Efficacy of bacteriophage treatment in reduction of *Salmonella* populations on poultry parts

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Masters of Science

Auburn, Alabama
December 13, 2014

Keywords: *Salmonella*, bacteriophage, antimicrobials

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Abstract

In 2011, the Centers for Disease Control and Prevention (CDC) released updated statistics detailing foodborne illness occurrence and attribution in the United States. From this compilation of data, the CDC concluded that approximately 1 out of 6 Americans has succumbed to illness which has been the direct result of consumption of various foodborne pathogens. These numbers translate into approximately 48 million people per year putting tremendous burden on this country's health care system. Consequently, non-typhoidal *Salmonella* was responsible for 35% of the hospitalizations due to foodborne illness (Scallan et. al., 2011). Due to the increased prevalence of *Salmonella* in poultry parts as determined by the base-line parts study conducted by USDA-FSIS, many processors are looking at new multi-hurdle approaches at controlling *Salmonella* prevalence on poultry products.

Bacteriophages are an older technology which has recently made waves in pathogen control due to the presence of many antibiotic-resistant strains of various foodborne pathogens. Recently, USDA-FSIS has given the use of bacteriophages in poultry processing GRAS status (USDA-FSIS, 2014). A new product produced by Intralytix called SalmoFresh™ has been designed to target the top *Salmonella* serotypes found on poultry products.

The first objective of this project was to determine the ideal concentration of SalmoFresh™ in order to give the optimal reduction of *Salmonella*. Reduction in

Salmonella populations was measured alongside 100ppm and 700ppm peracetic acid (PAA). Results suggested that that 9.602 log PFU/sample phage concentration would be optimal to use in a plant setting. The second objective of the project was then to combine the optimized SalmoFresh™ concentration with a 8) post-chill intervention. This was designed in order to give the best reduction in *Salmonella* cell counts in a plant setting. From this, the results suggested that 90ppm PAA in combination with a 9.602 log PFU/sample phage spray treatment gave the most significant reduction in *Salmonella* populations after a 24 hour period. In conclusion, experimental evidence suggests that using bacteriophage treatment in combination with currently used chemical interventions can provide optimal control of *Salmonella* on poultry parts. These results opens a new door as to adding a new multi-hurdle approach to foodborne illness control in the plant in various different locations.

Acknowledgements

I would like to thank several people, without whom, this project would not have been possible. I would first like to thank Dr. Shelly McKee whose guidance helped to shape myself into the best student possible. Secondly, I would like to thank my committee members Drs. Jean Weese and Tung-Shi Huang for their support and guidance throughout this process.

Some of the most important thanks I would like to extend to my friends and family. Without the combined efforts of Gretchen Nagel, Laura Bauermeister, Lei Zhang, and Xi Chen, this project would have never been a success. I would also like to thank my parents, Keith and Connie Newton, for their love and support of all of my endeavors. Most importantly, I must extend a special thank you to my wonderful husband, Caleb, and beautiful daughter, Ada, for their unending love, support, and motivation they provided me on a daily basis

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CHAPTER I

GENERAL INTRODUCTION

Currently, foodborne illnesses putting a huge economic strain on the United States economy. It is currently estimated that there are over 48 million cases each year which result in over 128,000 hospitalizations and 3,000 deaths (CDC, 2011). This equates to $51 billion per year in health-related costs (Scharff, 2011). Therefore, approximately 1 in 6 Americans 10) contracting a foodborne illness at some point in their life. However, *Salmonella* spp. are responsible for over 1 million of the foodborne illnesses reported in the United States per year (CDC, 2011). Moreover, *Salmonella* spp. incidences in poultry have been ranked as the number one pathogen/food combination risk (Batz et al, 2011).

Numerous outbreaks of *Salmonella* associated with poultry products have had a negative on the poultry industry. In 2010, there was a recall of approximately 500 million eggs in the United States due to *Salmonella*. In 2014 there was a recall of 33,840 pounds of mechanically deboned poultry meat (MDM) by Tyson which was due to the presence of *Salmonella* Heidelberg. This particular strain has been determined to originate in a Tennessee plant, but the same strain has since been found in 12 states in various poultry facilities. Recalls and outbreaks of *Salmonella* in poultry products have been growing in incidence over the years, which makes control of *Salmonella* a top priority for poultry processors.

In order to have more control over *Salmonella* prevalence in poultry products, processors have developed new and novel technologies coupled with the use of various antimicrobials in order to adhere to USDA-FSIS's new standards. Two of the most

commonly used antimicrobials that are utilized in US poultry plants are chlorine and peracetic acid (PAA). New technologies entering the market over the past 5 years include the Finishing Chiller® and COPE Unit, both manufactured by Morris & Associates. The Finishing Chiller® and COPE Unit are currently being used as post-chill antimicrobial interventions for whole carcasses and the subsequent poultry parts. Even with the use of these new technologies and antimicrobials, *Salmonella* still poses a threat to poultry processors country wide due to the increase in foodborne illness incidence rate in the United States. Therefore, in order to assure the safety of the US poultry supply and to meet new and anticipated *Salmonella* regulations, poultry processors are looking at alternatives or additional methods they can employ to reduce bacterial populations in the plant.

Currently, regulations established by USDA-FSIS have set the prevalence of *Salmonella* on whole carcasses to not exceed 7.5% or no more than 5 positives out of 51 samples. In 2013, USDA-FSIS conducted a study to determine levels of *Salmonella* on poultry parts and have published their current findings. Many in industry believe that this is a precursor to potential new regulations on poultry parts. Since the majority of the poultry that is consumed in the United States is in the form of poultry parts or further processed products, this is the next sector of the poultry market that needs to be addressed in regards to pathogen control (National Chicken Council, 2011). Poultry parts create a certain problem in the poultry industry due to the fact that cross-contamination is more likely to occur during processing.

Use of bacteriophages provide a new alternative for *Salmonella* control in the poultry industry. Several studies have validated the efficacy of bacteriophage treatment on poultry products and have demonstrated up to a 4-log reduction of *Salmonella* spp. on inoculated broilers (Spricigo et al., 2013; Wong et al., 2014). Therefore, the current research was conducted in order to validate the efficacy of a new bacteriophage treatment, SalmoFresh™, in reduction of the top foodborne illness Salmonella serotypes that are most commonly found on poultry parts. Results of the study indicate that the use of SalmoFresh™ in conjunction with low levels of antimicrobials, provide a significant reduction in *Salmonella* populations. When used with currently approved antimicrobials in a plant setting, SalmoFresh™ was able to deliver a 1.5 log reduction in *Salmonella* spp. with a sustained reduction of 1.5 log at 24 hours. This proven efficacy may provide beneficial pathogen reduction with anticipated standards to be released on poultry parts.

CHAPTER II

LITERATURE REVIEW

Food Borne Illness Statistics. In 2011, the Center for Disease Control (CDC) released statistics detailing foodborne illness occurrence and attribution in the United States. From this compilation of data, it was concluded that approximately 1 out of 6 Americans has succumbed to illness which has been the direct result of consumption of various foodborne pathogens. These numbers translate into approximately 48 million people per year resulting in tremendous burden in terms of overall economic losses in the United States. Out of the 48 million people, approximately 128,000 become hospitalized which has resulted in approximately 3,000 deaths (CDC, 2011). The newest set of foodborne illness attribution data reinforces how paramount control of foodborne pathogens in the poultry industry is. This is due to the fact that previous research showed that estimates of foodborne illness cases were much lower. (Scharff, 2012).

From similar data gathered by the CDC in 2009, it was determined that the same pathogens were responsible for 228,744 hospitalizations per year. Out of the total hospitalizations, 64% were caused bacteria, 27% by viruses, and only 9% from parasites. The pathogens that were responsible for the majority of human illnesses and

hospitalizations were nontyphoidal *Salmonella*, T. gondii, and *Campylobacter* spp. (Scallan, 2011).

A follow-up study was also conducted by the CDC in 2012, indicating that the incidence of foodborne illness caused by nontyphoidal *Salmonella* incidence in the United States has risen by 3% from 2011 (CDC, 2012). Approximately 1 million of all foodborne illness cases per year in the United States can be attributed to various subtypes of *Salmonella* (CDC, 2011). All of this data translates into an estimated burden of $51.0 billion dollars being paced on the United States health care system annually, with each average case per person costing an approximate $1,068 (Scharff, 2012). More specifically, nontyphoidal *Salmonella* falls above the overall mean, costing roughly $11,086 per case and accounts for a total of $11,391 million dollars on the US Health Care system (Scharff, 2012).

The concept of food safety started with the Federal Meat Inspection Act (FMIA) which was enacted in 1906. The purpose of this piece of legislation was to ensure quality meat products in the United States by mandating both ante- and post-mortem inspection of animals and their carcasses (Hulebak and Schlosser, 2002). It was at this time in the United States that the mainstream thought was that if diseased animals were to proceed through slaughter, then contaminated meat would be passed onto the consumer. Therefore, there was a greater importance placed upon antemortem inspection. As consumers' needs and wants changed, the need for a safe food supply

greatened. This boom in product diversity blossomed after World War II and into the 1970s. It was then that the United States Department of Agriculture Food Safety Inspection Services (USDA-FSIS), had to tackle the hurdle of microbiological control in meat and poultry products (Hulebak and Schlosser, 2002).

This desire to control foodborne pathogens eventually lead to the development and implementation of the hazard analysis critical control point (HACCP) program, enacted in 1996. The term "hazard" in the HACCP program is defined as anything that can be biological, physical, or chemical (Tompkin, 1994). The HACCP program, essentially, is a systematic way of executing adequate pathogen control in the plant. Although HACCP heavily relies on end product testing, it is important that there is a certain level of quality control that needs to be upheld and maintained throughout the plant (Jay et al., 2005). A basic HACCP plan consists of 7 principles: (1) Analysis of hazards and risks, (2) Determining critical control points (CCP's), (3) Determining critical limits at each CCP, (4) Establishment of monitoring systems at each CCP, (5) Developing backup systems at each CCP, (6) Protocol development at each CCP in order to monitor efficacy, and (7) Establishment of system of record keeping in order to document the implementation of a HACCP plan (Jay et al., 2005).

Developing a through, well-thought out HACCP plan is crucial for poultry processing plants due to the fact that it employs multiple monitoring points throughout processing in order to deliver the safest food to the consumer. In the past, many

producers relied upon end product testing to determine how safe a food product was. Since HACCP now employees multiple testing points throughout the production process, we see a better pathogen control overall in the plant. This not only produces safer food for domestic consumption but safer food for international distribution as well (Tompkin, 1994).

