Application of 1,3-Dibromo-5, 5-Dimethylhydantoin as a Post-Chill Immersion and Spray Application to Reduce *Campylobacter Jejuni* Populations on Poultry

by

Jacob Mitchel Smith

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Approved by

Manpreet Singh, Chair, Associate Professor Department of Poultry Science
Shelly McKee, Associate Professor Department of Poultry Science
Sacit Bilgili, Professor and Extension Scientist, Department of Poultry Science

ABSTRACT

Chemical antimicrobials are widely used throughout poultry processing to reduce and/ or eliminate poultryborne pathogens that could potentially cause illness in humans. Recent regulatory guidelines mandate that certain pathogens, specifically *Campylobacter* spp., must be monitored and maintained within regulatory limits. Therefore, *in-vitro* studies were conducted to assess the applicability of 1, 3-Dibromo-5, 5-Dimethylhydantoin (DBDMH) as an antimicrobial treatment against *Campylobacter jejuni*. Results from *in-vitro* studies showed that DBDMH was successful in reducing the survival populations of *C. jejuni* below detectable levels after 30 s of exposure. From this knowledge, *in-vivo* studies were carried out to compare the efficacy of multiple concentrations of DBDMH solutions to other commercially used antimicrobials (Peracetic Acid; PAA and Sodium Hypochlorite; SH). Various concentrations of DBDMH, PAA, and SH were used to treat *C. jejuni* inoculated fresh poultry carcasses using both spray and immersion applications. *In-vivo* studies demonstrated that DBDMH reduced the survival populations of *C. jejuni* on poultry carcasses; and has an improved efficacy ($P \leq 0.05$) when applied as an immersion application rather than a spray. When compared to other commercially available antimicrobials, DBDMH showed to be more effective than SH ($P \leq 0.05$), but not as effective as PAA. These studies demonstrated that DBDMH is an effective antimicrobial to reduce populations of *Campylobacter* on fresh poultry carcasses when used in short contact time exposures.

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CHAPTER 1: INTRODUCTION

Consumption of poultry and poultry products in the United States has risen steadily during the last decade. The commercial poultry industry is a highly competitive and efficiency driven agri-sector. The health and growth efficiency of broilers during live production, efficiency during processing, and consistency in quality during manufacture are given high priority. This industry is consistently challenged by factors such as changing economic conditions, awareness and misconceptions regarding foodborne illnesses, and changes in meat consumption patterns. The contamination of poultry products, both raw and value-added, is of prominent concern to consumers, regulators, and public health agencies.

Throughout the mid- to late 1990's, the United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) focused on regulating the prevalence of *Salmonella* on raw poultry products. Most recently, the industry has maintained steady control of *Salmonella* and attention has shifted in a new direction, toward *Campylobacter*. In the past year performance standards have been implemented concerning *Campylobacter* on raw poultry. *Campylobacter* are Gram-negative organisms that commensally colonize the avian intestinal tract and proliferate at temperatures around 37 to 42°C. More specifically, *Campylobacter jejuni, Campylobacter coli, and Campylobacter lari* are very commonly associated with poultry products. *Campylobacter* grows most preferably under microaerobic conditions (5% N_2, 10% O_2, 85% N_2), which is why the intestinal tract of commercial broilers is such a favorable environment for its

growth. A few types of laboratory media have been developed for the selective isolation of *Campylobacter* but those providing the best recovery of *Campylobacter* possess antibiotics for selectivity as well as an oxygen-scavenging component to decrease the amount of free oxygen in their incubation environment. As is the case with many other bacterial pathogens, *Campylobacter* can enter into a Viable But Nonculturable (VBNC) state. This is a highly debated subject and VBNC state *Campylobacter* will not exhibit growth on traditional laboratory media although the pathogen can return to a culturable state and retain pathogenicity. Studies to define the pathogenic mechanism of *Campylobacter* have been inconclusive although specific genes are needed for intestinal colonization. Furthermore, the infective dose of *Campylobacter* for humans has been reported to be as low as 500 organisms.

In the United States, *Campylobacter* has a considerable impact on public health each year. The Centers for Disease Control and Prevention (CDC) estimates over 800,000 episodes of foodborne illness are attributed to *Campylobacter* annually. In most cases, the symptoms are gastroenteritis for a few days but severe cases can lead to hospitalization and death. Retrospective public health studies have also linked campylobacteriosis with many other long-term conditions such as Guillain-Barre syndrome and reactive arthritis. Furthermore, recent pathogen-food pairings have attributed poultry to having the greatest public health impact of all food products. Thus, interventions to aid in the control of *Campylobacter* for the commercial poultry industry are warranted.

In the United States, the temperature of broiler carcasses is reduced using large, immersion chillers with capacities of up to 40,000 gallons. The immersion approach is in

contrast to alternative systems which utilize cold air at high flow rates to reduce the temperature of carcasses. The benefit of air chilling is that carcasses do not come into direct contact with one another and cross-contamination is kept to a minimum. On the other hand, the immersion approach allows carcasses to freely come into contact with one another and this can lead to cross-contamination.

In an attempt to reduce and eliminate *Campylobacter*, researchers have used a variety of antimicrobial treatments and application methods, especially during the immersion chilling process. The primary reasons for using antimicrobial treatments during immersion chilling is because of extended contact time, the overall washing effect on carcasses, and a further scrubbing effect that is provided to reduce attached bacteria. In laboratory settings, thermal and irradiation treatments have shown promising results to control *Campylobacter* inoculated products, but their use in commercial broiler processing facilities is limited. Chemical treatments such as Sodium Hypochlorite (Chlorine), Peroxyacetic Acid (Peracetic Acid), Cetylopyridinium Chloride (CPC), and TriSodium Phosphate (TSP) on the other hand have shown to be effective in reducing the survivability of *Campylobacter* on raw poultry products. Each of these treatments has their own advantages and disadvantages for processors, but ultimately the decision amounts to finding a safe, cost-effective, and reliable product that is a proven food safety solution.

Limited research has been carried out applying 1,3-Dibromo-5, 5-Dimethylhydantoin (DBDMH), especially as it relates to *Campylobacter jejuni* inoculated raw poultry products. This is a chemical antimicrobial treatment that is approved for use at multiple stages during poultry processing at concentrations up to 200

ppm. Compared to some other widely available antimicrobials, DBDMH can be easier to apply to poultry products and is also more stable in the presence of organic matter. Much attention has been given to the application of DBDMH as either a pre- or post-chill applications on poultry products. Therefore, the present study was undertaken to investigate the impact of DBDMH, peracetic acid, chlorine, and water (10°C) as both spray and immersion applications on broiler carcasses inoculated with *Campylobacter jejuni*.

CHAPTER 2: LITERATURE REVIEW

2.1 THE HISTORY OF *CAMPYLOBACTER*

It is now known that the first report regarding *Campylobacter*, at the time a nonculturable spiral-shaped bacterium, was observed and described by bacteriologist and pediatrician Theodor Escherich in 1886 after examining the colons of deceased children and reporting the cause of death as 'cholera infantum.' Recent translation of Escherich's findings (Escherich, 1886) by Kist (1986) aided in the historical background of *Campylobacter*. Although Escherich was not able to identify the organism as *Campylobacter*, Kist (1986) provided justification that the organism was, most likely, *Campylobacter* based on: (1) the typical spiral morphology, (2) the association with enteritis in neonates, infants and kittens, (3) the inability to grow on solid medium even though detected microscopically and (4) since no other bacteria with similar morphology has been associated with enteric human infection.

The first isolation of *Campylobacter* was reported from bovines and ovines experiencing spontaneous abortions in England (McFaydean and Stockman, 1913). Smith and Taylor (1919) named the apparently identical organism isolated earlier by McFaydean and Stockman *Vibrio fetus* after isolation a few years later from the same sources. *Vibrio*-like organisms were described by Smith and Orcutt (1927) after isolation from the diarrhea of cattle. An apparent connection between vibrios and bovine dysentery was described and characterized by Jones *et al*. (1931a,b,c). Similarly, Doyle (1948) was

able to isolate a vibrio, then called *Vibrio coli*, from the feces of pigs experiencing diarrhea.

The blood samples of women experiencing spontaneous abortions were another isolation source of these bacteria which would eventually be called Campylobacters (Vinzent *et al*., 1947). Later Florent (1953) was able to demonstrate that two similar vibrios were causing fertility problems in cows and ewes. The genus *Campylobacter* was created because of differences such as low DNA base composition, microaerophilic growth requirements and nonfermentative metabolism between *Campylobacter* and *Vibrios* (Sebald and Véron, 1963). A filtration step employed by Butzler *et al*. (1973) helped to separate and isolate Campylobacters from fecal samples but it was not until 1977 (Skirrow) that selective culture media was formulated for the routine isolation of 'Campylobacters.' Referred to as Skirrow agar this was a mixture of vancomycin (10 mg/L), polymyxin B (2.5 IU/mL), and trimethoprim (5 mg/L), which were added to basal medium and provided the means for growth and further studies of the organism.

The infective doses of organisms are studied for epidemiological purposes and to help determine their disease causing mechanisms. The infective dose of *Campylobacter jejuni* was documented firsthand by Robinson in 1981 after he consumed 500 organisms of a known culture of *C. jejuni* in 180 mL of pasteurized milk. In addition to providing insight as to the amount of cells required to elicit symptoms this experiment was also the first time Koch's postulates had been met for *C. jejuni* in humans.

After the development of selective isolation media many different Campylobacters were isolated and identified in the 1980s. This also led to the differentiation among the Campylobacters and hence the term "*Campylobacter*-like

organism" became widely used and phylogenetic data was also compiled (Lau *et al*., 1987). This helped facilitate the naming of the *Campylobacteraceae* family (Vandamme and DeLey, 1991). More recently the use of biochemical tests has enabled routine species-level differentiation of Campylobacters (On *et al.,* 2001).

2.2 *CAMPYLOBACTER* AND CAMPYLOBACTERIOSIS

The genus *Campylobacter* belongs to the family *Campylobacteriaceae* and consists of 17 species (Sebald and Véron, 1963). *C. jejuni*, *C. coli*, and *C. lari* comprise the thermotolerant group within this family and they are mesophilic, microaerophilic, Gram negative, motile, spiral rods. The Centers for Disease Control and Prevention (CDC) estimates that over 800,000 cases of campylobacteriosis occur in the U.S. annually and 80% of these are foodborne (Scallan *et al.,* 2011). Furthermore, in the U.S. it is the leading cause of hospitalizations (over 8,000 annually) and is associated with approximately 76 deaths each year (Scallan *et al.,* 2011).

