °C) until ready for use at the Department of Poultry Science Microbiology Laboratory at the Poultry Science Research Center on the Texas A&M University campus. Frames received directly from the supplier were tested for initial levels of *Salmonella* spp. and were below the level of detection (10 CFU/frame).

Non-inoculated frames were rinsed using a modified combined USDA-FSIS young chicken carcass rinse method and raw chicken parts sampling program *(20, 23)*. Frames were first individually weighed and the appropriate amount (4 lb/400 mL) of chilled BPW was placed into a poultry carcasses rinse bag *(20)*. Frames were rinsed and rinsate samples collected in accordance to the Raw Chicken Parts Sampling protocols for USDA-FSIS *(23, 25)*. Samples were refrigerated (1.6 °C) until serially diluted in PBS and plated on APC Petrifilm® or Xylose Lysine Tergitol-4 (XLT4; Becton, Dickinson and Co.).

Procedures for aerobic plate count and Salmonella inoculation on chicken frames. Flame-sterilized poultry shears were used to butterfly each frame at the sternum/keel bone. One non-inoculated control frame was immediately placed into a

labeled poultry carcass rinse bag (38 cm by 51 cm polyethylene, poultry rinse bag, 12 L capacity, VWR Int., Radnor, PA) to determine the initial aerobic plate count (APC) or naturally present *Salmonella*.

For *Salmonella* trials, the remaining frames were individually placed into poultry rinse bags and inoculated with 0.1 mL of the *Salmonella* cocktail (10^8 CFU/frame) containing SE and ST to achieve 10^5 CFU/ml per sample frame. The bag was twisted to seal and hand massaged for 30 s; the time was determined by preliminary data which showed a massage longer than 30 s resulted in breaking of small bones and loss of meat integrity; data not shown. After massaging, each frame was removed from its bag and placed onto sanitized grills and allowed to rest for at least 15 min to allow for bacterial attachment. After the rest period, three frames were separately placed into poultry rinse bags to enumerate the counts of *Salmonella* cocktail inoculum on the frames.

Description of frames treatment apparatus. The equipment used for chicken frame treatment was a $H_2O_2$and UV light- advanced oxidative processes (H_2O_2/UV-AOP) device (Fig 4-1) described by Al-Ajeeli et al. *(2)*. The segments of the prototype used to apply the treatments were enclosed and contained two spray chambers and two UV light chambers. After each spray chamber was a UV light chamber containing 8 UV-C lamps (254 nm; 8 to 12 mW/cm^2 , Sankyo Denki G30T8-Germicidal, Japan) with 4 lamps above the conveyer and 4 underneath the conveyer. Each frame spent approximately 5 s in each UV light chamber. The time for each frame to traverse the entire system and receive two applications of H_2O_2+UV or reverse osmosis water (RO) and UV light (H_2O+UV) was 40 s.