However, *Salmonella* prevalence in poultry continues to be an ongoing problem, especially as regulations continue to become more and more stringent. In the past few years the CDC has been able to link multiple outbreaks of Salmonellosis directly to poultry and poultry products. For example, in 2010, there was an enormous recall of over 500 million eggs due to an outbreak of *Salmonella*. Other outbreaks include a currently ongoing 20-state outbreak in September 2013 which has been linked to chicken meat, a 13 state outbreak in July of 2013 linked to chicken meat, a 6 state outbreak in November 2011 linked to chicken livers and a 34 state outbreak in August of 2011 linked to ground turkey meat. These outbreaks have resulted in recalls of 23,093 units (approximately 102,635 pounds) of rotisserie chicken, an undetermined amount of chicken livers and a recall of 36 million pounds of ground turkey.

Even more recently, in January 11, 2014 Tyson issued a recall of 33,840 pounds of mechanically separated chicken due to an outbreak of *Salmonella* Heidelberg originating from a Tennessee plant. Nine persons contracted Salmonellosis, which resulted in all 9 ill patients being hospitalized. Currently no deaths have been reported

which have been linked to this outbreak. The particular strain of *Salmonella* Heidelberg has since been reported to Pulse-Net and has made an appearance in 12 different states in various poultry facilities. This is currently an ongoing investigation in order to determine if this recall can be linked to several different, additional recalls involving the same S. Heidelberg strain (CDC, 2014). The majority of these recalls have all been initiated due to the presence of *Salmonella* Heidelberg, which has been one of the top pathogens emerging in the poultry industry. It is considered more serious than other *Salmonella* spp. due to the likelihood of antibiotic resistance. Recalls such as these have contributed to a huge economic loss to industry through product losses and litigation.

Healthy People 2010/2020 Guidelines. The United States Department of Health and Human Services issued the Healthy People 2010 Initiative. This initiative was a 10-year, science based plan aimed at increasing the health of all Americans (Anonymous, 2014). Part of the Healthy People 2010 Initiative was to reduce the prevalence of foodborne illness in the United States. There were copious amounts of targets and projected final goals were published in order to achieve this goal and to help assure a more safe food supply to American consumers.

The four major foodborne pathogens to be monitored by FSIS within these guidelines were *Campylobacter*, *Salmonella*, *Listeria*, and *Escherichia coli* 0157. CDC reported that in 2009 that the incidence of *Salmonella* was 15.19 per 100,000 people. However, the Healthy People 2010 Objective was to reduce baseline cases from 13.6

(from 1997) to 6.80 (CDC, 2010). These objectives were not met. In fact, the incidence of foodborne illness attributed to *Salmonella* increased 10.3% (CDC, 2012)

Due to the fact that 2010 pathogen control objectives were not met, the Healthy People 2020 Guidelines have since been issued. These new guidelines have set the bar for *Salmonella* reduction higher than the previous standards that were issued in 2010. The new food standards call for a 25% percent reduction of the current incidence level of salmonellosis. This number has the potential to reduce the economic burden that foodborne illnesses every year in the United States. If these standards were to be met, it could potentially reduce 4.6 million illnesses attributed to foodborne illnesses annually. Furthermore, this is a reduction of 68,000 hospitalizations and 1,470 deaths. This can possibly translate into an enormous potential savings of $421 million in healthcare costs (CDC, 2011).

USDA-FSIS New Compliance Guidelines. In order to combat the never-ending battle for ensuring a more safe food supply for Americans, the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) has also revamped and updated their performance standards for *Salmonella* prevalence in broilers. Previously in 1996, USDA-FSIS performance standards stated that *Salmonella* spp. prevalence should be no more than 20% on whole broiler carcasses (USDA-FSIS, 1996). The updated *Salmonella* spp. directives for 2010 have stated that *Salmonella* spp. prevalence cannot be more than 7.5%.

Since issuing performance standards for broiler carcasses, USDA-FSIS has decided to tackle the issue of poultry parts. This is, in part, due to the increased consumption of poultry parts in the United States and to meet consumer demand of more convenient, easier to prepare food items. The National Chicken Council reports that in 1965 the total consumption of chicken products was 33.7 pounds per capita. An estimated per capita consumption for 2014 is 85.0 pounds (National Chicken Council, 2014). Also, in 1962, only 15% of all chicken sold in the United States was marketed as cut up parts. Currently, the forecasted percentage of chicken in 2014 that is sold as poultry parts has climbed dramatically to 41% (National Chicken Council, 2011). Part of marked increase in the consumption of poultry and poultry products can be directly linked to the paradigm shift in American eating habits. Many Americans have altered their eating habits in order to live a healthier lifestyle, and poultry has been one of the proteins of choice to fill this niche.

The need to ensure the food safety of poultry parts prompted USDA-FSIS to conduct a base-line study on poultry parts. This baseline study was conducted from January – August 2012. In this study, USDA-FSIS took 2,496 samples of chicken parts from various points in the processing line. These samples were analyzed for several bacterium present such as *Salmonella* spp., *Campylobacter* spp., as well as generic *E. coli*. When all samples were analyzed, it was found that *Salmonella* prevalence on poultry parts is approximately 24.02% with a 95% confidence interval ranging from

19.24 – 28.79%. Further analysis and serotyping revealed that the most common serotypes found in and on poultry parts were Kentucky, Enteritidis, and Typhimurium. Overall, there were 657 samples (26.32%) that came back positive for some serotype of *Salmonella*. The poultry parts that were most likely to come back positive for *Salmonella* are chicken necks (USDA-FSIS, 2012).

Chicken Part by Type	Number of Samples	Number of *Salmonella* Positives	Percent *Salmonella* Positives
A - Breast	**776**	**210**	**27.06%**
B - Neck	**22**	**12**	**54.55%**
C - Leg	**584**	**141**	**24.14%**
D - Wing	**321**	**107**	**33.33%**
E - Half Carcass	**149**	**33**	**22.15%**
F - Quarter Carcass	**330**	**68**	**20.61%**
G - Giblets	**57**	**23**	**40.35%**
H - Other	**248**	**59**	**23.79%**
NP*	**9**	**4**	**44.44%**
Totals	**2,496**	**657**	

Table 1: *Salmonella* Percent Positive by Specific Chicken Part in RCPBS

According to the CDC, the top 5 *Salmonella* strains that are associated with foodborne illness are Heidleberg, Typhimurium, Newport, Javiana, and Enteritidis (CDC, 2011). In 2010, USDA-FSIS determined that the top four *Salmonella* serotypes present in broilers were Kentucky, Enteritidis, Typhimurium, and Heidelberg while Hadar is most present on turkey carcasses (USDA, 2010). While there are no current in-place performance standards in place for poultry parts or ground product, the poultry industry

is constantly working to anticipate moves in regulatory status based upon actions by USDA-FSIS. Therefore, anticipating the industries future needs and pending performance standards for parts and ground meat, the industry has found it crucial to investigate novel new technologies to reduce *Salmonella* levels on poultry parts and thus in ground product, such as modifications on poultry chillers and use of new antimicrobials.

***Salmonella* spp**. *Salmonella* is a Gram-negative, non-sporeforming rod that belongs to the family *Enterobacteriaceae*. It is a pathogen most commonly found in the intestinal tract of many animals and is most often transmitted via a fecal-oral route, even though *Salmonella* can be found almost anywhere and is ubiquitous in nature (Antunes et al., 2003). The majority of *Salmonella* spp. are fastidious organisms which are capable of being cultured on various media within 24-48 hours at 37°C. *Salmonella* spp. are generally not able to ferment various types of sugars, such as lactose or sucrose, but yet are able to ferment glucose and produce a gas byproduct (Jay et al., 2005).

Salmonella spp. grows best at a temperature that ranges from 35-42°C (Russell, 2012). Like many other microorganisms in the *Enterobacteriaceae* family, *Salmonella* has the ability to ferment carbohydrates into several different by-products, some of

these include gas and acid. *Salmonella* also has the ability to produce H_2S as a by-product of fermentation (Russell, 2012).

Due to *Salmonella's* route of contamination, it can also contaminate water sources, and therefore, the bacteria can be spread through the water to other organisms and become internalized by animals in this way. This unfortunate cycle of events further perpetuates the spread and continued infection cycle of the *Salmonella* organism (Jay et al., 2005). Over the years, there are several *Salmonella* serotypes that have a recurring presence in the poultry industry, because *Salmonella* colonizes in the broiler intestinal tracts. This is a main reason as to why this particular zoonosis is mostly commonly found in poultry and poultry products (Antunes et al., 2003). In 2010, from analyzing 6,828 samples, it was determined that the top four *Salmonella* serotypes that were present in whole broilers and confirmed from HACCP verification were Kentucky, Enteritidis, Typhimurium, and Heidelberg (USDA, 2010).

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

Table 2: Profile of Serotypes from Analyzed PR/HACCP Verification Samples by Calendar Year. Broilers (1998-2005 'A' Set Samples; 2006-2010 All Samples)

Salmonellosis. Food poisoning via *Salmonella* spp. is caused by the ingestion of bacteria that can reside and proliferate in foods via undercooking, cross-contamination, or general mishandling. The majority of persons who have contracted *Salmonella* often show systems of illness anywhere from 12-72 hours after first exposure (CDC, 2010). Infection often occurs with the consumption of anywhere from 10^3 cells per gram. Certain populations are particularly more prone to develop *Salmonellosis*. This group

includes children, the elderly, pregnant women, and any persons who are immunocompromised (CDC, 2010).

In order to cause a case of salmonellosis, the *Salmonella* serotypes have to become extremely adaptive to their environment. For example, they need to have a high acid-tolerance level to survive in the intestinal tract. Cases of salmonellosis will not occur until the bacterium has reached the intestines, therefore all *Salmonella* bacteria that are capable of causing illness have to be able to survive the harsh, acidic pHs of the human stomach (WHO, 2002). Furthermore, to cause a salmonellosis infection, bacteria will need to make their way to the small intestines and infiltrate Peyer patches that are located there (WHO, 2002).

Presently, there have been over 2300 serotypes of *Salmonella* that have been discovered, all of the 2300 serovars that have been currently discovered are virulent and capable of causing Salmonellosis in humans (Center for Food Security and Public Health, 2005). However, in some animals an infection of *Salmonella* may not be able to be diagnosed as they are part of their normal intestinal microflora.

The most common symptoms associated with salmonellosis are diarrhea, fever, and abdominal cramping (CDC, 2010). In addition to these main symptoms, others may include a generalized feeling of malaise, muscle weakness, as well as a feeling of drowsiness. The duration of symptoms may last anywhere from 2-3 days, and can often times result in hospitalization of certain compromised individuals. One may also

develop enteric signs of Salmonellosis and more systemic complications from the bacterium (Center for Food Security and Public Health, 2005).

Persons who are more prone and may have a harder time recovering from a *Salmonella* infection are those who are pregnant, the elderly, children, and any persons with a compromised immune system (CDC, 2010). However, salmonellosis is one of the most common foodborne illnesses that can be found in industrialized countries (Antunes et al., 2003). In 2013, the CDC reported that *Salmonella* causes an estimated 15.19 illnesses per 100,000 people in the United States. This has changed and in preliminary data for 2013, cases of salmonellosis have dropped to 15.19 illnesses per 100,000 people. The CDC also estimates that for every case of salmonellosis that is diagnosed, there are approximately 29 more which are not (CDC, 2013). However, the incidence is much higher for children who are more susceptible for *Salmonella* infections. Children under the age of 5 have an incidence rate of 69.5 infections per 100,000 children, much higher than the general population (CDC, 2011).