Symptoms commonly associated with campylobacteriosis include fever, headache, muscle pain, diarrhea that oftentimes contains blood and mucus, and severe abdominal cramps (Sahin *et al.*, 2012). Guillain-Barré syndrome (GBS) (McCarthy and Giesecke, 2001), and reactive arthritis (Hannu *et al.*, 2002) are chronic sequelae associated with *C. jejuni* infection, although neither disease state has yet to be completely understood. Indication of recent *C. jejuni* infection has been found in as many as 1 of 4 cases of GBS (McCarthy and Giesecke, 2001) and as many as 7% of patients with reactive arthritis (Hannu *et al.*, 2002).

Campylobacter also can enter the Viable But Nonculturable (VBNC) state and these are considered a degenerative rather than dormant stage (Debruyne *et al*., 2008).

This state is a highly debated topic and a state that many common foodborne pathogens enter (Oliver, 2010). Campylobacters entering this state have a lowered metabolic activity and are often able to regain pathogenicity after they are revived to a culturable state. Inducers of the VBNC state can be factors such as dramatic temperature changes, available nutrient changes, and oxygen availability to name a few (Oliver, 2010). VBNC-*Campylobacter* can also adhere to chicken skin at 4, 25, and 37°C (Jang *et al*., 2007) and their ability to attach to stainless steel coupons increases over time (Nguyen *et al*., 2010).

As a zoonotic pathogen, *Campylobacter* is widespread in food-producing animals. Improper handling and consumption of undercooked poultry products remain prevailing vehicles for campylobacteriosis (Batz *et al*., 2011) and much attention is given to postmortem, raw products. In commercial broilers, *Campylobacter* results from antemortem colonization with no apparent clinical symptoms in poultry, amplifying the problem of antemortem identification and intervention of *Campylobacter* (Hargis *et al*., 2001). Byrd *et al.* (2001) demonstrated that supplying lactic acid (0.44%) during feed withdrawal provided significant reductions in postharvest *Campylobacter* contamination. Competitive exclusion studies have shown significant reductions in colonization rates of *C. jejuni* (Soerjardi-Liem *et al*., 1984, Schoeni and Wong, 1994). Additional antemortem studies to reduce *C. jejuni* colonization of chicks have shown different degrees of success (Soerjadi, *et al*., 1982, Shanker, *et al*., 1988, Stern *et al*., 1995, Shanker and Sorrell, 1990,) and further emphasize the need for intervention strategies in broiler processing facilities.

2.2.1 THE PATHOGENESIS OF *CAMPYLOBACTER*

The pathogenic mechanism of *Campylobacter* has not been fully determined due in part to the lack of a reliable animal model (Fox, 1992). A few virulence factors that have been studied are its ability to invade cells, flagella and motility, and the toxins that it produces.

Campylobacter has the ability to invade *in vitro* cell lines (Konkel and Joens, 1989) and Konkel *et al*. (1992) also discovered it can translocate intestinal epithelial cells. Additionally, *Campylobacter* may interact with M cells (Microfold cells) along the epithelium of the Peyer's patch which may facilitate transport of the pathogen from the intestine (Walker *et al.*, 1988) although the internalization mechanism is not known.

The full length, in-tact, polar flagella of *Campylobacter* appears to be an important source of its invasion-translocation properties independent of the organism's motility (Grant *et al.*, 1993). From a genetic standpoint the *flaA* and *flab* genes are both involved in the expression of the flagellar filament. While these genes are located adjacent to one another in *C. jejuni* and *C. coli* (Nuijten *et al*., 1990) *flaA* appears to play a more important role in gut colonization than *flab* (Nachamkin *et al*., 1993). Furthermore, non-motile and adhesion-lacking strains appear to also be avirulent (Black *et al.*, 1988).

A recent study described the production of a cholera-like enterotoxin by *C. jejuni* (Daikoku *et al.*, 1990), but a similar study repudiated these findings (Perez-Perez *et al.*, 1992). Specific toxins have been described, such as Cytolethal Distending Toxin (Johnson *et al.*, 1988), however no toxin activity has been able to be recovered from the tissues of *Campylobacter* infected animals (Everest *et al.*, 1993). Cover *et al*. (1990)

showed that cytotoxic activity was found in patients both with and without campylobacteriosis, which led to uncertainties of the role of the toxin. Further studies need to be conducted in order to provide a clear role of this toxin and the pathogenesis of *Campylobacter* in general.

2.2.2 SELECTIVE MEDIA FOR *CAMPYLOBACTER* ISOLATION

Consistent laboratory culturing and research into *Campylobacter* was not possible until the evolution of selective culture media, the first of which was developed by and named for Skirrow (1977). Direct enumeration of *Campylobacter* from contaminated sources such as poultry carcass rinses is a highly valuable tool for both researchers and regulatory agencies alike. Two commonly used media types are Campy-Cefex agar and modified charcoal, cefoperazone, desoxycholate agar (mCCDA), the selectivity of which is determined by the antibiotics supplements used. Oyarzabal *et al*. (2005) found that although Campy-Cefex provided the best isolation and enumeration of *Campylobacter* it was statistically similar to other frequently used agars, such as mCCDA. Of equal importance to the selectivity of the media is the atmosphere provided for the agar plates to grow *Campylobacter*. This should be a microaerophillic gas mixture containing 10% CO_2, 5% O_2, and 85% N_2 with an incubation period ranging from 24 to 48 h.

Developed by Stern *et al*. (1992), Campy-Cefex agar consists of Brucella agar base, ferrous sulfate (0.05%), sodium pyruvate (0.05%), sodium bisulfite (0.02%). It is supplemented with lysed horse blood (5%) and cefoperazone (33 mg/L) and cyclohexamide (200 mg/L) after sterilization. Lysed horse blood assists in scavenging oxygen while plates are being incubated. Cefoperazone inhibits the growth of other Gram-negative intestinal microflora that can be found alongside *Campylobacters*.

Charcoal based agars, such as mCCDA, are also supplemented with cefoperazone sodium salt (32 mg/L) after sterilization for the same characteristics as those in Campy-Cefex. Charcoal is an equally acceptable source to create oxygen scavenging conditions (Oyarzabal *et al.*, 2005) but unlike blood its addition is not required after sterilization.

2.3 POULTRY PROCESSING

The conversion of live poultry to foodstuffs is the process referred to as poultry processing. More specifically, this process involves converting the muscle tissues of live broiler chickens to meat intended for human consumption. Poultry processing is a highly automated and multi-step process that originates with commercially housed broilers and culminates with many different products in numerous market-ready forms. The study and quantification of the microflora, particularly its potential reduction, is a very important aspect of the final products and can be affected by many different elements throughout the growout and processing of broilers.

Environmental and management impacts related to commercial poultry husbandry have been extensively studied to optimize growth rate while maintaining the highest quality and welfare conditions possible. Similarly, the areas of nutrition, genetics, and disease control have been equally vital to the development and progress of the commercial broiler industry. In addition to the technical developments in the area of poultry science have been, another correspondingly important factor in the growth of this industry has been vertical integration. Vertical integration involves bringing together the various aspects of poultry production under one independent business structure and includes, although is not limited to, breeding, incubation, husbandry, processing, marketing, and sales (Fletcher, 2004). Overall, vertical integration has provided

tremendous flexibility in improving the operational efficiency due to more control over the costs associated with poultry production.

Poultry processing begins long before carcasses are transported to processing facilities. Feed and water are withdrawn so that the intestinal tract is devoid of any undigested material at the time of processing. Ideally, this time is limited to between eight and ten hours before the bird is to be processed.

At the broiler processing plant, the primary processing begins with unloading the carcasses from the transportation vehicle. They are then hung onto individual shackles in an environment with dark lighting to minimize bird activity; followed by stunning using a saline solution to render them insensitive to pain, killed by bleeding followed by scalding and defeathering. Usually, mechanical removal of head, feet, and viscera follows and carcass chilling ensues. An inside-outside bird washer (IOBW) is commonly used prior to the chilling process. Antimicrobials can be used in the IOBW, but it is important to note that the mechanical force from the sprayers can also account for bacterial reduction. Chillers and IOBWs have been studied extensively in laboratory and commercial settings and are typically steps in which processors are able to achieve bacterial reduction at higher rates compared to other steps when these processes are managed appropriately.

Post-chill immersion interventions, also referred to as finishing chillers, which specifically target *Salmonella* and *Campylobacter* are gaining popularity due to more stringent performance standards set forth by the USDA-FSIS (United States Department of Agriculture-Food Safety Inspection Service, 1996, 2009, 2011, 2012).

2.4 POULTRY CONTAMINATION BY *CAMPYLOBACTER* AT THE PROCESSING PLANT

It is generally accepted that *Campylobacter* colonize the avian gut as a commensal organism, therefore contamination of poultry products during primary processing occurs in a number of different ways. *Campylobacter* occurs in the feces of live poultry therefore contamination of the external surface of the chickens before processing is highly plausible. Steps such as scalding, picking, eviscerating, cropping, and chilling are all chances in which cross contamination can reasonably occur. All of these steps serve as a probable source of contamination and cross-contamination because bacteria can be transmitted by equipment that preforms this task from one carcass to the next. For example, transmission of bacteria during chilling is plausible because a carcass which is *Campylobacter* negative could come into contact with a carcass which is positive during the immersion process (Potturi-Venkata, *et al.*, 2007). All of these steps can lead to contamination of fresh poultry products at the retail level which, if handled improperly, can be vectors for foodborne illnesses. A study evaluating the contamination of poultry products by *Campylobacter* at retail reported greater than 80% prevalence (Kramer *et al.*, 2000).

In 1996, the USDA-FSIS implemented a "zero tolerance" pre-chill performance standard for visible fecal material as part of their final ruling on the implementation of the Hazard Analysis and Critical Control Point (HACCP) systems for meat and poultry production. HACCP is a systematic and preventative approach to food safety that identifies physical, chemical, and microbiological hazards. HACCP utilizes Critical Control Points (CCP) where hazards can either be reduced or eliminated to monitor process control (USDA, 1996). Processors were troubled by establishing measures to

comply with the pre-chill zero visible fecal contamination regulations and resulted in increasing carcass washers and wash systems and some plants even increased water use by as much as 50% after the implementation of HACCP (Jackson *et al.*, 1999). The primary source of concern to processors stemmed from the idea that the application of pre-chill performance standards ignored the antimicrobial effects of the immersion chilling process (Bilgili *et al.*, 2002). To make an objective assessment of the scientific basis of a zero-tolerance standard for ingesta, Bilgili *et al.* (2002) demonstrated that there is a lack of correlation between the pre-chill presence of visible ingesta and microbial contamination on poultry carcasses post immersion chilling in seven different commercial processing facilities.

The research and investigation of intervention strategies to more efficiently produce safe and wholesome meat and poultry products with minimal water use is especially necessary to allow poultry processors to economically produce such products. Many antimicrobial intervention strategies have been researched and are currently in use by processors (McKee, 2010).