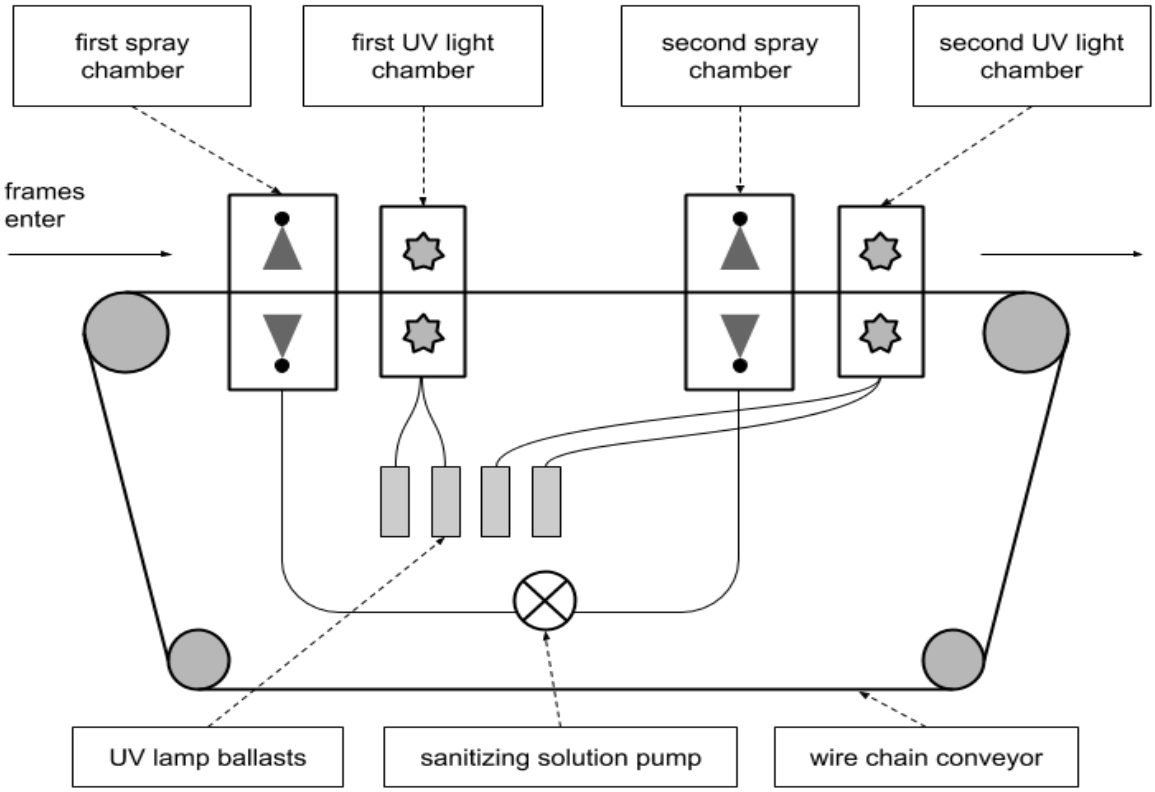

Figure 4-1. Schematic of chicken frame sanitation device allowing for application of hydrogen peroxide application followed by ultraviolet light exposure via operator control.

Sanitizing treatment application. The two H_2O_2 solutions were prepared by diluting a 35% concentrated stock (Brainerd Chemical Company, Inc., Tulsa, OK) with reverse osmosis (RO) water. In sets of three, butterflied frames were placed slightly apart, sternum side down, onto the conveyor of the H_2O_2/UV-AOP. Ultraviolet lamps were turned on and the spray pump was primed with appropriate solution *(2, 9)*. Following application of a designated treatment, frames were individually aseptically removed from the conveyer onto a sterile surface. Each frame was rinsed with 10 mL of sterile RO water using a hand-held water spray bottle to reduce the tissue damage of the frames by rinsing

off the visual bubbling caused by the H_2O_2 reaction with organic material. Each frame was retrieved aseptically with poultry carcass rinse bags to begin frame rinse procedure. Once all samples were completed for a given treatment, another treatment was performed. The H_2O_2/UV-AOP system was rinsed with RO water and sanitized with a 3% bleach solution (Commercial Concentrated Bleach, The Clorox Company, Oakland, CA) to prevent cross contamination between treatments. Treatments were run in order as listed in Table 4-1.

Experimental design. Preliminary experimentation with concentrations of 3.0% and 3.5% H_2O_2 concentrations previously researched on eggs were insufficient to achieve *Salmonella* reductions compared to positive controls (6.7 $\log_{10}$/frame) on chicken frames; data not shown *(2)*. Subsequent experiments focused on higher H_2O_2 concentrations of 5.0% and 7.0% which was hypothesized to result in significant bacterial reductions of ST and SE on chicken frames. As the frames exited the apparatus, bubbling was observed on the surface of the frames, indicating the breakdown of H_2O_2 into O_2+H_2O_2 was continuing. Therefore, a manual H_2O rinse was added to assist in the termination of the reaction and reduce the damage to the tissue of the frames by rinsing off residual H_2O_2 *(18)*.

Table 4-1. Experimental design groups for *Salmonella*-inoculated or non-inoculated frames.

Treatment	**Device Used**	**H_2O_2 % Conc.**	**Final Manual H_2O Rinse**	**UV**	**Total frames per group**
[1]Control	-	0.0	-	-	9
[2]Manual H_2O Rinse	-	0.0	+	-	9
[3]H_2O + UV	+	0.0	+	+	9
[4]5.0% H_2O_2 + UV	+	5.0	+	+	9
[5]7.0% H_2O_2 + UV	+	7.0	+	+	9
Total					**45**

[1]Control: no treatment; [2]Manual H_2O Rinse: no treatment, final hand H_2O spray; [3]H_2O + UV: device used with H_2O, UV light, and final hand H_2O spray, no H_2O_2; [4]5.0% H_2O_2 + UV: device used with 5.0% H_2O_2, UV light, and final hand H_2O spray; [5]7.0% H_2O_2 + UV: device used with 7.0% H_2O_2, UV light, and final hand H_2O spray