Overall, salmonellosis can be caused by several factors, all of which can usually be tracked back to poor hygiene among consumers, food not cooked to the proper temperatures, temperature abuse of the food, or improper handling (Davis et al., 2010). If, in fact, the *Salmonella* infection does persist in healthy peoples or those who are more susceptible, there are several choices in antibiotics. The ones most commonly

used are ampicillin, amoxicillin, gentamicin, trimethoprim/sulfamethoxazole, and fluoroquinolones (Center for Food Safety and Food Health, 2005).

Current Poultry Processing Methodology. More often than not, animal hides and feathers can be contaminated with various bacteria upon entering the slaughter house. Prior to plant arrival, birds are withheld feed from anywhere to 8-10 hours prior to slaughter. This serves as the first barrier against bacteria contamination in the slaughter house. This feed withdrawal period allows for the intestines to empty to the point to where there is minimal fecal matter present within them at the time of slaughter. Any longer than an eight hour feed-withdrawal time may result in watery intestines that may puncture more readily during various steps of the slaughter process (McKee, 2012). Any less than eight hours will significantly increase fecal matter presence in the intestines and results in possible cases of recontamination. There is a zero tolerance for fecal material on poultry entering the chiller and fecal material is known to contribute to cross-contamination of pathogens.

The poultry slaughter process has become highly automated in the recent years. By eliminating human hands at unnecessary points, this process helps to maximize product safety by reducing cross-contamination. During the poultry slaughter process, there are several steps that can serve as vehicles for cross-contamination. The two major steps in the slaughter process where cross-contamination of the carcass can occur are during feather picking and evisceration (McKee, 2012).

Once birds have been delivered, placed on the line, properly stunned, and allowed to adequately bleed out, they are sent to the scalding tank. During the scalding process, birds are placed in hot water of either 53-55°C for 120 seconds (a soft scald) or at 62-64°C for 45 seconds (a hard scald) (Sams and McKee, 2010). In industry production, a hard scald is more common than soft scalds due to a more complete removal of the cuticle, which gives the product more consumer appeal and has more potential for further processed products.

However, the scalding process will usually consist of multiple tanks in which the temperature can be slightly adjusted to reflect the needs and wants of the producer. In these multiple tanks, water flows in a counter-current direction. This counter-current water flow insures that at any given point in time, broiler carcasses are coming in contact with the cleanest water at all possible times (Sams and McKee, 2010). Despite using high temperatures in the scalding process, the bacterial load can often be high here due to possible fecal matter covering the bird upon arrival to the processing plant. Therefore, scalders can be considered as a minor point of cross-contamination during the slaughter process through the scalder water (Davis et al., 2010). Poultry processors have a choice of utilizing two different scalding temperatures, depending on the desired product of the manufacturer. A hard-scald consists of sending whole broilers through various tanks with a counter-current water flow with temperatures ranging 140-145°F for approximately 45 seconds. A soft-scald uses the same methodology except

temperatures are kept lower (at approximately 128°F) and held for a longer period of time (120 seconds). However, due to the counter-current water flow combined with the high temperatures, the scalding process remains a point of minimal cross-contamination in the poultry plant (McKee, 2012).

After scaling, broilers are then sent to the picker. A picker entails the broiler passing through a tunnel that consists of several rows of rubber "fingers" which are constantly rotating. Picker fingers take feathers, whose attachment to the cuticle have been loosened during the scalding process, and finish the removal process. Due to the fact picker fingers are made out of rubber, they often become cracked and damaged because of their constant use. These cracks, which are often times not visible, are a major source of cross-contamination for broiler carcasses (Sams and McKee, 2010). Research has shown that picker fingers can serve as a significant spot for cross-contamination of *Salmonella* spp. (Davis et al., 2010). Some think that the cross-contamination is, in part, due to the fact that the carcass temperatures are warm upon exiting the scalder. The higher temperature allows feather follicles to expand and more open. When the feather follicles are open, certain bacteria, including *Salmonella* spp., can intercalate underneath the skin via the feather follicles (Davis et al., 2010). Later in the slaughter process, when the carcass passes through the primary chiller and are exposed to lower temperatures, the feather follicles will constrict. This constriction can

cause the bacteria to become trapped, making it harder for antimicrobials to reduce their populations (McKee, 2012).

Another point of pathogen contamination during the slaughter process is during evisceration. During evisceration, broilers vents are opened via a pneumatic "vent gun" and then the body cavity is sliced open until the bottom of the breast bone. This allows for the most complete removal of viscera. From the collective viscera, the edible viscera is then harvested, after inspection, and then packaged separately. Edible viscera, commonly referred to as the "giblets", consists of the heart, liver, and gizzards (Sams and McKee, 2010).

It is during the evisceration process where intestinal integrity becomes an important factor in carcass contamination. As previously discussed, birds that are withheld feed for 8-10 prior to slaughter maximize intestinal integrity and have a smaller chance of intestinal breakage and therefore fecal contamination. However, when birds have been on feed withdrawal for 10+ hours, their intestinal integrity are lessened and the chance for intestinal breakage are dramatically increased (Davis et. al., 2010). During evisceration when viscera is removed, birds as well as equipment are generally sprayed with a water plus an antimicrobial solution. The antimicrobial solution that is most commonly used in this step of processing is chlorine (Sams and McKee, 2010).

Once birds are void of all edible and inedible viscera, they are then sent through a manual inspection station. It is here that each bird is visually inspected for any sort of

fecal contamination on the external surface of the carcass. If any sort of fecal material is detected, birds are often times removed from the line and sent to a reworking station or a washing cabinet. When carcasses are sent on through a washing cabinet, carcasses are frequently treated with chlorinated water other antimicrobial compounds (McKee, 2012).

Birds then travel through the "inside/outside" (I/O) bird wash. Chlorine (20 ppm), peracetic acid (50-100 ppm) or Cetyl peridinium chloride (3000 -6000 ppm) is are generally used for an I/O bird wash application. From an industry survey, McKee discovered that peracetic acid, followed by chlorine, are the two most commonly used antimicrobials at this step (McKee, 2011). The pressure in the sprayers needs to be high enough to remove any sort of matter than may be left on the carcasses up to this point in the slaughter process (Davis et al., 2010). Pressure must be continually monitored and adjusted due to the fact that high pressure may, in fact, drive bacteria into the carcass skin (Brashears et al., 2001). This will lead to higher microbial counts on finished carcasses. The purpose of the inside/outside bird wash is to remove any additional debris that may be present on the bird before entering the pre chiller. The I/O bird wash is generally the first multi-hurdle intervention point in the processing line where antimicrobials are directly applied to carcasses.

After the I/O bird wash, carcasses enter the pre-chiller. The pre-chiller serves to begin the cooling process of the carcasses. The pre-chiller's temperatures can range

from 7-12°C and the carcasses are immersed for approximately 10 to 15 minutes (Sams and McKee, 2010). The purpose for gradual reduction in chiller temperature is to prevent shocking the carcasses when entering the primary chiller. Exposure to lower temperatures, without a gradual introduction, can lead to negative effects on the quality of the meat. Once the carcasses exit the chiller, their average temperate can range anywhere from 30-35°C (Sams and McKee, 2010). This reduction in temperature also helps to lighten the cooling load on the primary chiller.

Primary chillers serve to (1) meet USDA chilling guidelines and (2) to reduce initial bacteria loads on broilers. Chillers utilize a water immersion technique over air chilling. When birds enter the chiller, the water temperature is usually around 4°C and 1°C at the exit. The current, most commonly used antimicrobial in the chiller is peracetic acid which has been proven to impart less sensory and meat quality defects versus the traditional use of chlorine (Nagel, 2013). However, chlorine is often times used but it cannot exceed a concentration of 50 ppm in the chiller. Chlorine is most effective when kept at a concentration above 25 ppm (Davis et al., 2010). In addition, for the usage of chlorine to be most effective, contact times of 1 – 1.5 hours are usually required (McKee, 2011). This is another step in the multi-hurdle approach to microbial control within the plant.

In an experiment conducted by Bauermeister et al. (2008), the two most common antimicrobials, chlorine and peracetic acid (PAA), were tested at various

concentrations in a chiller application. Chlorine was tested at 0.003% and PAA was tested at 0.0025%, 0.01%, and 0.02%. Carcasses were inoculated with 1mL of 10^8 CFUs/mL *Salmonella* Typhimurium and then allowed to be immersed in the various solutions designed to mimic a primary chiller application. It was determined that 0.02% PAA gave the greatest reduction in bacterial loads.

Various different designs can be utilized for a primary chiller, all of which serve to agitate the carcasses. By agitating the carcasses, the carcass temperature drops at a much more efficient rate. Aeration of chiller adds additional agitation and helps to prevent thermal layering of carcasses. By eliminating this thermal layering effect, boiler carcasses are chilled to the appropriate temperatures within 4 hours of processing. One potential source of cross-contamination in a primary chiller is due to carcass overloading. This results in broilers not moving at an adequate rate which results in piling. Piling of carcasses is a potential source of cross-contamination.

Once birds exit the final chiller, a new technology termed a Finishing Chiller® can be used as another antimicrobial intervention step. The Finishing Chiller® has a much smaller footprint than that of a primary chiller, only holding approximately 400-600 gallons of water with a dwell time of less than 30 seconds (McKee, 2011). A Finishing Chiller® applies antimicrobials to the finished carcass. These antimicrobial concentrations can be considerably higher than that of a chiller system. This is able to

be achieved due to the short contact time that the carcasses will have inside the chiller.

Figure 1. Morris & Associates Finishing Chiller®

Due to antipiated standards on poultry parts to be issued in 2014, Morris and Associates has dervelped the COPE (Continuous Online Pathogen Elimation) Unit. This piece of equipment is similar to the finishing chiller, yet has a smaller footprint in the processing plant. The COPE's design is that so it has a short contact time and exhibits

the first-in first-out methodology as the finishing chiller. It's intended usage is for bone-in and boneless poultry parts. Current validation studies have demonstrated its efficacy in reducing *Salmonella* Typimurium as well as *Campylobacter jejuni* on poultry parts (Zhang et al., 2013). Additionally, these signficant pathogen reductions has been validated to significantly reduce pathogen presence in ground poultry meat made with treated parts (Chen et al., 2013).

Figure 2: Morris & Associates COPE (Continuous Online Pathogen Elimination) Unit

Current Antimicrobial Interventions Used in Poultry Processing. The majority of foodborne pathogens that can infect humans, find a natural host in animals (CDC, 2011).

In regards to broilers, Salmonella can become prevalent in various parts of the bird and broilers have proven to be a natural reservoir. Due to this fact, there are multiple antimicrobials which have been approved for usage during the poultry processing process. These antimicrobials are listed in the FSIS directive 7120.1 revision 18 (USDA-FSIS, 2014).