2.5 USDA-FSIS *CAMPYLOBACTER* PERFORMANCE STANDARDS

HACCP is an integral part of managing a successful food safety system for any food product. A multi-hurdle approach is an important factor in HACCP systems and helps to control harmful microorganisms in raw meat and poultry products. These practices can have substantial effects on helping processors successfully achieve USDA-FSIS performance standards for both *Campylobacter* and *Salmonella*.

From July 2007 to June 2008 the USDA-FSIS conducted a Nationwide Microbiological Baseline Data Collection Program, examining 6,550 carcass rinse

samples, which revealed that the post-chill prevalence of *Campylobacter* was 40.23% positive. This data also showed that 99.8% of the samples taken were below 3 $\log_{10}$ CFU/mL. This sampling led to the implementation of *Campylobacter* performance standards on July 1, 2011 (75 FR 27288). For young chickens post-chill *Campylobacter* should not be greater than 10.4% positive. The sampling technique used utilizes a 1 mL portion of a 400 mL carcass rinse microbiologically plated onto four separate agar plates (0.25 mL per plate) to detect higher levels of contamination. A carcass is considered positive for *Campylobacter* by USDA-FSIS if at least one colony appears on one of the four Campy Cefex plates.

Along with the new *Campylobacter* performance standards, the USDA-FSIS has also issued updated *Salmonella* performance standards (USDA, 2009). While these performance standards seem to be logical means to reduce cases of human salmonellosis, and the new *Campylobacter* performance standards by the same logic should reduce human campylobacteriosis, these rates have seen very little impact over the course of the introduction of these performance standards (Russell, 2012).

2.6 INTERVENTIONS TO CONTROL *CAMPYLOBACTER*

Many interventions strategies have been investigated to eliminate *Campylobacter* both ante-mortem and post-mortem. The bactericidal activity of intervention treatments relies heavily on the disruption of an organism's physiological processes and treatments in experimental research are validated based on the $\log_{10}$ scale. These intervention processes can be categorized as: thermal, irradiation-based, and chemical based.

2.6.1 THERMAL INACTIVATION

Campylobacter is not only found in raw poultry products, they are also commonly isolated from raw, unpasteurized milk (Dupont, 2007). Since *Campylobacter* proliferates at 42-43°C the thermal tolerance and inactivation of these organisms have been studied in liquid milk products. High temperature short time (HTST) pasteurization of raw milk at 60°C provided a 4 $\log_{10}$ reduction in *Campylobacter* with only 16 s of exposure (D'Aoust *et al.*, 1988). This method is practical for liquid products, but raw poultry products cannot be treated in the same manner. If poultry carcasses or parts are exposed to heat the meat proteins will denature leading to either partial or complete cooking of the meat. In turn, this will render the products either partially or fully cooked depending on the product matrix. This would decrease consumer acceptability of raw poultry products.

Thermal treatments tend to bridge the gap between product safety and quality concerns in poultry processing. After utilizing what is known as a second scalding on broiler carcasses, Berrang *et al*. (2000) found that such a treatment gentle enough not to alter carcass appearance or meat quality would not effectively lower *Campylobacter*, *E. coli*, or coliform bacterial counts. In a similar experiment Murphy *et al*. (2004) showed that the size and shape of poultry products had a tremendous effect on the inactivation of *Salmonella* and *Listeria* in chicken meat during thermal treatment.

2.6.2 IRRADIATION TREATMENTS

Irradiation treatments are a useful means to reduce and eliminate spoilage and pathogenic microorganisms from food products. The USDA-FSIS approved the use of such treatments twenty years ago (USDA, 1992) but consumer acceptability and lack of appropriate scientific knowledge on the subject prevents such treatments from being

widely accepted. Raut *et al*. (2012) were able to achieve complete elimination of *Campylobacter* inoculated broiler meat (10^5 CFU/g) with gamma radiation (1 kGy) and after enrichment of samples no injured *Campylobacter* cells were able to be resuscitated. A study to calculate the theoretical population killed by gamma irradiation treatments was performed and showed that 10.64 $\log_{10}$ CFU/g C. *jejuni* could be killed in a ground beef matrix (Clavero *et al*., 1994).

Patterson (1995) investigated the sensitivity of *Campylobacter* to irradiation treatments and found that there were many differences among *C. jejuni, C. coli,* and *C. lari*. Patterson also tested three strains of *C. jejuni* in this experiment and found that even within the same species the sensitivities were significantly different. Furthermore, the comparative data generated in this study revealed that *Campylobacters* are more sensitive to radiation than *Salmonella* and *Listeria monocytogenes* irradiated under the same conditions.

2.6.3 Chemical Decontamination Strategies

Chemical antimicrobial treatments are evaluated for many different factors and their use on meat, poultry, and other food products should be approved by the appropriate regulatory agencies. While the worker safety and production of harmful effluents should be monitored and minimized for environmental concerns, another very important concern is that a chemical antimicrobial should not have any organoleptic effects on the products they are being used to treat.

In the commercial poultry chilling process, the chemical intervention strategies used have evolved over time. Historically, chlorine and chlorine-based compounds have been used on poultry products. However, more recent findings suggest a shift away from

Chlorine-based compounds to compounds such as Peroxyacetic Acid (PAA) (McKee, 2010).

2.6.3.1 SODIUM HYPOCHLORITE

The use of Sodium Hypochlorite (SH; Chlorine) as a disinfectant during immersion chilling is underscored by its low cost and widespread availability. Free chlorine is the amount of chlorine available in an aqueous solution to eliminate microorganisms (Russell and Axtell, 2005). Free chlorine is highly sensitive to the complex matrix in commercial chill tanks and reacts with blood, fat, fecal materials, and proteins (Russell and Axtell, 2005). The bactericidal activity of chlorine competes with the chemical reactions occurring for the free chlorine in the chill tank and without free chlorine the bactericidal effect of chlorine is halted (Tsai *et al.*, 1992). The USDA-FSIS regulates use of chlorine in poultry chillers with the maximum allowable limit set at 50 ppm however, a typical poultry chiller can have a chlorine demand of 1,000 to 2,000 ppm and this cannot be overcome by 50 ppm chlorine in the chiller (Russell and Axtell, 2005). Lillard (1979) reported that the efficacy of chlorine solutions is highly dependent upon pH and a solution greater than pH 7 and high organic load decreases the efficacy of chlorine.

$$\mathbf{Cl_2 + H_2O \rightarrow HOCl + Cl}$$

Figure 1: Reaction of Chlorine with Water to generate Hypochlorous Acid

Bauermeister *et al.* (2008b) reported a reduction of 12.8% in *Campylobacter*-positive carcasses post-chill after treatment with 30 ppm chlorine during chilling. Inside outside bird washers (IOBW) are another method of application commonly used in commercial broiler processing facilities. Irrespective of antimicrobial technology used in

an IOBW, it must be noted that the mechanical action of an IOBW should be considered when comparing this application method to others. Using an IOBW in pilot plant conditions, Northcutt *et al.* (2007) demonstrated 1.6 $\log_{10}$ CFU/mL reductions in *Campylobacter* when treated with 50 ppm sodium hypochlorite. Further studies on chicken wings reported a 3 $\log_{10}$ CFU/mL reduction in *C. jejuni* after a 30 min exposure in 50 ppm chlorine (Park *et al.*, 2002). In a commercial processing facility reductions of 12.8% in *Campylobacter*-positive carcasses have been documented after immersion chilling using 30 ppm chlorine (Bauermeister *et al.*, 2008b).

2.6.3.2 PEROXYACETIC ACID

Peroxyacetic acid, also known as Peracetic Acid (PAA), is an aqueous mixture of peroxyacetic acid, hydrogen peroxide, acetic acid, and 1-hydroxyethylidene-1, 1-diphosphonic acid. The simultaneous oxidative and acidic properties of PAA are responsible for characterizing its antimicrobial effects. Oxidation comes from peroxyacetic acid, hydrogen peroxide, and peroxyoctanoic acid and this disrupts the cell membrane and alters cellular protein synthesis. The acidification of the carcass surface assists in the penetration of undissociated acids into the bacterial cell. Figure 2 shows the chemical reaction carried out when Acetic Acid and Hydrogen Peroxide are mixed to form the bactericidal species Peracetic Acid.

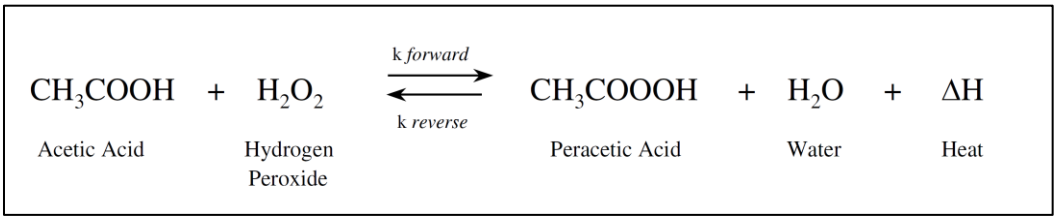

Figure 2: PAA equilibrium chemistry (FMC Microbial Control, 2012)

The USDA-FSIS permits the use of PAA at concentrations not to exceed 220 ppm on poultry products in extended dwell time chillers and at concentrations not to exceed 2,000 ppm short dwell time post-chill dip tanks. Previous studies have shown to reduce *Campylobacter*-positive carcasses by 43.4% post-chill in a commercial broiler processing facility (Bauermeister *et al.*, 2008b). In a pilot plant inoculation study, Bauermeister *et al.* (2008a) reported a 1.5 $\log_{10}$ CFU/mL reduction of *C. jejuni* using 200 ppm PAA in a pilot plant inoculation study. *C. jejuni*-containing biofilms were tested and found to be highly susceptible to PAA treatments, although they could not be completely eliminated (Trachoo and Frank, 2002).

2.6.3.3 BACTERIOPHAGES

Bacteriophages, also known as phages, have the ability to infect bacteria and most bacterial species have their own unique bacteriophages (Loc Carillo *et al*., 2005). Adoption of phage therapy has proven to be effective (Sulakvelidze *et al*., 2001); however widespread use has not been adopted. In the U.S., FDA approval and GRAS (Generally Recognized as Safe) status has been granted to *Listeria* bacteriophage mixtures intended as antimicrobials (FDA, 2011).

Reducing the bacteria shed by broilers before they enter a commercial processing facility is the most ideal approach to meeting *Campylobacter* performance standards. One such approach involves the use of lytic bacteriophages to reduce the gut colonization of *Campylobacter*. Loc Carrillo *et al*. (2005) and Wagenaar *et al*. (2005) separately demonstrated that in an experimental environment this methodology is able to achieve between 2 and 3 $\log_{10}$ reductions in *Campylobacter* shedding. While this method seems to be an effective way to reduce *Campylobacter* there are concerns that bacteriophage

resistant *Campylobacter* will be selected for and be more difficult to eliminate using chemical decontamination methods in the processing plant environment.