Enumeration of surviving microbes following treatment. Serial dilutions from the rinsate of each treatment were performed in PBS as necessary for the enumeration of *Salmonella* survivors. APC were enumerated using 3M® Aerobic Count Plate (ACP) Petrifilm® and *Salmonella* were enumerated using XLT4 agar plates. All plates were incubated at 37 °C and colonies counted after 48 h (APC) or 36 h (XLT4) *(1)*. Countable colony forming units for *Salmonella* on XLT4 differed depending on the strain present. *Salmonella* Typhimurium showed as smooth, round, clear colonies with black centers. *Salmonella* Enteritidis showed as smooth, round, red or yellow colonies with black centers.

Statistical analysis of data. Three trials were completed in identical fashion with 3 replicates for each treatment per trial (n=9). Values were statistically analyzed to determine significant differences in *Salmonella* or APC reduction as a function of the given treatment. All data from frames APC and *Salmonella* enumeration were separately analyzed by treatment using a two-way analysis of variance (ANOVA) using JMP v14.0 software (SAS Institute Inc., Cary, NC). Means were then separated using Student's t-test ($\alpha = 0.05$). Any significant interactions between treatments were reanalyzed as a one-way ANOVA using the Statistical Analysis System (SAS, version 9.4, SAS Institute Inc.)

Results and Discussion

Reduction of aerobic bacteria by AOP application. A significant difference ($P<0.05$) between the control and 7.0% H_2O_2 treatment with an APC reduction of 1.6 $\log_{10}$ CFU/frame was observed (Table 4-3). Even though the manual H_2O rinse, H_2O + UV, and 5.0% H_2O_2 + UV treatments did result in a reduction of APC by 0.1, 0.5, and 0.8 $\log_{10}$

CFU/frame, respectively, the reductions were not different from the control or the 7.0% H_2O_2 + UV treatment.

Moore et al. *(15)* inoculated chicken frames with *S.* Heidelberg (SH) and dipped them into various USDA-approved antimicrobials prior to blending the frames to obtain a material similar to MSC. No differences were found in the reduction of aerobic bacteria with the use of 0.1% PAA, 0.6% etylpyridinium chloride(1-hexadecylpyridinium chloride, CPC), 0.3% propionic acid, or 1.5% lactic acid compared to the control. However, a study by Chen et al. *(6)* with chicken parts (breast and thigh with skin) dipped into various antimicrobials, rinsed, and then ground, showed the use of PAA (0.07%, 0.10%) significantly lowered ($P \leq 0.05$) the aerobic bacteria counts when compared to 0.35% CPC, 0.60% CPC, and 0.003% chlorine. Chen et al. *(6)* concluded the use of PAA on parts before grinding would increase shelf life when compared to other treatments analyzed during 10 days of storage. The current study demonstrated the ability to reduce aerobic bacteria on frames before continuing onto further processing. This reduction of APC on frames prior to further processing could improve shelf-life of MSC.

Table 4-2. Least square means of aerobic bacteria (APC) and *Salmonella* counts of recovered bacteria from chicken frames.

Treatment[1]	Mean $\log_{10}$ CFU/frame	
	APC	***Salmonella***
Control	$3.6^{a} \pm 0.1$	$5.6^{a} \pm 0.2$
Manual H_2O Rinse	$3.5^{ab} \pm 1.0$	$5.2^{b} \pm 0.3$
H_2O + UV	$3.2^{ab} \pm 1.2$	$5.0^{c} \pm 0.2$
5.0% H_2O_2 + UV	$2.8^{ab} \pm 1.0$	$4.5^{d} \pm 0.4$
7.0% H_2O_2 + UV	$2.1^{b} \pm 0.8$	$4.6^{d} \pm 0.3$

[1]Each treatment (n=9); denotes use of H_2O_2-AOP prototype machine; values not sharing lowercase letters (a, b, c, d) differ at $p<0.05$.