One of the most common antimicrobials currently used in poultry processing is peracetic acid (McKee, 2011). Peracetic acid (PAA) consists of an equilibrium of acetic acid (an organic acid) and hydrogen peroxide, which is an oxidizer (Baldry and Fraser, 1988). Acids have the ability to penetrate the cell membrane, acidifying the cellular internal environment and thus hindering basic cellular metabolism (Marriott and Gravani, 2006). Peracetic acid is allowed up to 220ppm peracetic acid plus 110ppm hydrogen peroxide in a chiller application and then up to 2000ppm in a post chill dip (USDA-FSIS, 2014). A study conducted by Bauermeister et al. (2008) compared an 85 ppm peracetic acid usage alongside a normal 30 ppm chlorine usage in poultry chillers in their effectiveness of pathogen reduction on broiler carcasses, namely *Salmonella* and *Campylobacter* species. The study demonstrated a 92% reduction in *Salmonella* populations when 85 ppm peracetic acid was used vs only a 57% reduction in *Salmonella* populations when 30 ppm acidified chlorine was used (Bauermeister et al., 2008).

In a Finishing Chiller®, PAA has been approved for use up to 2000-ppm for a dip application (USDA-FSIS, 2014). Since this concentration is considerably higher than what

can be used in a primary chiller, the FMC-Corporation holds the patent for this application. Even though the patent for the use of higher levels of PAA have been approved, levels of approximately 1200ppm have been shown to be the most effective in the eradication of 99% of *Salmonella* spp. that may be present on broiler carcasses (Davis et al., 2010).

Peracetic acid has also increased in popularity in the past few years due to the fact that Russia, one of the United States largest export markets, has recently outlawed the usage of chlorine as an antimicrobial treatment on their poultry parts. Therefore, this decision Russia made has equaled a paradigm shift in the antimicrobial intervention choice amongst US poultry processors, making peracetic acid the most commonly used intervention chemical of choice in the United States (McKee, 2012).

Chlorine is another commonly used antimicrobial. It has been approved for usage for the treatment of poultry carcasses as well as poultry parts and other organs at levels of 50-150 ppm for pre-chill application (USDA-FSIS, 2014). However, in a primary chiller the chlorine concentrations should remain around 30 ppm (Russell, 2012). In solution, chlorine reacts with water in order to form hypochlorous acid and hypochlorite ions. However, hypochlorous acid is most effective at pathogen reduction. The amount of hypochlorous acid is dependent upon the pH. The pH of the system needs to be acidified around pH of 6. At lower pH's, hypochlorous acid can form yet is less stable. Extremely low pH can promote chlorine gas formation which is extremely toxic. Higher

pH promotes the formation of hypochlorite ions. This is a more stable form, but pathogen reduction efficacy is greatly lessened. However, concentration of chlorine is measured in ppm of the amount of free chlorine in the system (McKee, 2012).

Free chlorine in a processing system can be effected by several factors. One of the highest concern is the organic load of the water. Organic load can be defined as any sort of blood, fat, and protein that can end up in poultry wash water. In order to minimize the amount of organic in either a primary or Finishing Chiller®, all other equipment on the processing line must be adequately calibrated. It is imperative that birds are as clean as possible when they reach the primary chiller, adequately bleed out, and scalder temperatures properly adjusted. Scalders which have not been properly adjusted will lead to unsaturated fatty acids being leached out of the broilers and therefore end up in a lower yield (McKee, 2012). These fatty acids will be leached into chiller water and bind up free chlorine levels (Russell, 2012). A scalder whose temperature is set too high will liquefy the unsaturated fats present in poultry and this will translate into a higher fatty residue making it into the primary chiller. In addition, used chiller water free chlorine levels must be monitored and cannot exceed over 5 ppm per USDA-FSIS regulations. However, despite chlorine's popularity in the United States, it cannot be used as an antimicrobial intervention in Europe and Russia (Goode et al., 2003).

Cetylpyridinium chloride (CPC) is another antimicrobial which is utilized in the poultry processing industry. CPC has been approved for application as a spray mist prior to broiler carcasses or poultry parts entering into a chiller or it can be used as a liquid application like in a drench cabinet. It has also been approved for dip take applications but cannot exceed 0.8% by weight (USDA-FSIS, 2014). However, all CPC which is used during processing has to be recycled and recaptured post-treatment due to the fact that current environmental regulations prevent CPC from being discharged into wastewater. Therefore, processing plants that implement CPC usage for poultry processing have to have a water-filtering (with the use of activated charcoal) system in place to prevent CPC from entering the waste water. During every step of processing, there are always antimicrobial agents in place in hopes to reduce bacterial loads, especially *Salmonella*, from one waypoint to the next.

At the most basic level, bacteriophages are viruses which are designed to infiltrate, infect, multiply, and then kill target bacterial cells. Bacteriophages are some of the most abundant and oldest living microorganisms and are ubiquitous in nature and outnumber prokaryotes 10 to 1 (Connerton and Connerton, 2005; Denes and Wiedmann, 2014). They were first used in a scientific endeavors by Felix d'Herelle who utilized them to treat severe dysentery (Garcia et al., 2008). Bacteriophage life cycles are very short and range anywhere from 20 to 60 minutes in total and pose no harm to

eukaryotic cells (O' Flaherty et al., 2009; Garcia et al., 2008). Therefore, their safety for usage in a wide variety of applications cannot be understated or underplayed.

Even though scientists have known about the benefits bacteriophage technology for some time, it is not until recently with the increasing prevalence of antibiotic resistant bacteria that the avenue of bacteriophage use has been opened (Connerton and Connerton, 2005). In recent years, there have been numerous cases of antibiotic resistance among several bacteria. For example, *Staphylococcus aureus, Salmonella* spp*., Mycobacterium tuberculosis, Acinetobacter, Escherichia coli, Streptococcus pneumoniae, Campylobacter jejuni, Pseudomonas aeruginosa, Haemophilus influenza,* and *Clostridium difficile* have all been linked to antibiotic resistant outbreaks (O' Flaherty et al., 2009). Due to these antibiotic resistant outbreaks, many avenues for bacteriophage usage have developed.

Usage of bacteriophages on food products as well as live broilers to reduce bacterial loads has been approved by USDA and FDA (O' Flaherty et al., 2009; USDA-FSIS, 2014). One type of bacteriophage that is commonly used to combat various foodborne illness causing bacteria are lytic bacteriophages. Lytic bacteriophages seek out specifically targeted bacterial cells, infect, and then multiply within their host's cytoplasm due to the fact they themselves lack any sort of replication system. They redirect the host cell metabolism in order to replicate themselves. This process ends in the lysis of bacterial cells and the release of new bacteriophages (Toro et al., 2005;

Oliveria et al., 2012; Dalmasso et al., 2014). The lysis of bacterial cells is due to the release of endolysins. These endolysins break down the peptidoglycan within the cell walls of the host bacteria during their termination phage. This causes an increase in osmotic pressure which results in the destruction of the cell wall, releasing new bacteriophages in to the matrix (Oliveria et al, 2012).

In specific regards to *Salmonella* control, there have been several studies proving the efficacy of bacteriophage treatments in controlling populations on meat and ready-to-eat products. An experiment was conducted by Goode et al. (2007) concluded that when a phage treatment was applied to broiler skin, they noticed a 99% reduction in *Salmonella enterica* serovar Enteritidis when compared to broiler skin in which there was no phage treatment applied (O' Flaherty et al., 2009). In a separate study conducted by Bielke et al. (2003), the effect of bacteriophages was explored on broiler carcasses inoculated with *Salmonella enterica* serovar Enteritidis or *Salmonella* Typhimurium. These carcasses were treated with 5 mL of bacteriophage treatment. Their results showed that there were no detectable levels of *Salmonella enterica* left on the carcasses.

Spricigo et al. found that a significant reduction in *Salmonella* populations on inoculated poultry breasts and pig skin with bacteriophage treatment. Pig skin was irradiated and treated with 70% ethanol to insure no residual background bacteria present on the samples. Skin samples were inoculated with a 10^4 *Salmonella* cocktail

and incubated at 33°C for 30 minutes. Samples were analyzed 3 and 6 hours post treatment as well as at 3 and 6 days. There was an overall reduction of 2.2-4.0 log dependent upon the particular *Salmonella* strain (2013). Poultry breasts were analyzed after inoculation via 10^6 dip application of *Salmonella* Typhimurium and Enteritidis. Breasts were then treated with 100mL a 10^9 PFU/mL treatment and samples analyzed at 0, 1, 2, 5, and 7 days. Each time samples were analyzed there was an average 1.4-2.2log reduction observed (2013)? A similar study was conducted by Hungaro et al. (2013) who compared bacteriophage treatment against other commercially used antimicrobials. A 10^5 inoculum of *Salmonella* enteritidis dip treatment was used on broiler skin. After inoculation, the skin was exposed to either a 200ppm sodium dichloroisocyanurate solution, 100ppm PAA, a 2% lactic acid treatment, or bacteriophage cocktail containing 10^9 PFU/mL. From this, no significant difference in bacterial reductions was observed.

Furthermore a study conducted by Higgins et al. (2005) found that treating commercially collected broiler rinse water with a bacteriophage solution found significant reduction in of *Salmonella* Enteritidis in the poultry plant. *Salmonella* Enteritidis, at a level of 3 log, was added to aliquots of broiler rinse water. Rinse water was then treated with 6 or 10 log PFU/mL of bacteriophage. All samples were held for 24 hours at refrigeration temperature (4°C) to, once again, simulate USDA-FSIS sampling and shipping procedures. It was found that there was a reduction of 50-100% of

Salmonella levels post-enrichment. Additionally, the research group inoculated broiler carcasses which were pulled off the processing line (prior to chilling) with 6 log of *Salmonella* Enteritidis. Bacteriophage treatments were applied via a hand-held sprayer with 10 log PFU/mL and carcass rinses were collected. These carcass rinses were held for 24 hours at refrigeration temperature (4°C) and then enriched. An 85% reduction in *Salmonella* Enteritidis levels were observed upon final examination.

Additionally, a live-bird study was conducted by Wong et al. (2014) that concluded that bacteriophages can be effective in eliminating *Salmonella* populations from the intestinal tract of broilers. The research group inoculated live broilers with 10^{10} CFU/mL *Salmonella* Typhimurium. After treatment with a bacteriophage solution, they observed a 5-6 log reduction in *Salmonella* colonization of the intestines. Similar results were observed in a study conducted by Fiorentin et al. (2005). Day-old broiler chicks were inoculated with 100µL of 8 log *Salmonella* Enteritidis. At 7-days they were then given a cocktail of bacteriophage treatment at 11 log PFU/mL. Birds were then necropsied at 0, 5, 10, 15, 20, and 25 days post-treatment and cecal contents analyzed for *Salmonella* prevalence. The prevalence of *Salmonella* was determined to be significantly lower than that of the control and non-treated broilers.