2.6.3.4 1,3-DIBROMO-5, 5-DIMETHYLHYDANTOIN

Recently, the application of the novel antimicrobial 1,3-Dibromo-5,5 Dimethylhydantoin (DBDMH) has been evaluated for its efficacy against *Salmonella* and *Escherichia coli* on inoculated cutaneous beef trunci muscle sections and beef hearts, showing 0.7 and 1.1 $\log_{10}$ reductions, respectively (Kalchayanand *et al.*, 2009). When DBDMH is mixed with water this yields two molecules of HOBr, the active antimicrobial compound and the byproduct of this reaction is DMH (Figure 3). Like SH, DBDMH is a halogen compound, however it works in a wider pH range so there is no need to acidify the product when preparing it for use as is needed with SH (Elanco Food Solutions, 2010). Solutions of DBDMH are permitted to be used at levels no higher than 100 ppm on poultry carcasses but it is permitted for use up to 300 and 500 ppm throughout beef and swine processing, respectively (USDA, 2012). Additionally, DBDMH can be used in water supplied to ice machines to make ice intended for use in poultry and beef processing (USDA, 2012).

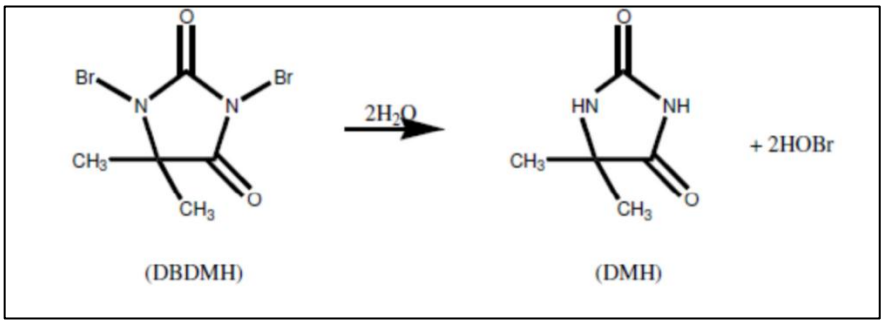

Figure 3: Reaction of one molecule of DBDMH yields two molecules of HOBr (McReynolds *et al.*, 2011; unpublished data)

Previous research has also shown that DBDMH is an effective method to protect against in-plant biofilm formation. Furthermore, DBDMH does not cause corrosion to plant equipment and concrete floors (Elanco Food solutions, 2010). Since DBDMH is a dry, solid nugget the method of application is another of its unique properties. Free flowing water is run over the dry nuggets in a closed system to produce the desired DBDMH solutions. Reductions of *E. coli, Salmonella,* and *Campylobacter* have been reported as high as 4.7, 4.5, and 4.7 $\log_{10}$ CFU/carcass using only 78 ppm DBDMH (Elanco Food Solutions, 2010). A comparative study investigating DBDMH as a spray and immersion application has yet to be conducted against *C. jejuni* inoculated poultry carcasses has not been performed.

2.7 FOOD HANDLING AT HOME AND *CAMPYLOBACTER* CONTAMINATION

Retail poultry carcasses contain a high prevalence of *Campylobacter* (Dickins *et al.*, 2002). Therefore improper preparation or mishandling of contaminated products in consumers' homes can be attributed to a substantial proportion of the annually reported foodborne diseases. According to food safety observational studies, consumers frequently fail in their efforts at safe domestic food-handling practices. In fact, one observational study concerning raw chicken and consumer food-handling techniques revealed extensive *Campylobacter* cross-contamination during food preparation (Redmond and Griffith, 2003). An improvement in domestic food-handling practices and behaviors is likely to reduce the risk and incidence of foodborne illnesses simultaneously.

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CHAPTER 3: PRELIMINARY TRIALS EVALUATING THE EFFECTS OF 1,3-DIBROMO-5, 5-DIMETHYLHYDANTOIN ON *CAMPYLOBACTER JEJUNI*

3.1 ABSTRACT

Recent pathogen-food pairings attribute *Campylobacter* contaminated poultry to more illnesses than any other bacteria-food combination. Therefore, novel methods to reduce bacterial accumulation on fresh poultry carcasses provide a means to potentially reduce this overall burden. *In-vitro* trials were performed to evaluate the effect of 1,3-Dibromo-5, 5-Dimethylhydantoin (DBDMH) on *Campylobacter jejuni* inoculated poultry carcasses. Carcasses were inoculated with 10 mL of *C. jejuni* (ca.7 $\log_{10}$ CFU/mL) and allowed 30 min for bacterial attachment. Inoculated carcasses were then subjected to one of three different treatments: immersion in a 33-gallon plastic container with 78 L of antimicrobial treatment, immersion in an individual 5-gallon bucket for up to 120 s, or spraying with 460 mL of antimicrobial treatment. The results from these studies provided insight as to the overall reductions that DBDMH solutions can achieve with respect to *C. jejuni* in a laboratory setting which could potentially translate to reductions when used in the commercial poultry processing industry. Coupling the data in the current study with data from commercial plant trials will help validate and justify the use of DBDMH as an alternative to current antimicrobials that are used in the United States poultry industry.

Keywords: 1, 3-Dibromo-5, 5-Dimethylhydantoin, poultry processing, *Campylobacter*, antimicrobials, carcass chilling

3.2 INTRODUCTION

The United States broiler processing industry is a large, highly competitive, and efficiency-driven industry. Maximizing food safety is a prominent goal the industry is tasked with primarily because of pathogenic organisms such as *Campylobacter* and *Salmonella* which live commensally in the avian gut. The primary method of reducing and eliminating foodborne pathogens in the commercial poultry industry is through reducing the temperature of broiler carcasses using large immersion chillers with capacities of up to 40,000 gallons. This provides an opportunity to expose carcasses to antimicrobial solutions, but managing large volumes of solutions can prove to be difficult because of the organic load of these chillers. Therefore post-chill immersion interventions, also referred to as finishing chillers are gaining popularity due to more stringent performance standards set forth by the USDA-FSIS (United States Department of Agriculture-Food Safety Inspection Service, 1996, 2009, 2011, 2012) to control *Salmonella* and *Campylobacter*.

The application of novel antimicrobial solutions, such as DBDMH on fresh poultry carcasses has not been documented in the past. Therefore research on means by which DBDMH can be applied and evaluated on fresh poultry carcasses is necessitated. Previous research has shown the ability of DBDMH solutions to reduce *Escherichia coli* by up to 1.1 $\log_{10}$ CFU/mL on inoculated beef parts. However, these results may not translate to reductions of *Campylobacter* and the use of DBDMH must first be validated for its efficacy against such foodborne pathogens on poultry carcasses. These studies will provide a means to move forward with trials in commercial poultry processing facilities to provide an indication of the real-world applicability of DBDMH solutions.

3.3 MATERIALS AND METHODS

Bacterial Culture

Campylobacter jejuni (ATCC BAA 1062) was cultured in 1 L of *Campylobacter* Enrichment Broth (Acumedia, MI) and grown microaerobically (5% O_2, 10% CO_2, 85% N_2) for 18 h at 42°C. Following this, 20 mL of the *Campylobacter* enrichment broth was dispensed into a 50 mL centrifuge tubes and centrifuged at 1069 x g for 10 min at 4°C (Sorvall Legend RT+ Centrifuge, Thermo, CT; F13-14x50c rotor, Piramoon Technologies, CA) and the supernatant was decanted. The pellet was then suspended in 10 mL of Phosphate Buffered Saline (PBS; Acumedia, MI) for a final bacterial population of approximately 7 $\log_{10}$ CFU/mL.

Chicken Carcasses

Freshly processed and chilled chicken carcasses which had not been treated with any antimicrobials were obtained from the Auburn University Poultry Research Unit and held at 4°C until further use.

Carcass Inoculation

Individual carcasses were inoculated with 10 mL of *C. jejuni* (ca. 10^7 $\log_{10}$ CFU/mL) in a laminar flow biosafety cabinet (NuAire™ Biological Safety Cabinets, NuAire Laboratory Equipment Supply, MN). The carcasses were placed in a sterile carcass rinse bag and the culture was applied to the breast and backside using a serological pipette. Each carcass was thoroughly massaged inside the carcass rinse bag and allowed 30 min for bacterial attachment under the laminar flow biosafety cabinet. Non-inoculated carcasses served as the negative control in the study, while inoculated but untreated carcasses served as the positive controls.

Preparation of Treatment Solutions

Each treatment solution was prepared and held at 10°C until application. The treatment solutions used for our study consisted of DBDMH at concentrations of 50, 75, 100, 200 and 300 ppm; PAA at levels of 100 and 200 ppm; SH at levels of 25 and 50 ppm (pH 6.0); and a water treatment. The positive control sample was not subjected to any treatment and was rinsed using the USDA-FSIS whole carcass rinse procedure following the 30 min bacterial attachment. The concentration of each solution was determined using the Hach® Pocket Colorimeter II Test Kit (Hach® Company, CO) for Hypobromus Acid and Hypochlorous acid and the LaMotte Test Kit (LaMotte Company, MD) for PAA.

Treatment Application—33-gallon Plastic Container

Inoculated carcasses (n=13) were treated by immersing them in a 33-gallon plastic container filled with 78 L of treatment solution. One carcass (t=0 s) was immersed and removed immediately after treatment application. Carcasses were mixed within the container for the duration of the experiment using a pitchfork. Every 30 s for 120 s three carcasses were removed and a USDA-FSIS whole carcass rinse was performed.

Treatment Application—Immersion in Individual Buckets and Spray

Inoculated carcasses (n=9 per application method) were applied using two separate methods: spraying with 460 mL (~62 s of continuous spraying) using a 2-gallon Garden Sprayer producing approximately 20 psi of pressure (The Fountainhead Group, NY) and a static immersion application was carried out in 6.05 L of solution in individual 5-gallon buckets for 120s with samples taken every 30s. The garden sprayer was equipped with a fan shaped nozzle, and this application was carried out under a fume

hood (BMC Laboratory Fume Hoods, MI) by suspending carcasses on a traditional poultry processing shackle within the cabinet. The fan nozzle covered an area approximately 15 cm wide by 3 cm tall and the distance between the carcass and spray wand was held constant at approximately 15 cm.

Microbiological Analysis

Following treatment, carcasses were rinsed for 1 min with 200 mL of 0.1% sterile Buffered Peptone Water (Acumedia, MI) using the USDA-FSIS whole carcass rinse method for bacterial recovery. The rinsate was recovered in sterile flasks, serial dilutions were prepared, and plated (0.25 mL) onto Campy Cefex Agar (Acumedia, MI) followed by incubation at 42°C for 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). At 48h typical mucoid *C. jejuni* colonies were counted and reported as $\log_{10}$ CFU/mL of rinsate. *Campylobacter jejuni* confirmation was carried out by examining typical *Campylobacter* colonies using phase-contrast microscopy and observing corkscrew-like motility.