***Salmonella* reduction by H_2O_2 + UV combined application.** Decreased prevalence of *Salmonella* on chicken frames that are used for MSC may reduce the risk of human foodborne salmonellosis. All treatments performed were able to reduce ($P<0.05$) inoculated *Salmonella* compared to the control (Table 4-2). The manual H_2O rinse showed a significant ($P<0.05$) decrease of 0.4 $\log_{10}$ CFU/frame from the control which indicated the manual H_2O rinse mechanically removed *Salmonella* (Table 4-2). All treatments using the H_2O_2/UV-AOP prototype showed a significant ($P<0.001$) decrease compared to the control (Table 4-2). The H_2O_2/UV-AOP *Salmonella* reductions from the control were 0.6 $\log_{10}$ CFU/frame (H_2O+UV), 1.1 $\log_{10}$ CFU/frame (5.0% H_2O_2+UV), and 1.0 $\log_{10}$ CFU/frame (7.0% H_2O_2+UV). The use of 5.0% and 7.0% H_2O_2+UV produced significant ($P<0.001$) reductions of 0.7 and 0.6 $\log_{10}$ CFU/frame, respectively, from the manual H_2O rinse. The 5.0% and 7.0% H_2O_2 +UV treatments produced significant ($P<0.05$) reductions of 0.5 $\log_{10}$ CFU/frame and 0.4 $\log_{10}$ CFU/frame, respectively, from the use of RO H_2O+UV in the sprayers. The further reduction of *Salmonella* spp. with the application of either concentration of H_2O_2 indicates the utilization of a full coverage H_2O spray + UV is effective at reducing *Salmonella* compared to the control. The use of the sanitizer in combination with UV light increases the reduction of *Salmonella* on the frames. When comparing the two concentrations of H_2O_2 (5.0% and 7.0%) there was no significant difference in observed pathogen reductions (P=0.30), indicating the higher concentration of H_2O_2 did not produce an additional effect over 5.0% H_2O_2 (Table 4-2).

Overall, the mechanical action of the sprayers was able to remove *Salmonella* with RO water alone indicating the ability to remove *Salmonella* cells that had loosely attached onto the meat, bone, cartilage, or skin of the frames. The addition of H_2O_2 and UV light damaged the DNA and other microbial components (membrane, lipid, and proteins) of the *Salmonella,* inhibiting its ability to sustain homeostasis and capability to remain attached to the frame. Further research is needed to determine the role of UV light without the addition of H_2O_2on chicken frames. Previous research on shell eggs with UV light reduced bacterial counts by 2.07 $\log_{10}$ CFU/egg *(26)*.

Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)*Moore et al. *(15)* showed 0.1% PAA and 0.1% lauric arginate ester (LAE) each significantly reduced inoculated SH on chicken frames after grinding by 0.9 $\log_{10}$ CFU/g while 0.1% PAA produced an additional reduction of 0.5 log CFU/g after the product was refrigerated for 24 h. They also found 0.005% chlorine (sodium hypochlorite) to be ineffective. Chen et al. *(6)* found significant reductions of ST with the use of 0.35 and 0.6% CPC (0.8 log CFU/g), 0.07% PAA (1.5 log CFU/g), and 0.1% PAA (1.3 log CFU/g) on ground chicken parts. These studies further indicate the ability for a pre-processed antimicrobial intervention will further reduce the incidence rate of *Salmonella* before entering into a further processed step.

USDA-FSIS currently tests for prevalence of *Salmonella* in comminuted chicken products with a maximum acceptable detection frequency of 25.0%. However, there are no regulations for MSC until further testing has been completed by USDA-FSIS *(22)*. *Salmonella,* which can be present in the bone, may cause cross-contamination on the final

product containing MSC*(11)*. The current study was able to reduce aerobic bacteria counts with H_2O_2+UV by up to 1.5 $\log_{10}$ CFU/frame. Initial contamination of carcasses and cross-contamination concerns are addressed by adding antimicrobials throughout the processes, decreasing the microbial counts on a raw product which increases the product's overall shelf life *(17)*. The findings from this study also indicate that 5.0% and 7.0% H_2O_2 + UV with the use of the H_2O_2/UV-AOP prototype machine is effective at reducing *S.* Enteritidis and *S.* Typhimurium on chicken frames. Five and 7.0% H_2O_2 produced similar numerical reductions of *Salmonella* on chicken frames. In addition, H_2O + UV light with the use of the H_2O_2/UV-AOP device reduced *Salmonella* on inoculated frames through mechanical rinsing, although not to the same extent as 5.0% and 7.0% H_2O_2 + UV. Thus, application of this antimicrobial process using the apparatus can reduce *Salmonella* contamination on chicken frames. Further research is needed to validate the efficacy of these findings in a commercial setting as well as the impact on moisture retention and color in further used products. In addition, performing a risk assessment to determine if the reduction of *Salmonella* on frames pre-grinding reduces disease risk would be beneficial.

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