Currently there are several bacteriophage products available for market and are considered safe for human consumption. Some examples of these are ListSheild™, EcoShield™, and SalmoFresh™; all which are considered to have GRAS status and safe

for human consumption (USDA-FSIS, 2014). In addition to seeing results in bacterial reductions, there have been studies proving that bacteriophage application on food products can and will lengthen the self-life of various products (O' Flaherty et al., 2009). The use of bacteriophages in the food industry has many benefits. One is that bacteriophages are fairly inexpensive to produce, making treatment of bulk food amounts to be a more viable option for food processors. The specificity of bacteriophages means that it will not adversely effect any naturally occurring gut microflora. Additionally, phages themselves are described as self-limiting. Simply put, the replication and multiplication of these phages is host-dependent. Therefore, if there are no target bacteria available for host-replication, bacteriophage concentrations will not increase (Connerton et al., 2005).

One downside to phage usage is that many phages are specifically designed to target one specific serotype. Due to the nature of bacteriophages, it is often beneficial to use cocktails in an application (Joerger, 2003). However, to place bacteriophage use practically in a plant it would ideally be done immediately prior to packaging. This is due to the possibility that bacteria can become resistant to the bacteriophages themselves. As bacteria replicate, the proteins present on their cellular membrane can change and therefore the bacteriophage cannot bind to the bacteria, producing bacteriophage resistant bacteria (Connerton et al., 2005). In addition, bacteriophage survival rates are drastically reduced when exposed to various levels of PAA and other commercially used

antimicrobials (Sukumaran and Sharma, 2014). Due to the fact that peracetic acid has been demonstrate to have no residual activity on poultry carcasses, this further substantiates as to why bacteriophage treatment should be applied as close to packaging as possible. Since cross-contamination of poultry parts has been a major point of concern amongst poultry processors, ideally parts would be treated with a phage treatment immediately prior to packaging. Therefore, any residual phage activity would continue to reduce *Salmonella* populations during transport and possibly provide further *Salmonella* reduction in ground and comminuted products.

It is here, however, that poultry parts have the potential to be treated with a possible bacteriophage cocktail that may help to reduce not only *Salmonella* populations, but possibly *Campylobacter*, *Listeria*, and *E. coli* populations as well. Reductions in *Campylobacter* populations have been seen in various different studies. Frost et al. (1999) administered bacteriophages to broilers in the hopes of reducing *Campylobacter* jejuni levels in cecal contents. Within 48 hours, it was determined that treated broilers had a 3 log reduction in *C. jejuni* levels. This technology also has potential to be used in plants that produce a variety of ready-to-eat (RTE) products as bacteriophages have little no impact on the organoleptic properties of products and have potential to extend the shelf-life of certain products. In contrast, traditional bacterial reduction methods often used in industrial practices such as steam, dry heat, and UV light often cause deterioration in the sensory and organoleptic properties of

products (Garcia et al., 2008). In addition, any product exposed to a bacteriophage treatment can bear the "all natural" label (Garcia et al., 2008; Kocharunchitt et al., 2009). This is something that many producers want and desire on their product label, as natural labels become more of a marketing tool and something that many producers are trying to target this specific clientele. There is also room for expansion of this product into various food-products such as ready-to-eat produce, beef, pork, and other RTE products.

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CHAPTER III

MATERIALS AND METHODS

Bacteriophage Concentration Optimization. A total of 640 lbs (290.3 kg) of bone-in chicken parts were sampled (40 lbs per treatment x 8 treatments x 2 replications). A total of 160 samples were analyzed (10 samples per treatment x 8 treatments x 2 replications). Treatments consisted of two water chill treatments of 700ppm and 100ppm peracetic acid (Spectrum; FMC, Philadelphia, PA) and four spray bacteriophage treatments of varying concentrations. Concentrations of bacteriophage used were calculated at 9.904 log PFU/sample, 9.602 log PFU/sample, 9.301 log PFU/sample, and 8.602 log PFU/sample.

For PAA treatments, a concentration of 700ppm and 100ppm were used. The use of PAA served to mimic reductions that would typically be seen in a poultry plants with the use of a Finishing Chiller®. For these treatments, 49.2 liter solutions of 700 ppm and 1000 ppm PAA (Spectrum; FMC, Philadelphia, PA) were prepared. Once again, chilled water (4°C) was used to bring each mixture of PAA to the correct concentration.

Positive control samples were used in order to determine the recovery of *Salmonella* on each sample. Positive controls consisted of parts that have been inoculated, but not been exposed to any antimicrobial intervention. This served as a mechanism to measure bacterial reductions by. Negative controls were included in order to determine the presence of background *Salmonella* on the parts prior to

treatment. Negative controls consisted of non-inoculated poultry parts exposed to no antimicrobial interventions.

Bacteriophage concentrations were obtained by from a stock solution of SalmoFresh™ (Intralytix, Baltimore, MD) and diluted to generate 9.904 log PFU/sample, 9.602 log PFU/sample, 9.301 log PFU/sample, and 8.602 log PFU/sample concentrations. Chilled, sterile water (4°C) was used to obtain the appropriate concentration of bacteriophage. After allowing for bacterial attachment following inoculation, 9mL of each of the phage concentrations were applied via spray to each parts sample. Once phage treatment was applied, parts were allowed to set for an additional hour at room temperature (23°C) prior to rinsing.

The *Salmonella* inoculum used was a mixture of various different strains. Serotypes included were Typhimurium, Hadar, Heidelberg, Kentucky, and Enteritidis. These strains were chosen as they are the ones most present in poultry that are most often implicated in foodborne illness (CDC, 2011). One milliliter of each serotype was obtained from a frozen culture, added to 10mL tryptic soy broth and incubated at 37°C for 24 hours. Each corresponding culture was streaked for isolation on xylose lysine tergitol 4 agar (XLT4, Acumedia Manufacturers Inc., Baltimore, MD). Plates were then incubated at 37°C for 24 hours. An isolated colony from each plate was streaked for isolation once again on XLT4 for verification. Plates were incubated again at 37°C for 24 hours. One isolated black colony from each serotype was added to 100mL tryptic soy

broth, incubated at 37°C for 24 hours. From this, a stock solution of $10^{7.5}$ cells/mL *Salmonella* inoculum was prepared. Inoculum levels were then verified through direct plating.

During each replication, bone-in chicken parts were commercially obtained the day prior to research and stored at 4°C for 24 hours prior to inoculation. Chicken parts were weighed into 4 lb. samples containing breasts, thighs, wings, and drums and placed into sterile rinse bags for subsequent inoculation per guidelines set forth in the USDA Raw Chicken Survey (USDA, 2012). Each 4-lb. parts sample was inoculated with a spray application of 1 ml of 7.5 log CFU/ml *Salmonella* inoculum. Poultry parts were then allowed to set for 20 min to allow adequate time for bacterial attachment before treatment (Nagel et al, 2013).

Samples were placed into solution and agitated using a sterile paddle for a dwell time of 30 seconds. Parts were then aseptically removed and placed into sterile rinse bags. Parts were sampled using a modified USDA method of sampling poultry parts (USDA-FSIS, 2012). The modification made was the use of 200mL rinsates instead of traditional 400mL. This was impart due to the splitting as samples into 2-2lb. (0.91 kg) samples as well as following current research methodology that reduction in rinsate volume does not impact *Salmonella* recovery (Cox et al., 1980; Nagel et al., 2013).

All parts were divided into 2-2 lb. samples into sterile rinse containers. One 2-lb. parts sample was rinsed using 200 mL of buffered peptone water (BPW; Accumedia

Manufacturers Inc., Baltimore, MD) for the 0 h analysis. The second 2-lb.parts sample was then analyzed at 24 h post-treatment. Samples were analyzed in this manner in order to best mimic USDA-FSIS sampling methodology in which samples are placed on ice, transported, and then analyzed 24-hours after. Rinsates from 0 h were stored at 4°C and plated again at 24 h to determine further *Salmonella* reduction.

Determination of Efficacy of SalmoFresh™ Application paired with antimicrobial application in a Primary Chiller application. A total of 135 4-lb (217.7 kg) batches of poultry parts were sampled (5 4-lb. samples x 9 treatments x 3 replications). Treatments consisted of water chill treatments of 95ppm peracetic and 30ppm chlorine. There was an additional water chill treatment to which no antimicrobial compounds were added. Overall, treatments consisted of water chill, water chill plus bacteriophage spray, 95ppm PAA chill, 95ppm PAA chill plus bacteriophage spray, 30ppm chlorine chill, 30ppm chlorine chill plus bacteriophage spray, a bacteriophage spray treatment only as well as a positive and negative control. The bacteriophage concentration used for the spray application was calculated at 9.602 log PFU/sample.

For chlorine and PAA chill treatments, a concentration of 30ppm and 95ppm was used, respectively. Appropriate amounts of chlorine or PAA was added to 49.2L of chilled water (4°C). Chlorine concentration was measured via Aquachek Water Quality Test Strips (HACH Company, Loveland, Co.) and the pH of the system was adjusted to 6.0

using a 1N HCl solution. PAA concentrations were measured using a titration system administered by a drop test kit (LaMotte Co., Chestertown, Md.).

Positive control samples were used in order to determine the amount of *Salmonella* inoculated per sample. Positive controls consisted of parts that were inoculated, but not been exposed to any antimicrobial intervention. This served as a mechanism to compare bacterial reductions. Negative controls were included in order to determine the presence of background *Salmonella* on the parts prior to treatment. Negative controls consisted of non-inoculated poultry parts exposed to no antimicrobial interventions.

Bacteriophage concentrations were created from a stock solution of SalmoFresh™ (Intralytix, Baltimore, MD) and diluted to generate a 9.602 log PFU/sample. Chilled, sterile water (4°C) was used to obtain the appropriate concentration of bacteriophage. After allowing for bacterial attachment following inoculation, 9mL of each of the phage concentrations were applied via spray to each sample.

The *Salmonella* inoculum used was a mixture of various different strains. Serotypes included were Typhimurium, Hadar, Heidelberg, Kentucky, and Enteritidis, as these strains are the ones most present in poultry which are implicated in foodborne illness (CDC, 2011). One milliliter of each serotype was obtained from a frozen culture, added to 10mL tryptic soy broth and incubated at 37°C for 24 hours. Each

corresponding culture was streaked for isolation on xylose lysine tergitol 4 agar (XLT4, Acumedia Manufacturers Inc., Baltimore, MD). Plates were then incubated at 37°C for 24 hours. An isolated colony from each plate was streaked for isolation once again on XLT4 for verification. Plates were incubated again at 37°C for 24 hours. One isolated black colony from each serotype was added to 100mL tryptic soy broth, incubated at 37°C for 24 hours. From this, a stock solution of $10^{8.5}$ cells/mL *Salmonella* inoculum was prepared. Inoculum levels were then verified through direct plating.