3.4 Results and Discussion

Mean survival populations ($\log_{10}$ CFU/mL of rinsate) for immersion of *C. jejuni* inoculated fresh poultry carcasses in a 33-gallon plastic container filled with 78 L of antimicrobial solution are presented in Figure 4. This data also shows that DBDMH solutions do show the ability to reduce survival populations of *C. jejuni* on inoculated carcasses. This data also shows that this application did not show an effect of time of exposure. Figure 8 dos show that the higher concentration solutions (300 ppm) did show lower survival populations than the 50 ppm, 75 ppm, and control solutions. Therefore,

alternative application methods were utilized to further characterize the ability of DBDMH to reduce *C. jejuni* inoculated fresh poultry carcasses.

One such method is presented in Figure 5 and represents the mean survival populations of *C. jejuni* inoculated fresh poultry carcasses treated with various antimicrobial solutions utilizing immersion in a 5-gallon bucket filled with 6.05 L of treatment solution. The data shows that inoculating with *ca.* 7 $\log_{10}$ CFU/mL of *C. jejuni* provides survival populations of approximately 5.6 $\log_{10}$ CFU/mL when rinsed with 200 mL of 0.1% sterile Buffered Peptone Water. Survival populations of *C. jejuni* were similar for all DBDMH treatments (50, 75, 100, 200, 300 ppm) and PAA 100 at all time periods sampled and no time effect was observed. The SH treatment showed survival populations higher than the positive control and PAA 200 showed the lowest overall survival populations as compared to the positive control.

Figure 6 represents the mean survival populations for the spray application method of the antimicrobials in this study. No differences were observed among DBDMH solutions and with respect to the positive control. The lowest survival populations were observed using the PAA 200 treatment, although this was marginal when compared to the positive control. The spray application method also showed that SH treatments achieve similar results when compared to DBDMH solutions.

The data obtained in these preliminary studies led to knowledge about the impact DBDMH solutions have on *C. jejuni* inoculated fresh poultry carcasses. The use of the 33-gallon plastic container to apply DBDMH was not further studied because the results were not clear and no strong time effect was observed. This study led to the use of DBDMH and other antimicrobials as immersion, in individual 5-gallon buckets, and

spray, using a pump-style garden sprayer, applications. Using these methods also provided a means of comparison between the two methods to provide insight as to which application method could be more effective at reducing survival populations of *C. jejuni*.

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Figure 4: Effect of varying concentrations of 1,3-Dibromo-5, 5-Dimethylhydantoin (DBDMH) solutions on the survival populations (mean ± standard deviation) of *Campylobacter jejuni* (Log_{10} CFU/mL of rinsate) over 120 s following immersion in a 33-gallon plastic container of DBDMH

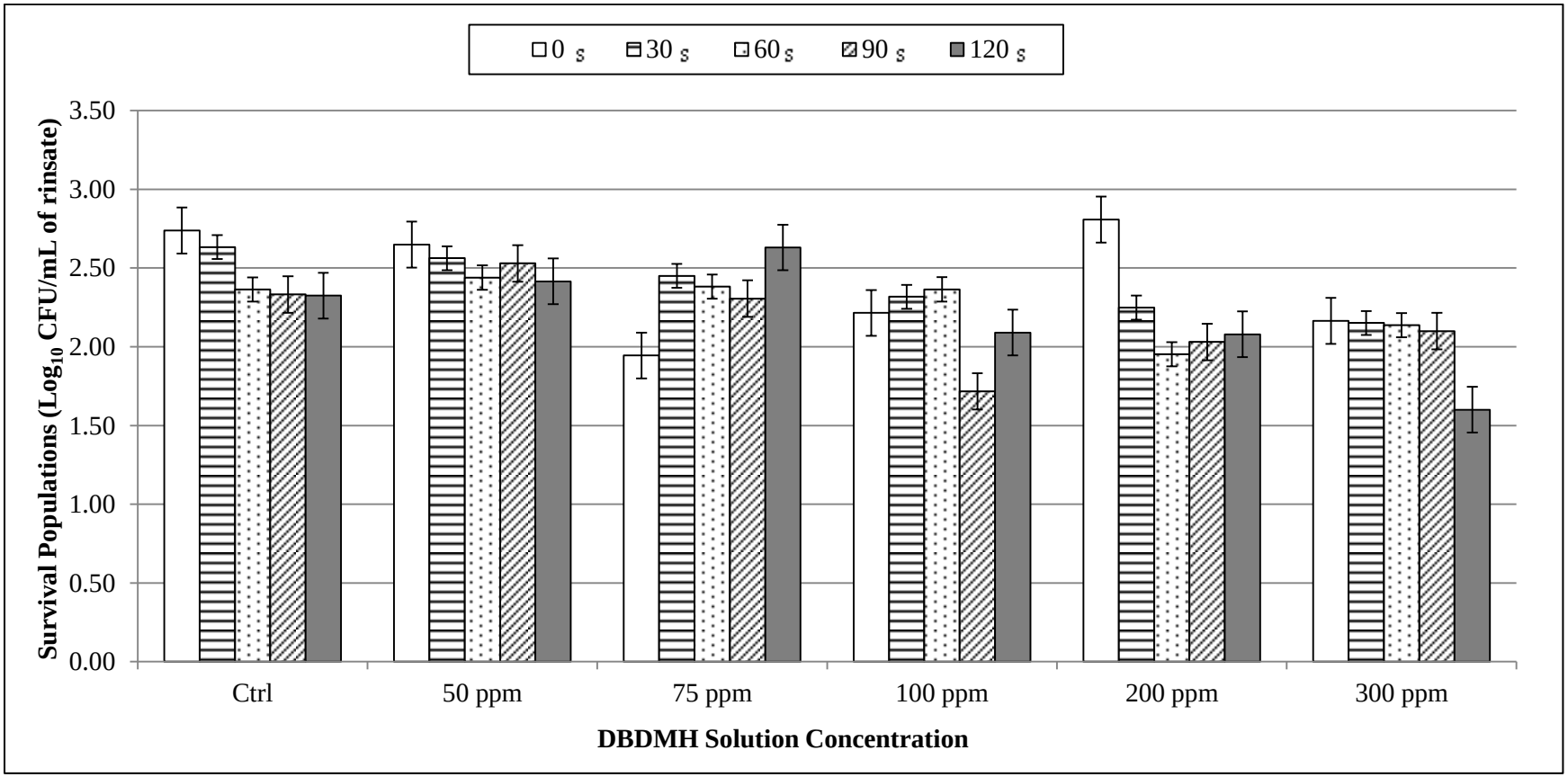


Figure 5: Survival populations (mean ± standard deviation) of Campylobacter jejuni (Log10 CFU/mL of rinsate) on chicken carcasses following immersion application of various antimicrobial solutions

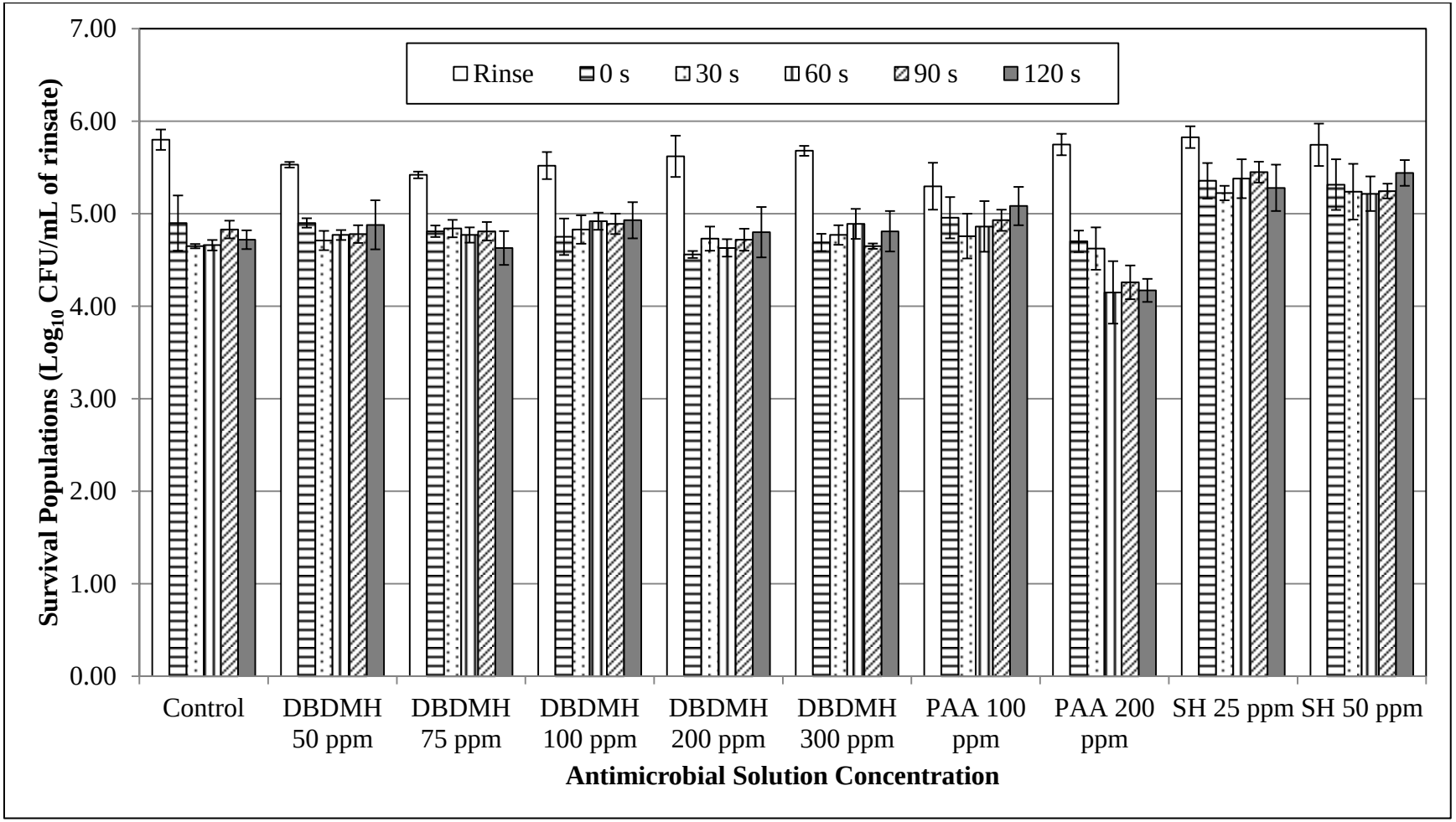
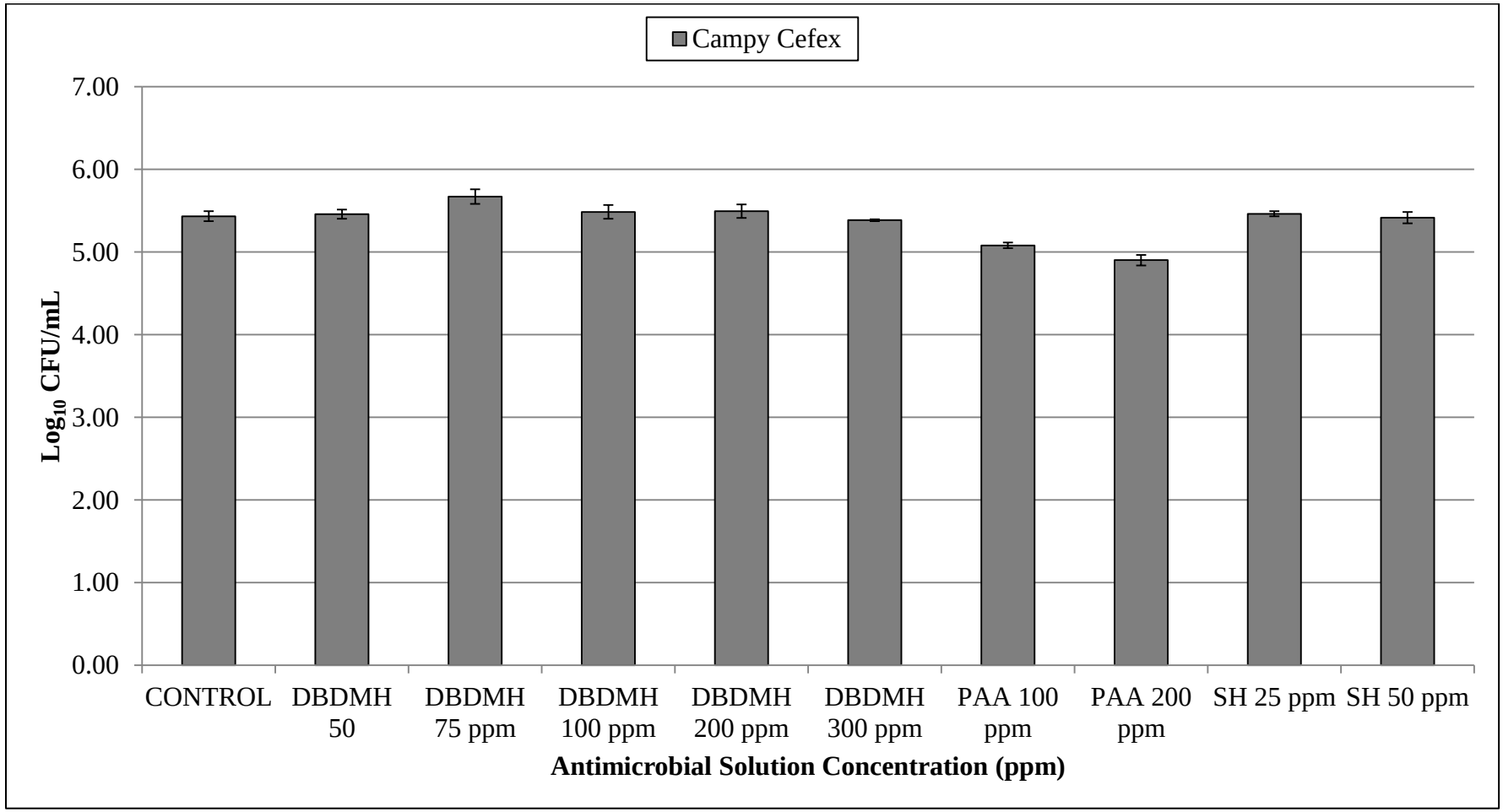


Figure 6: Survival populations (mean ± standard deviation) of *Campylobacter jejuni* (Log_{10} CFU/mL of rinsate) on chicken carcasses following spray application with various antimicrobial solutions



CHAPTER 4: APPLICATION OF 1,3-DIBROMO-5, 5-DIMETHYLHYDANTOIN AS A POST-CHILL IMMERSION AND SPRAY APPLICATION TO REDUCE *CAMPYLOBACTER JEJUNI* POPULATIONS ON POULTRY

4.1 ABSTRACT

Current regulatory guidelines to control poultry-borne pathogens, more specifically *Campylobacter jejuni*, necessitate the need for novel applications of antimicrobials during poultry processing. The objective of this study was to evaluate and compare the efficacy of 1, 3-Dibromo-5, 5-Dimethylhydantoin (DBDMH; 50, 75, 100, 200 and 300 ppm), Peracetic Acid (PAA; 100 and 200 ppm), and Sodium Hypochlorite (SH; 25 and 50 ppm) as antimicrobial interventions to reduce *C. jejuni* on fresh poultry using two separate application methods (spray and immersion). Broiler carcasses were inoculated with 10 mL of *C. jejuni* (ca.7 $\log_{10}$ CFU/mL) and allowed 30 min for bacterial attachment, followed by either a 60 s immersion or 62 s (460 mL) spray treatment with DBDMH, PAA, and SH. Following treatment, the carcasses were rinsed and the rinsate was plated (0.25 mL) onto *Campylobacter* Cefex agar and incubated microaerobically at 42°C for 48 h. Immersion application significantly ($P \leq 0.05$) reduced *C. jejuni* as compared to the spray application, however, within the DBDMH treatments, a dose-response was not observed and survival populations were lowest with 200 ppm DBDMH. Results also suggested that PAA in an immersion application showed the lowest survival populations ($P \leq 0.05$) of *C. jejuni* irrespective of the application method. This study demonstrated that DBDMH, like other commercially available antimicrobials, can reduce

populations of *C. jejuni* on fresh poultry carcasses when used as short exposure applications.

Keywords: 1, 3-Dibromo-5, 5-Dimethylhydantoin, poultry processing, *Campylobacter*, antimicrobials, carcass chilling

4.2 INTRODUCTION

As a zoonotic pathogen, *Campylobacter* is a common poultry commensal and a well-known cause of human gastroenteritis worldwide. Estimates for the U.S itself account for in excess of 800,000 episodes of foodborne illnesses annually (Scallan *et al*., 2011). Recent pathogen-food pairings attribute *Campylobacter* contaminated poultry to more illnesses than any other bacteria-food combination, and contaminated poultry alone has the greatest public health impact among all foods (Batz *et al*., 2011). To combat this issue, the United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) implemented stringent *Campylobacter* performance standards (USDA, 2009). The compliance to such standards is projected to result in the reduction of more than 5,000 *Campylobacter* infections in the United States annually (USDA, 2011a).

Although the specific pathogenic mechanism of *Campylobacter* has not been fully characterized (Fox, 1992) its acute diagnosis includes symptoms such as fever, headache, muscle pain, diarrhea that frequently contains blood and mucus, and severe abdominal cramps (Sahin *et al.*, 2012). Not only does campylobacteriosis cause acute gastro-intestinal symptoms, but in uncontrolled cases it is also linked with chronic conditions such as reactive arthritis (Hannu *et al*., 2002) and Guillain-Barré syndrome (McCarthy and Giesecke, 2001). *Campylobacter* can also enter the Viable But Nonculturable

(VBNC) state that can be induced by factors such as dramatic environmental conditions and after this they are able to regain culturability and pathogenicity (Oliver, 2010).

Campylobacter is seamlessly introduced into the commercial poultry processing facility through the gut contents of live poultry and horizontal transmission throughout plants occurs in a variety of ways. The highly automated, stainless steel equipment involved in the processing (scalding, picking, eviscerating, cropping) of broilers serve as the primary method of cross-contamination since contact of potentially *Campylobacter*-free carcasses with ingesta or other materials from *Campylobacter*-contaminated carcasses readily occurs (Potturi-Venkata *et al.*, 2007). Furthermore, Nguyen *et al.* (2010) showed that the ability of VBNC-*Campylobacter* to survive and adhere to stainless steel coupons increases over time. The USDA-FSIS introduced a "zero-tolerance" pre-chill performance standard for visible fecal material in an attempt to reduce the spread of pathogenic bacteria such as *Campylobacter* and *Escherichia coli* (USDA, 1996). Immersion chilling is an especially important site of cross-contamination because of the volume of carcasses that come into contact with one another and the extended duration of this process.

The most effective method to achieve reductions in poultry-borne bacterial pathogens during commercial poultry procurement is a multi-hurdle approach, where many intervention points are used along the processing line to reduce the chances of pathogens surviving. This approach is unique in each poultry processing plant and can utilize multiple inside-outside bird washers (IOBW) and or dip tanks before and after chilling. Carcass chilling also represents an opportunity to reduce and or eliminate pathogens such as *Campylobacter*. Immersion chilling utilizes a counter-current system,

whereby water flows in opposite direction to carcass movement and therefore continuously exposing carcasses to cleaner water. Therefore immersion chilling is the primary site for utilization of many antimicrobial treatments, including Sodium Hypochlorite (SH), Peroxyacetic Acid (PAA), Cetylpyridinium Chloride (CPC), TriSodium Phosphate (TSP), and 1,3-dibromo-5, 5-dimethylhydantoin (DBDMH) at varying concentrations (USDA, 2012). While PAA is allowed to be used at levels up to 2,000 ppm for shorter periods in post-chill dip tanks PAA can also be used at lower concentrations (up to 220 ppm) in extended dwell time chillers (USDA, 2012). The use of SH is driven by its low cost and extensive availability although it is only effective at pH levels below 7.0 (Lillard, 1979). Additionally, SH cannot be used at a level greater than 50 ppm in the chiller and it is not widely used in finishing chiller applications (USDA, 2012). According to McKee (2010) PAA is the most frequently used antimicrobial in both pre- and post-chill applications in poultry processing plants.

Data available on the ability of PAA and SH to reduce *Campylobacter* populations on poultry shows that these antimicrobials can be used as effective treatments if managed properly. Using a pilot plant IOBW, Northcutt *et al*. (2007) demonstrated that 1.6 $\log_{10}$ CFU/mL reductions were achieved using 50 ppm SH. Likewise, the incidence of *Campylobacter*-positive carcasses was reduced by 12.8% and 43.4% using 30 ppm SH and 85 ppm PAA in a commercial chiller tank, respectively (Bauermeister *et al*., 2008b). The sensory properties of both of these treatments have been studied, with no negative effects reported on poultry carcasses (Bauermeister *et al*., 2008a). Additionally, these products do not pose any environmental concerns and no special precautious need to be taken while application in commercial poultry processing plants.