Poultry parts were weighed out in 4-lb (1.8kg) increments. Each 4 lb. batch of poultry parts contained, at the minimum, two bone-in breasts, thighs, wings, and drums. Parts were placed into sterile rinse bags for inoculation. All bone-in chicken parts were commercially obtained the day prior to sampling and stored at 4°C for 24 hours prior to inoculation.

All 4-lb. bags of poultry parts per replication were inoculated with a spray application of 1 ml of $10^{8.5}$ CFU/ml *Salmonella* inoculum. The inoculum consisted of combined strain of Enteritidis, Typhimurium, Hadar, Kentucky, and Heidelberg. Poultry parts were then allowed to sit for 20 minutes for bacterial attachment (Nagel et al, 2013).

Once parts were inoculated, they were placed into a mesh netting and closed. The mesh netting served to keep all parts contained during treatment. Once parts were contained within mesh netting, they were placed into a 13-gallon (49.2 liter) container

with the following antimicrobial treatments, 90 ppm PAA, 35 ppm Chlorine, and water treatment. Each treatment was chilled to 4°C. Chlorine concentration was measured via Aquachek Water Quality Test Strips (HACH Company, Loveland, Co.). PAA concentrations were measured using a titration system administered by a drop test kit (LaMotte Co., Chestertown, Md.). Parts were treated for 1 hour with intermittent agitation applied every 10 minutes. The pH of all treatments were recorded (HACH Company, Loveland Co.). The average pH of the PAA treatments was approximately 4.0 and the chlorine treatment was at 6.0, respectively. The pH of the chlorine treatment was adjusted to pH 6.0 with 1N HCl.

Bacteriophage concentrations were diluted from stock SalmoFresh™ (Intralytix, Baltimore, MD) to a 9.602 log PFU/sample concentration. Once the poultry parts had were removed from their respective antimicrobial treatment dips, they were placed into sterile rinse bags, and then 9mL of the phage concentrations was sprayed onto the parts. Chilled, sterile water (4°C) was used to obtain the correct concentration of bacteriophage. Once the phage treatment was applied, the parts were allowed to sit for an additional hour before analysis.

All 4-lb. (1.8 kg) samples of poultry parts were analyzed by the guidelines set forth in the USDA-FSIS Poultry Parts Baseline Study (USDA-FSIS, 2012). Each 4-lb. (1.8kg) batch of poultry parts was rinsed with 400 mL of buffered peptone water. The rinsate was not reduced in order to adhere to USDA-FSIS testing methodology. Rinsates which

were used for PAA and chlorine treatments contained 0.1% sodium thiosulfate. The addition of this was to stop any residual oxidative antimicrobial effects that may alter *Salmonella* counts (Kemp and Schneider, 2000). Rinsates were analyzed at 0 hours, held at refrigeration (4°C) temperatures overnight, and analyzed again at 24 hours. Negative controls were included in order to confirm the absence of background *Salmonella* on the parts in question.

Enumeration of *Salmonella* levels. *Salmonella* reduction on chicken parts was determined by direct plating methods. Serial dilutions were performed and 0.1mL was spread-plated on xylose lysine tergitol 4 agar (XLT4, Acumedia Manufactures Inc., Baltimore, MD) and incubated at 37C for 24 h until the presence of black colonies were detected Bacterial populations were recorded and then converted to log colony forming units per sample

Statistical Analysis. For each experiment, three replications were conducted. For each objective, all bacterial counts were transformed into log colony forming units per sample, using either 200 or 400 mL of rinsate, respectively. For all plates with no colonies present, 0.5 $\log_{10}$ CFU was used for analysis (McKee et al, 2008). Data were analyzed in a 2x2 factorial arrangement of antimicrobial treatment and trial. All data were analyzed using the least square means including standard error using the General Linear Model of SAS (SAS Institute, 2003). A P-value ≤ 0.05 was used to determine significance among means.

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CHAPTER III

RESULTS AND DISCUSSION

Currently, two main antimicrobials that are used in the poultry processing industry. The most commonly used one is peracetic acid (PAA). Peracetic acid is made via a combination of acetic acid and hydrogen peroxide. Therefore, when the compound decomposes it consists of acetic acid, hydrogen peroxide, as well as oxygen and water (Kitis, 2003). PAA has been hailed as the most popular antimicrobial intervention due to the fact that it gives a yields a better reduction in *Salmonella* populations versus other antimicrobials.

Usage levels are determined by the desired application. For example, PAA levels in a primary chiller will remain low while usage levels for a Finishing Chiller® are typically between 600-800ppm. These higher concentrations are able to be utilized due to the shorter contact time the antimicrobial has with the actual broiler carcass (Nagel et al, 2013). Chlorine is the second most commonly used antimicrobial in processing plants. It is most effective when kept at concentrations between 25 and 50 ppm, but levels cannot exceed 50ppm (Davis et al, 2010).

Alternative antimicrobial treatments that have been gaining in popularity is the use and application of bacteriophages. Bacteriophages offer several benefits that other traditional antimicrobials do not. The main reason for their appeal is that they are

considered as a generally recognized as safe substance (GRAS) by USDA-FSIS and the product can be labeled as “all-natural” (USDA-FSIS, 2014). To determine the efficacy for reduction of various *Salmonella* serotypes (Kentucky, Typhimurium, Heidelberg, Enteritidis, Hadar) on poultry parts, various concentrations of SalmoFresh™ bacteriophage were evaluated at 0 and 24 h in a spray application and compared to treatment with peracetic acid (PAA; 100ppm and 700 ppm). Once an optimal phage concentration was determined, the phage was likewise evaluated in combination with a chlorine and PAA dip.

The first objective of this study was to determine the optimal phage concentration to be applied to the poultry parts for reduction of *Salmonella* populations. For 0 h analysis, both of the PAA treatments resulted in the best ($P \leq 0.05$) reduction of *Salmonella* populations on the inoculated parts. The 100 ppm PAA treatment resulted in a 0.88 log reduction while the 700 ppm PAA treatment gave a 1.70 log reduction. Out of the various phage concentrations evaluated, the 9.904 log PFU/sample and 9.602 log PFU/sample and phage concentration provided the best ($P \leq 0.05$) reduction of *Salmonella* populations on inoculated parts giving approximately a 0.6 and 0.25 log reduction when compared to the positive control. The non-inoculated control was below the detection limit of 0.69 log indicating very low levels or no background S*almonella* spp. on the poultry parts prior to testing. Treatment with both

9.301 log PFU/sample, and 9.602 log PFU/sample phage resulted in no reduction of *Salmonella* populations on the parts tested with no differences (P>0.05) found (figure 1). The non-inoculated control was below the detection limit of 5 CFU/mL. This equates to no or very low levels of *Salmonella* present on poultry parts before the study started.

Rinsates were analyzes again at 24 h. This was to accurately determine if the bacteriophage application would have any sustained effect on *Salmonella* populations. This would give a more accurate description of what consumers would be encountering at the market level and to best replicate USDA-FSIS sampling procedures. When the 0 h analysis parts samples were analyzed again after 24 h to determine any residual activity, both the 9.602 log PFU/sample and 9.904 log PFU/sample phage concentrations showed a greater than 1-log decrease in *Salmonella* populations on the parts when compared to the positive control which is greater than the reductions found when analyzed at 0h. No differences (P>0.05) were found between the positive control and the 8.602 log PFU/sample phage treatment. In addition, the 09.301 log PFU/sample phage treatment showed a less than 1 log reduction in *Salmonella*. The 100 ppm and 700 ppm PAA treatments provided a reduction of 1.30 log and 1.94 log of *Salmonella*, respectively (Figure 3).

Finally, additional parts samples were analyzed at 24h post inoculation with the various *Salmonella* serotypes and treatment with bacteriophage. It was at 24h that

there was a reported significant reduction of 0.70 log when parts were treated with the 9.904 log PFU/sample phage and 0.47 log with the 9.602 log PFU/sample phage concentration when compared to the positive control (Figure 3). The 09.301 log PFU/sample phage treatment provided a less than 0.5 log reduction in *Salmonella* while no differences ($P>0.05$) existed between the 8.602 log PFU/sample phage treatment and the positive control. Furthermore, the PAA treatments provided the optimal reduction ($P\leq0.05$) of the *Salmonella* populations on the parts. The 100 ppm PAA treatment provided nearly a 1 log reduction while the 700 ppm PAA treatment gave a greater than 1.5 log reduction compared to the positive control. Therefore, for the remainder of the study PAA was combined with phage treatment to determine the efficacy of reduction of *Salmonella* populations on the inoculated parts.

Due to PAA's known efficacy against *Salmonella* and other foodborne microorganisms, the results of the first part of this study echoed previous research performed by Bauermeister et al. (2008) and Nagel et al. (2013). According to Bauermeister et al. (2008), PAA performed better than the traditional use of chlorine in a primary chiller. Nagel et al. (2013) compared higher levels of PAA to other various antimicrobials in a Finishing Chiller® application which has a much shorter contact time (generally less than 30s). Like results suggest here, higher concentrations of PAA in both studies gave the best reduction in *Salmonella* populations. Additionally, research has

shown that PAA is not only effective at reducing overall bacterial loads on whole broiler carcasses but on poultry parts and ground products as well (Chen et al., 2014; Zhang et al., 2013).

The efficacy of bacteriophage treatments on the reduction of *Salmonella* populations is dependent on several different factors, such as the PFU/sample or PFU/mL of bacteriophage concentration as well as initial bacteria inoculum levels (Connerton et al., 2005). The sustained activity demonstrated by analyzing rinsates again at 24h has been seen in various different research. Spricigo et al. (2013) demonstrated this sustained reduction in *Salmonella* populations on inoculated pig skin with bacteriophage treatment. Pig skin was treated with bacteriophage after inoculation with a *Salmonella* cocktail. When analyzed, additional bacterial reductions of 2.2-4.0 log was observed up to 6 days post-treatment.

Conversely, Hungaro et al. (2013) compared bacteriophage treatment against other commercially used antimicrobials in a similar application. A 5 log cfu/ml inoculum of *Salmonella* enteritidis was used on broiler skin. After inoculation, the skin was exposed to either a 200ppm sodium dichloroisocyanurate solution, 100ppm PAA, a 2% lactic acid treatment, or bacteriophage cocktail containing 9 log PFU/mL. From this, no significant difference in bacterial reductions was observed up to several days after treatment.

Although the 9.904 log PFU/sample phage concentration provided the best *Salmonella* reduction (P≤0.05) on parts treated with varying concentrations of phage, the 9.602 log PFU/sample phage concentration was selected to evaluate in combination with chlorine and PAA treatment. This was due to the fact that the reduction in *Salmonella* populations seen with both concentrations are thought to be adequate to eliminate the remaining bacterial cells at the end of any post-chill interventions. Therefore, the second objective of the study was to test the efficacy of the 9.602 log PFU/sample SalmoFresh™ concentration in combination with chlorine and PAA dips to simulate a primary chiller application. This entailed testing a spray application of bacteriophage following an antimicrobial dip for 1h. The antimicrobial dips included a solution of 35 ppm chlorine and 90 ppm PAA, with and without bacteriophage treatment. A chilled water dip was likewise utilized as a control to determine if there were any washing effects. The negative control again was below the detection limit of 0.69 log.