The use of Cetylpyridinium Chloride (CPC) at levels not to exceed 0.8% by weight (USDA, 2012) is also permitted on pre-chill poultry carcasses, although if immersion chilling does not follow exposure carcasses must be rinsed with potable water (USDA, 2012) due to the likelihood of residues remaining after exposure. Riedel and coworkers (2009) demonstrated that exposure of inoculated chicken skin to 0.5% CPC for 1 min yielded a more than 4.2 $\log_{10}$ CFU/mL reduction of *C. jejuni*, although after 24 h of refrigerated storage the same piece of skin yielded 2.26 $\log_{10}$ CFU/mL of *C. jejuni*. Another antimicrobial that has been used in poultry processing is TriSodium Phosphate (TSP). Treating poultry carcasses with TSP is approved as both a pre- and post-chill antimicrobial intervention; however, its use is not permitted for more than 15 s using an 8-12% solution in a range of 45 to 55°F (USDA, 2012). Moreover, Somers *et al.* (1994) showed that 5.5 $\log_{10}$ CFU/mL of planktonic *C. jejuni* cells were reduced by treating with 1% TSP and *C. jejuni* was not detectable when biofilms were treated with 8% TSP. This demonstrates that *C. jejuni* is highly sensitive to the USDA-FSIS approved concentrations of TSP and Hollender *et al.* (1993) reported that the use of TSP on poultry carcasses does not significantly affect the sensory properties of carcasses. However, TSP is likely to alter waste-water pH and create strong alkaline conditions. Another novel intervention that has potential to be used in poultry processing is DBDMH, whose antimicrobial effects are exhibited as hypobromus acid (HOBr) in water. This is also a compound which has less sensitivity to organic matter than SH and is approved as an intervention in both pre- and post-chill spray and immersion applications in poultry processing (USDA, 2012). There is limited data available regarding the use of DBDMH on poultry; however at 75 ppm DBDMH has shown to reduce *Salmonella* and *E. coli*

0157:H7 inoculated beef parts by 0.7 and 1.1 $\log_{10}$ CFU/cm^2, respectively (Kalchayanand *et al.*, 2009). Therefore, the current study was undertaken to evaluate the efficacy of DBDMH as a post-chill intervention strategy to reduce *Campylobacter jejuni* inoculated chicken carcasses. Our objectives were to compare spraying and immersion applications of DBDMH and other commercially used antimicrobial intervention strategies.

4.3 MATERIALS AND METHODS

Bacterial Culture

Campylobacter jejuni (ATCC BAA 1062) was cultured in 1 L of *Campylobacter* Enrichment Broth (Accumedia, MI) and grown microaerobically (5% O_2, 10% CO_2, 85% N_2) for 18 h at 42°C. Following this, 20 mL of the *Campylobacter* enrichment broth was dispensed into a 50 mL centrifuge tubes and centrifuged at 1069 x g for 10 min at 4°C (Sorvall Legend RT+ Centrifuge, Thermo, CT; F13-14x50c rotor, Piramoon Technologies, CA) and the supernatant was decanted. The pellet was then suspended in 10 mL of Phosphate Buffered Saline (PBS; Accumedia, MI) for a final bacterial population of approximately 7 $\log_{10}$ CFU/mL.

In-vitro Study

In-vitro experiments were performed in triplicate and carried out by adding 4 mL of *Campylobacter jejuni* to 36 mL of treatment solution. For these experiments two DBDMH treatment solutions were used, 12 and 31 ppm along with a positive control. Following this 0.25 mL was plated in duplicate onto Tryptic Soy Agar containing sheep's blood (5%) every 30 s for 300 s.

Chicken Carcasses

Freshly processed and chilled chicken carcasses (n=3 per treatment) which had not been treated with any antimicrobials were obtained from the Auburn University Poultry Research Unit and held at 4°C until further use.

Carcass Inoculation

Individual carcasses were inoculated with 10 mL of *C. jejuni* (ca. 10^7 $\log_{10}$ CFU/mL) in a laminar flow biosafety cabinet (NuAire™ Biological Safety Cabinets, NuAire Laboratory Equipment Supply, MN). The carcasses were placed in a sterile carcass rinse bag and the culture was applied to the breast and backside using a serological pipette. Each carcass was thoroughly massaged inside the carcass rinse bag and allowed 30 min for bacterial attachment under the laminar flow biosafety cabinet. Non-inoculated carcasses served as the negative control in the study, while inoculated but untreated carcasses served as the positive controls.

Preparation of Treatment Solutions

Each treatment solution was prepared and held at 10°C until application. The treatment solutions used for our study consisted of DBDMH at concentrations of 50, 75, 100, 200 and 300 ppm; PAA at levels of 100 and 200 ppm; SH at levels of 25 and 50 ppm (pH 6.0); and a water treatment. The positive control sample was not subjected to any treatment and was rinsed using the USDA-FSIS whole carcass rinse procedure following the 30 min bacterial attachment. The concentration of each solution was determined using the Hach® Pocket Colorimeter II Test Kit (Hach® Company, CO) for Hypobromus Acid and Hypochlorous acid and the LaMotte Test Kit (LaMotte Company, MD) for PAA.

Treatment Application

Inoculated carcasses were treated using two separate methods: spraying with 460 mL (~62 s of continuous spraying) using a 2-gallon Garden Sprayer producing approximately 20 psi of pressure (The Fountainhead Group, NY) and a static immersion application was carried out in 6.05 L of solution in a 5-gallon bucket for 60 s. The garden sprayer was equipped with a fan shaped nozzle, and this application was carried out under a fume hood (BMC Laboratory Fume Hoods, MI) by suspending carcasses on a traditional poultry processing shackle within the cabinet. The fan nozzle covered an area approximately 15 cm wide by 3 cm tall and the distance between the carcass and spray wand was held constant at approximately 15 cm.

Microbiological Analysis

Following treatment, carcasses were rinsed for 1 min with 200 mL of 0.1% sterile Buffered Peptone Water (Accumedia, MI) using the USDA-FSIS whole carcass rinse method for bacterial recovery. The rinsate was recovered in sterile flasks, serial dilutions were prepared, and plated (0.25 mL) onto Campy Cefex Agar (Accumedia, MI) followed by incubation at 42°C for 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). At 48h typical mucoid *C. jejuni* colonies were counted and reported as $\log_{10}$ CFU/mL of rinsate. *Campylobacter jejuni* confirmation was carried out by examining typical *Campylobacter* colonies using phase-contrast microscopy and observing corkscrew-like motility.

Statistical Analysis

Results were reported as survival populations of *C. jejuni* ($\log_{10}$ CFU/mL of rinsate). All experiments were performed in triplicate with three sub-samples per

replication (total n=9 carcasses for each treatment). Means were separated and the differences ($P \leq 0.05$) among and between treatments and treatment application methods were analyzed using the General Linear Model procedure in the SAS statistical software, version 9.1 (SAS Institute Inc., Cary, NC).

4.4 Results and Discussion

The results in Figure 6 represent the mean survival populations of *Campylobacter jejuni* for the *in-vitro* study. The 12 ppm DBDMH solution reduced *C. jejuni* populations to a non-detectable level at 30 s of exposure. The detection limit was 0.3 $\log_{10}$ CFU/mL for this method. *C. jejuni* was not detectable for any time periods using the 31 ppm DBDMH solution and the positive control indicated survival populations of approximately 6.5 $\log_{10}$ CFU/mL for the duration of the study. These results indicate that even low concentrations of DBDMH solutions are able to reduce *C. jejuni* to non-detectable levels after exposure for short periods of time. Aside from these results, the presence of organic material and the intricate nature of chicken skin will aid in determining the reductions in bacterial accumulation that can potentially be achieved with DBDMH solutions.

The mean survival populations of *C. jejuni* ($\log_{10}$ CFU/mL of rinsate) for each antimicrobial treatment applied as an immersion are shown in Table 1. Populations of *C. jejuni* on carcasses treated with 50, 75, 100, 200, and 300 ppm DBDMH were 0.8, 0.8, 0.61, 0.94, and 0.68 $\log_{10}$ CFU/mL lower ($P \leq 0.05$) than the positive control carcasses, respectively. Additionally, there were no significant differences ($P > 0.05$) within the five DBDMH treatment groups when used as an immersion application. This data revealed that there was no apparent dose-response when using higher concentrations of DBDMH

compared to lower concentrations (Table 1). The SH treatment group showed only a marginal effect (*P > 0.05)* on the survival populations of *C. jejuni* as compared to the positive control. However, using the 50 ppm SH solution as an immersion treatment did show a decrease (*P > 0.05)* in survival populations of *C. jejuni* compared to the 25 ppm solution. Table 1 shows that the survival populations of *C. jejuni* obtained following immersion application of PAA at 100 and 200 ppm were significantly lower (*P ≤ 0.05)* as compared to the positive control and the 25 and 50 ppm SH treatments. Furthermore, there were no significant differences *(P > 0.05)* between the immersion application of PAA and DBDMH at 100 and 200 ppm (Table 1). There were no viable *Campylobacter* counts detected on any negative control samples.

Mean survival populations utilizing the spray application method of the antimicrobials in this study are presented in Table 2. This data illustrates that with the exception of PAA treatments of 100 and 200 ppm, no significant differences ($P > 0.05$) were observed between any of the treatments and the positive control (5.43 ± 0.12 $\log_{10}$ CFU/mL). However, Northcutt *et al.* (2007) reported that SH at 50 ppm was able to achieve a 1.5 $\log_{10}$ CFU/mL lower count of *Campylobacter* using a pilot-plant carcass washing cabinet. These differences in the results can be attributed to the use of a different spray application method in our study as compared to Northcutt *et al.* (2007). The spray application method in the present study was not able to achieve the same amount of reductions as reported by Northcutt *et al.* (2007) primarily because the present study did not use the same volume of treatment solution nor was the treatment applied in the same manner. However, the data from the present study can be useful in providing information on the efficacy of application methods of different antimicrobial products in the poultry

processing plants. The spray application method showed that PAA was able to significantly reduce ($P \leq 0.05$) the survival populations of *C. jejuni* on chicken carcasses as compared to the positive control and other antimicrobial treatments.

The data in Table 3 shows a comparison between spray and immersion application methods within treatment groups. Overall, the survival populations of *C. jejuni* for the immersion application were lower ($P \leq 0.05$) than the spray application within each concentration of an antimicrobial treatment on the chicken carcasses. The SH 25ppm, SH 50ppm, and PAA 100 ppm treatments showed no significant differences ($P > 0.05$) in the survival populations of *C. jejuni* between the two application methods. Overall, immersion application method exhibited lower survival populations when compared to the spray application irrespective of the antimicrobial treatment applied (Table 3). Furthermore, the present study indicates an immersion time of 60 s is an efficient method to decrease the populations of *C. jejuni* on fresh poultry carcasses. The efforts of adding antimicrobial solutions into extended dwell time chill tanks for up to 2 h in commercial poultry plants may be reduced considerably while still having similar effects on reducing *C. jejuni* numbers on fresh poultry, thus saving production time and expenses. The application of post-chill intervention strategies has the potential to be a more manageable and effective way to reduce bacterial concentrations rather than managing antimicrobial solution concentrations extended dwell chillers. In our study, it was shown that water treatments alone were more effective ($P \leq 0.05$) than the SH immersion application method, however, no significant differences ($P > 0.05$) were observed between the water and the SH spray application treatments. Since SH can be difficult to manage due to the pH requirements and organic load sensitivity its

effectiveness as an antimicrobial can be limited in chiller applications during poultry processing.