The results of the 0 h analysis indicated that 9.602 log PFU/sample phage treatment combined with 90 ppm PAA gave the best reduction (P≤0.05) in *Salmonella* populations than the other treatments evaluated, providing approximately a 1.5 log reduction. The treatment of 35 ppm chlorine plus phage and water plus phage gave approximately a 1.2 and 1.3 log reduction, respectively, when compared to the positive

control (figure 4). The 9.602 log PFU/sample phage spray resulted in less than 0.5 log reduction compared to the positive control when applied alone. Furthermore, no differences ($P>0.05$) existed between the water, 35 ppm chlorine, and 90 ppm PAA dip, all resulting in approximately a 1 log reduction in *Salmonella* populations.

When the inoculated parts were analyzed at 24 h, the results indicated that treatment with 90 ppm PAA plus 9.602 log PFU/sample bacteriophage gave the best results ($P\leq0.05$) of the various treatments with a 1.5 log reduction in *Salmonella* populations when compared to the positive control (Figure 5). At 24 h, no differences ($P>0.05$) existed between the water plus phage treatment or the 35 ppm chlorine plus phage treatment and resulted in approximately a 1 log reduction. The 90 ppm PAA dip resulted in approximately a 1 log reduction whereas no differences ($P>0.05$) existed between the 9.602 log PFU/sample phage, water dip, and 35 ppm chlorine dip which all resulted in a less than 1 log reduction. What is most important in regards to these results is the sustained bacteriophage activity. Based on research conducted in our lab, PAA has been shown to have no residual activity post-treatment, therefore all additional reductions observed after initial treatment are due to sustained bacteriophage activity. Ideally, sustained antimicrobial activity is desired in eliminating bacterial populations in food products in an industrial setting.

Both chlorine and peracetic acid have their own unique mechanisms by which that are able to reduce bacterial populations. Peracetic acid has been widely known for its efficacy is reducing bacterial loads, as well as viral, fungal, and yeast, populations for a broad number of applications (Kitis, 2003). It is currently used as an antimicrobial intervention by the majority of poultry processors in the United States (McKee, 2011). Additionally, PAA is not as affected by organic load such as chlorine (Kitis, 2003).

The mechanism by which PAA works in order to eliminate bacterial populations is that PAA serves as a strong oxidizer, stronger than that exhibited by chlorine (Kitis, 2003). It has also been proven to be more effective than hydrogen peroxide at various microorganisms because it has a lower effective dosage than that required of hydrogen peroxide (Kitis, 2003). According to a study conducted by Bauermeister et al, when examining the effects of PAA on the elimination of *Salmonella* on broiler carcasses, it was determined that there was a 92% reduction of *Salmonella* populations when a concentration of 85ppm was used. This was used in direct comparison to a concentration of 30ppm chlorine which illustrated only a 57% reduction in *Salmonella* populations (2008). Due to this marked difference in bacterial reduction with the use of PAA, this is why this is the most commonly used antimicrobial in poultry processing plants to date (McKee, 2011).

Chlorine is approved for application for the treatment of poultry carcasses as well as poultry parts and other organs at levels of 50-150 ppm for pre-chill application (USDA-FSIS, 2014). However, in a primary chiller the chlorine concentrations should be approximately 30ppm to be the most effective (Russell, 2012). In order for chlorine to exhibit the most bactericidal activity, the pH of chlorine solution needs to remain at approximately 6. Deviation from this pH can negatively affect the efficacy of the chlorine treatment. When chlorine is added into an aqueous solution, it reacts with water forming hypochlorous acid. Hypochlorous acid is the active antimicrobial compound, and it is approximately 80 times more effective than a hypochlorite ion (which can be formed when the pH of the chlorine system is not adequately adjusted) (Kim et al, 2000). Bacteria are destroyed when using chlorine as a disinfectant by effectively inhibiting glucose oxidation (Kim et al, 2000). However, one major downfall of using chlorine has an antimicrobial is that its efficacy is lessened with the amount of organic load is present in the system. Organic load present in your chiller water is an inevitable in poultry processing due to the fact that some unsaturated fats present in poultry meat will be liquefied during the scalding process (Russell, 2012). However, in the current study, chlorine was more effective than water at reducing *Salmonella* populations due to the fact that all poultry was exposed to the 1-1.5 hours necessary for optimal effectiveness (McKee, 2011).

Bacteriophages are some of the oldest living organisms on the planet. Since their discovery, technology has evolved to the point where they can now be customized and utilized as antimicrobial in various applications. With the development of SalmoFresh™ and its standing as a GRAS substance by USDA, it is gaining ground in the poultry processing industry. One of the main reasons bacteriophage usage has been increasing in the past 10 years is due to the fact that there is a rise in antibiotic resistant bacterial strains (Connerton and Connerton, 2005). Lytic bacteriophages, the type that is used in the SalmoFresh™ product, kill their specific host bacteria by over-taking their cellular metabolism. Once the bacteriophage merge inside the bacterium, they replicate until the bacterial host cell lyses and then releases more bacteriophages into the system. This helps to propagate and maintain bacteriophage levels (Toro et al., 2005).

A study conducted by Bielke et al, in which bacteriophages were used to treat broiler carcasses which were inoculated with *Salmonella enterica* serovar Enteritidis or *Salmonella* Typhimurium showed that there were no detectable levels of *Salmonella* spp. residing on the carcass after analysis (Bielke, 2007). This was after an application of 9 log PFU/carcass treatment administered via a spray application as well. An additional study demonstrated the effectiveness of bacteriophage therapy in reducing *Salmonella* populations on poultry breasts. Poultry breasts were inoculated with a 6 log *Salmonella* Typhimurium and Enteritidis cocktail inoculum. The breasts were then treated with

bacteriophages (9 log PFU/mL) through a dip application and samples were subsequently analyzed at 1, 2, 5, and 7 days post application when samples were held at refrigeration temperatures (4°C). When bacterial colonies were numerated, there was a 1-2.2 log reduction upon each analysis (Spricigo et al, 2013). This experiment clearly demonstrates the desired sustained bacteriophage activity shown in this current study.

Overall, this study indicated that there is a significant difference in the reduction of *Salmonella* populations when a bacteriophage application is used in conjunction with antimicrobials in a primary chiller setting. By placing bacteriophage application here, it is possible to reduce the incidence of cross-contamination and lower *Salmonella* counts on poultry parts in the plant. This is paramount to poultry processors due to the fact that there are anticipated *Salmonella* performance standards to be issued by USDA-FSIS in the near future. In addition, this can also allow poultry processors to put the "natural" label on their product as they are not considered to be a chemical intervention (Kocharunchitt et al, 2009). Studies have also been conducted which conclude that bacteriophage application has no adverse effect on the organoleptic properties of the final product that one may see with use of commercial antimicrobials (Hungaro et al, 2013). One major hurdle that the industry will have to overcome is a more cost-effective way of manufacturing such a treatment (Oliveria et al., 2012). Even so, this

may provide the industry a way to meet anticipated *Salmonella* performance standards and combat the emerging problem of antibiotic-resistant strains of *Salmonella*.

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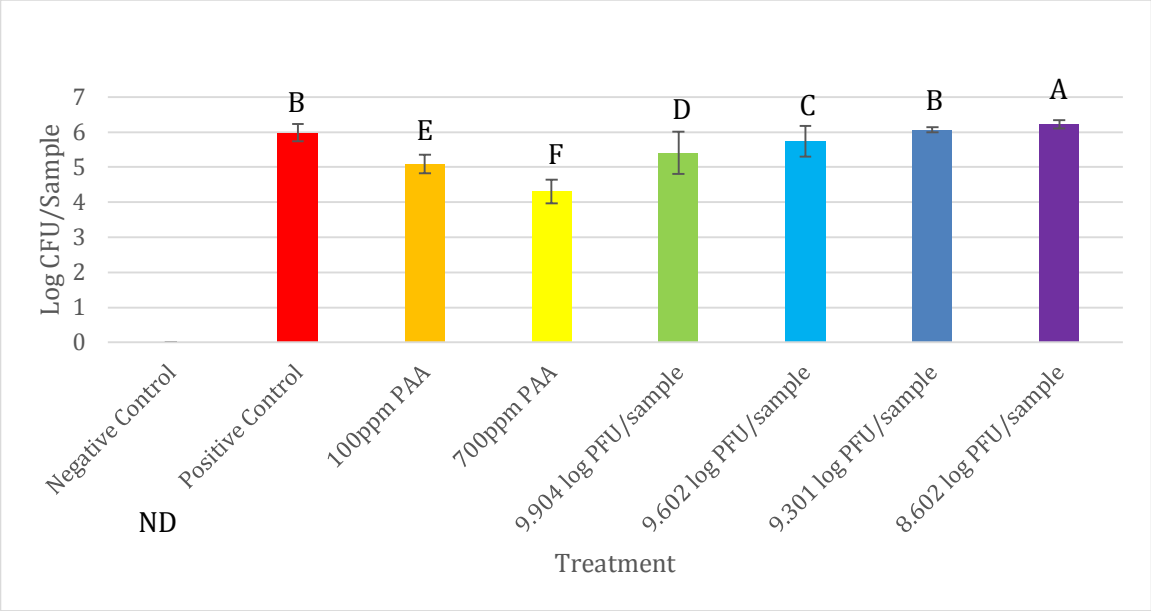
Figure 1: *Salmonella* spp. recovered at 0 hours from inoculated poultry parts (n=160) treated with various concentrations of bacteriophage and peracetic acid

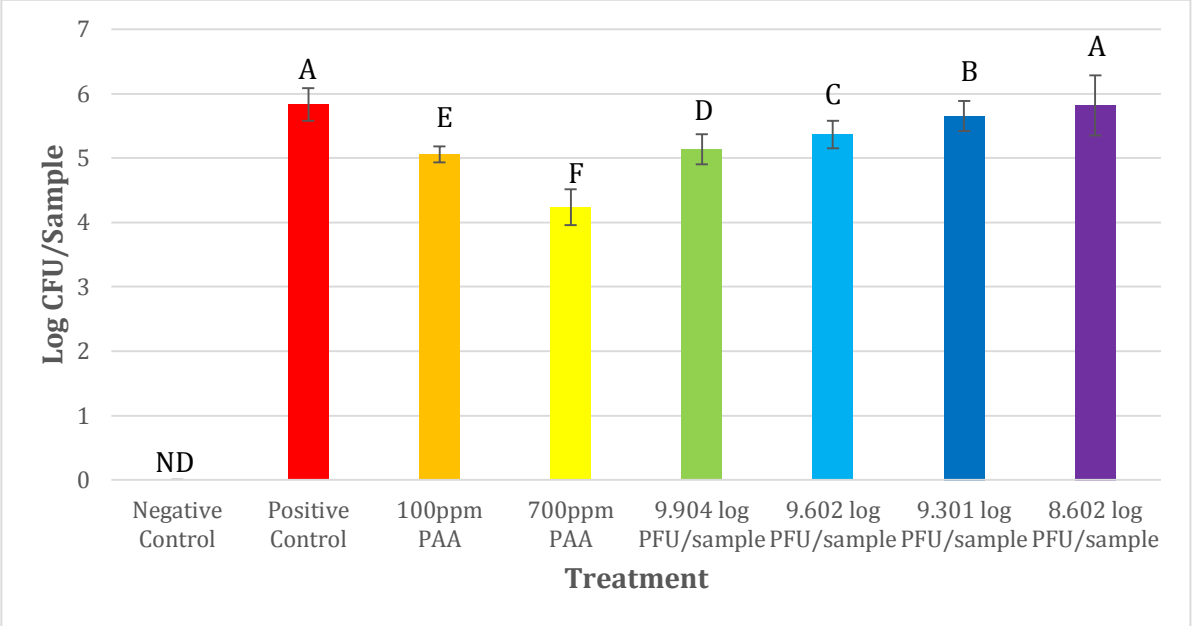


[1] Reported as mean log colony-forming units of *Salmonella* spp. per sample for each treatment group

ND = Not detectable; $\log_{10}$ CFU < 0.69

[a-f] Means with no common letter differ significantly (P≤0.05)

Figure 2: *Salmonella* spp. recovered at 24 hours from inoculated poultry parts (n=160) treated with various concentrations of bacteriophage and peracetic acid

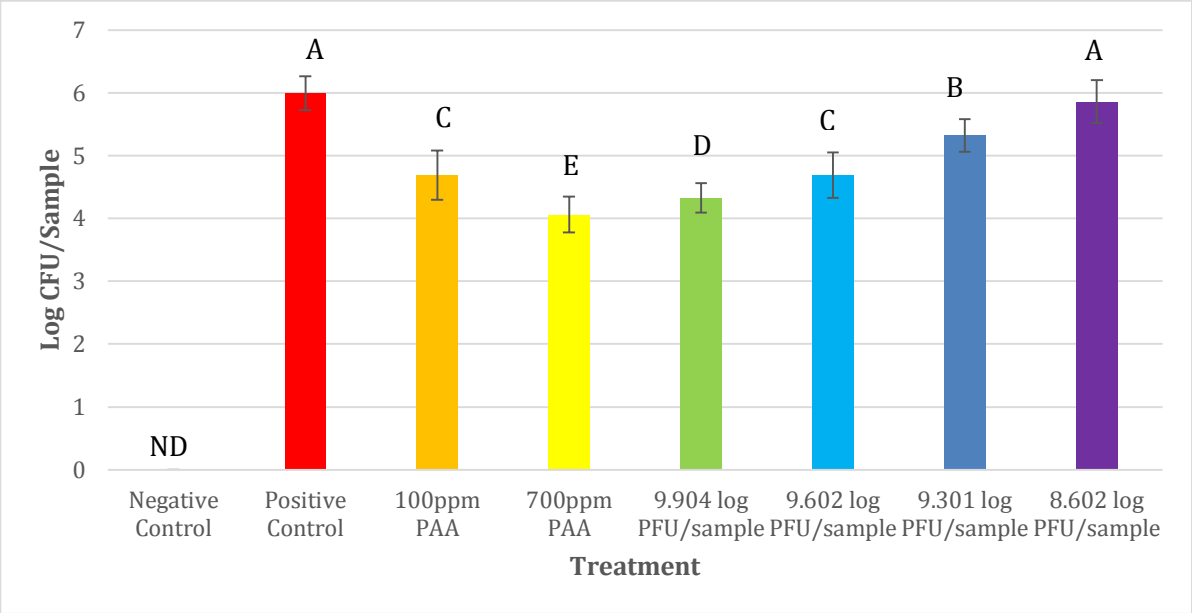


[1] Reported as mean log colony-forming units of *Salmonella* spp. per sample for each treatment group

ND = Not detectable; $\log_{10}$ CFU < 0.69

[a-f] Means with no common letter differ significantly (P≤0.05)

Figure 3: *Salmonella* spp. recovered from 0 hour samples analyzed again at 24 hours from inoculated poultry parts (n=160) treated with various concentrations of bacteriophage and peracetic acid

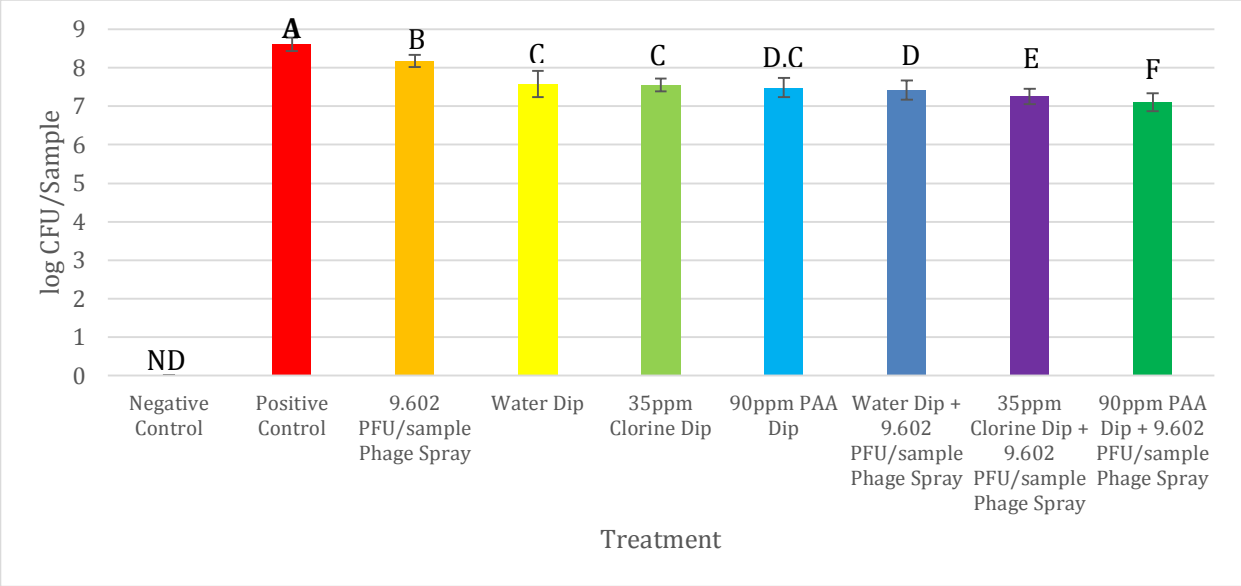


[1] Reported as mean log colony-forming units of *Salmonella* spp. per sample for each treatment group

ND = Not detectable; $\log_{10}$ CFU < 0.69

[a-f] Means with no common letter differ significantly (P≤0.05)

Figure 4: *Salmonella* spp. recovered from 0 hour samples from inoculated poultry parts (n=135) treated 9.602 log PFU/sample SalmoFresh™ with and without various antimicrobial interventions

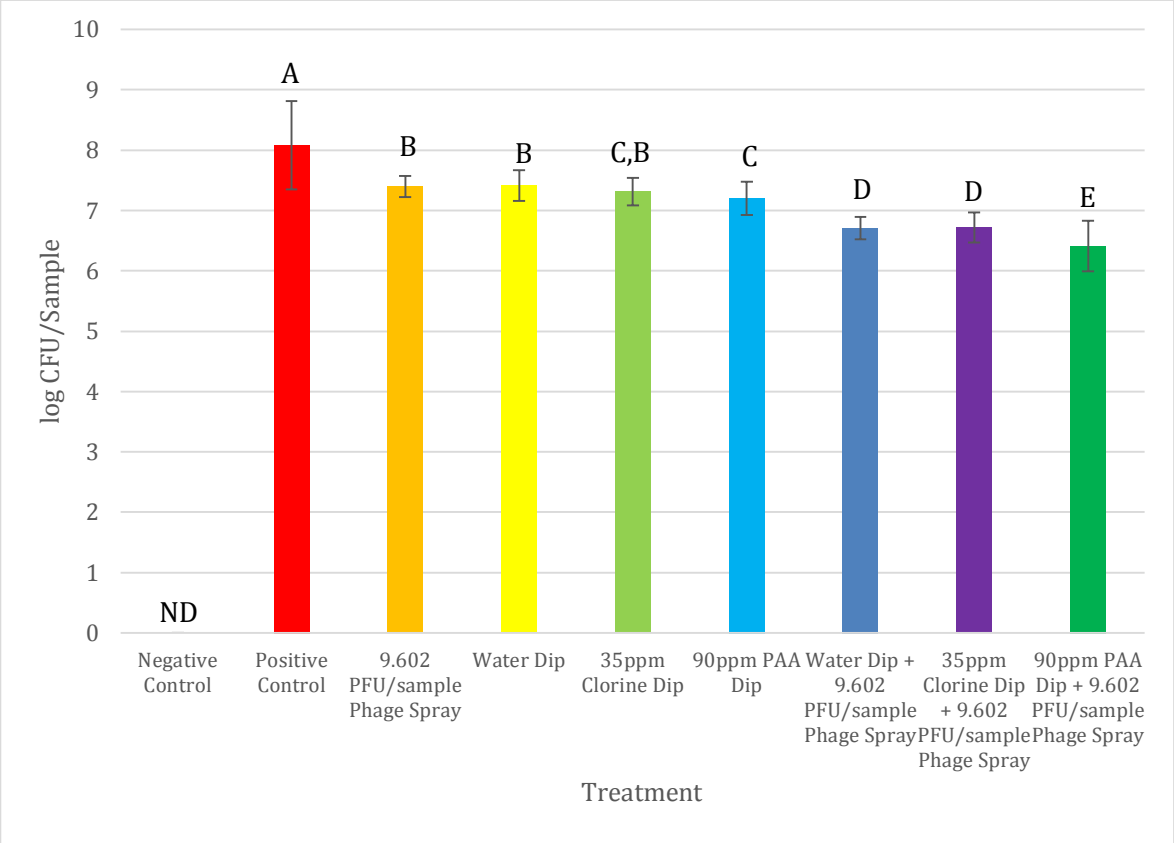


[1] Reported as mean log colony-forming units of *Salmonella* spp. per sample for each treatment group

ND = Not detectable; $\log_{10}$ CFU < 0.69

[a-f] Means with no common letter differ significantly (P≤0.05)

Figure 5: *Salmonella* spp. recovered from 24 hour samples from inoculated poultry parts (n=135) treated 9.602 log PFU/sample SalmoFresh™ with and without various antimicrobial interventions



[1] Reported as mean log colony-forming units of *Salmonella* spp. per sample for each treatment group

ND = Not detectable; $\log_{10}$ CFU < 0.69

[a-e] Means with no common letter differ significantly (P≤0.05)

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