Previous studies have shown that PAA treatments at 200 ppm have resulted in a 1.5 $\log_{10}$ CFU/mL reduction of *C. jejuni* on chicken carcasses (Bauermeister *et al.*, 2008a). Results from our study showing a 1.42 $\log_{10}$ CFU/mL reduction in the populations of *C. jejuni* on chicken carcasses following immersion application of 200 ppm PAA are in agreement with reports by Bauermeister *et al.* (2008a). Furthermore, the use of DBDMH in post-chill applications has the ability to reduce the overall survival populations of *C. jejuni* on fresh poultry carcasses by up to 0.91 $\log_{10}$ CFU/mL when compared to positive control carcasses. Previous studies have not evaluated the impact of DBDMH on *C. jejuni*, but reported reduction of *Salmonella* by 0.7 to 2.3 $\log_{10}$ CFU/cm^2 on inoculated beef parts (Kalchayanand *et al*., 2009). The efficacy of DBDMH against *C. jejuni* reported in our study is generally in agreement with Kalchayanand *et al*. (2009), although differences can be attributed to difference in the meat matrix of chicken carcass versus beef parts as well as the pathogen studied. The DBDMH solutions were stable as compared to SH (data not shown) in our study, indicating its better applicability in chillers that have high organic load. Hence, in commercial poultry processing plants the use of DBDMH will lead to a reliable pathogen reduction system. While conducting experiments for this study, no odors were detected while working with DBDMH which is more desirable in a plant environment and is favorable to plant workers when compared to SH solutions.

Decreasing the survival populations of *C. jejuni* on fresh poultry is a food safety goal that the commercial poultry industry has been tasked with accomplishing (USDA,

2009). One method to achieve and fulfill such goals is to employ antimicrobials accessible to processors. The present data shows that completely eliminating *C. jejuni* is a challenging task and maintaining a multi-hurdle approach in commercial poultry processing facilities will prove to be the most effective method to achieve performance standards set forth by the USDA-FSIS for this pathogen. Therefore, further research needs to be conducted for the use of DBDMH in conjunction with other widely used antimicrobials, as well as against other poultryborne pathogens at various stages of poultry processing to maximize the efficacy of such antimicrobials and enhance safety of fresh poultry.

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Figure 7: The effect of two concentrations of 1,3-Dibromo-5, 5-Dimethylhydantoin (DBDMH) on the survival populations (Log_{10} CFU/mL) of *Campylobacter jejuni* over 300 s

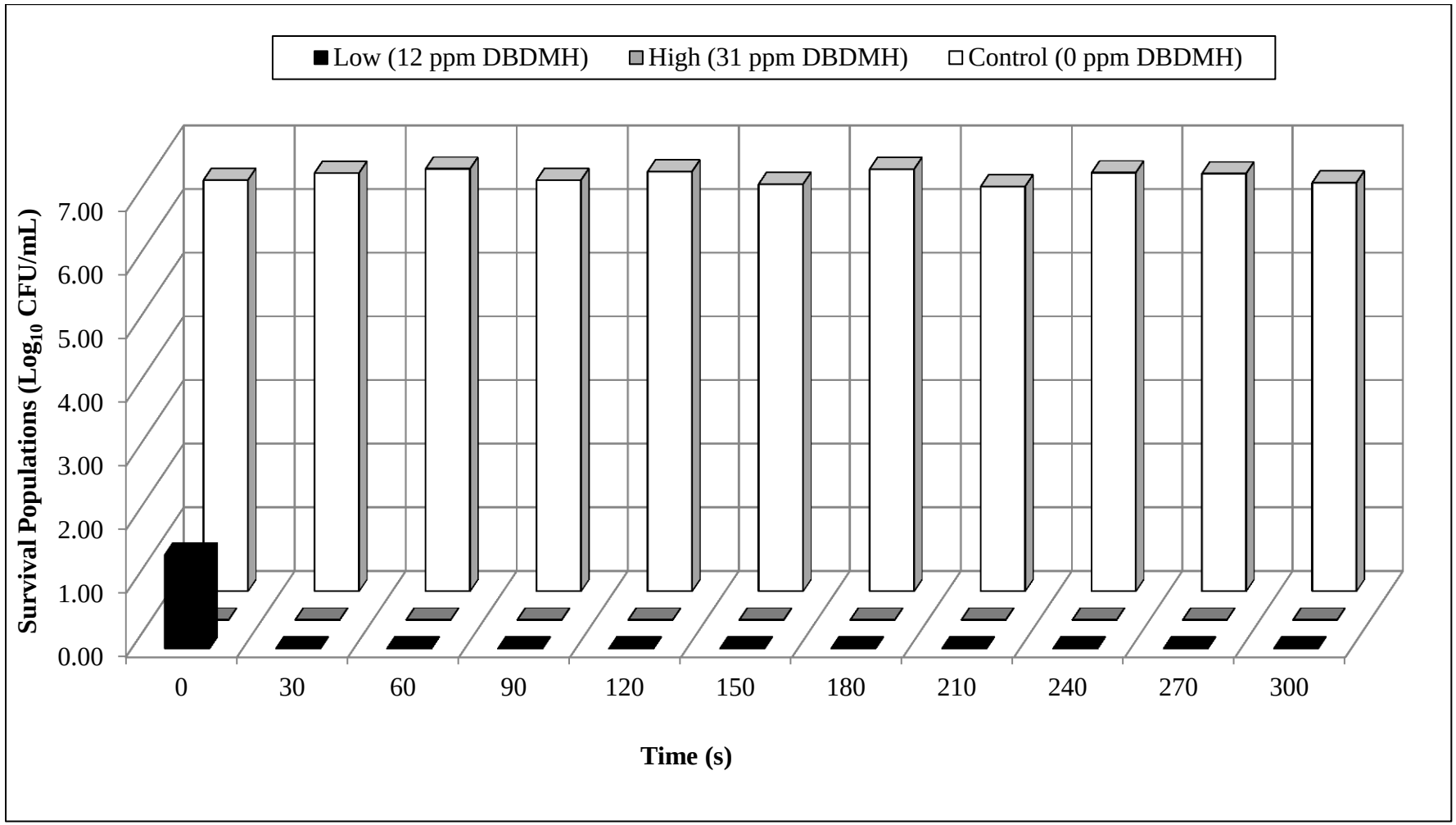

Table 1: Survival populations[¥] (log_{10} CFU/mL of rinsate) of *Campylobacter jejuni* on chicken carcasses following immersion application with various antimicrobials

Treatment	Immersion Application
Negative Control	ND
Positive Control	5.57 ± 0.26^{x}
Water Treatment	4.66 ± 0.20^{y}
DBDMH 50 ppm	4.77 ± 0.21^{y}
DBDMH 75 ppm	4.77 ± 0.19^{y}
DBDMH 100 ppm	4.96 ± 0.34^{y}
DBDMH 200 ppm	4.63 ± 0.26^{y}
DBDMH 300 ppm	4.89 ± 0.21^{y}
SH 25 ppm (pH 6.0)	5.38 ± 0.16^{x}
SH 50 ppm (pH 6.0)	5.22 ± 0.60^{x}
PAA 100 ppm	4.86 ± 0.48^{y}
PAA 200 ppm	4.15 ± 0.45^{z}

[¥]Mean ± Standard deviation

n=9 carcasses per treatment

x, y, and z superscripts indicates significant differences ($P \leq 0.05$) between treatment

ND = Not Detectable

DBDMH = 1,3-Dibromo-5, 5-Dimethylhydantoin

SH = Sodium Hypochlorite

PAA = Peracetic Acid

ppm = parts per million

Table 2: Survival populations[¥] (log_{10} CFU/mL of rinsate) of *Campylobacter jejuni* on chicken carcasses following spray application with various antimicrobials

Treatment	Spray Application
Negative Control	ND
Positive Control	5.43 ± 0.12^{x}
Water Treatment	5.43 ± 0.12^{x}
DBDMH 50 ppm	5.46 ± 0.11^{x}
DBDMH 75 ppm	5.67 ± 0.17^{x}
DBDMH 100 ppm	5.48 ± 0.16^{x}
DBDMH 200 ppm	5.49 ± 0.16^{x}
DBDMH 300 ppm	5.38 ± 0.02^{x}
SH 25 ppm (pH 6.0)	5.45 ± 0.06^{x}
SH 50 ppm (pH 6.0)	5.41 ± 0.14^{x}
PAA 100 ppm	4.97 ± 0.05^{y}
PAA 200 ppm	4.82 ± 0.06^{y}

[¥]Mean ± Standard deviation

n=9 carcasses per treatment

x and y superscripts indicate significant differences ($P \leq 0.05$) between treatments

ND = Not Detectable

DBDMH = 1,3-Dibromo-5, 5-Dimethylhydantoin

SH = Sodium Hypochlorite

PAA = Peracetic Acid

ppm = parts per million

Table 3: Comparison of spray and immersion application of various antimicrobials on the survival populations[¥] of *Campylobacter jejuni* (log_{10} CFU/mL of rinsate) on broiler carcasses

Treatment	Immersion Application	Spray Application
Negative Control	ND	ND
Positive Control	5.57 ± 0.26^{x}	5.43 ± 0.12^{x}
Water Treatment	4.66 ± 0.20^{y}	5.43 ± 0.12^{x}
DBDMH 50 ppm	4.77 ± 0.21^{y}	5.46 ± 0.11^{x}
DBDMH 75 ppm	4.77 ± 0.19^{y}	5.67 ± 0.17^{x}
DBDMH 100 ppm	4.96 ± 0.34^{y}	5.48 ± 0.16^{x}
DBDMH 200 ppm	4.63 ± 0.26^{y}	5.49 ± 0.16^{x}
DBDMH 300 ppm	4.89 ± 0.21^{y}	5.38 ± 0.02^{x}
SH 25 ppm (pH 6.0)	5.38 ± 0.16^{x}	5.45 ± 0.06^{x}
SH 50 ppm (pH 6.0)	5.22 ± 0.60^{x}	5.41 ± 0.14^{x}
PAA 100 ppm	4.86 ± 0.48^{x}	4.97 ± 0.05^{x}
PAA 200 ppm	4.15 ± 0.45^{y}	4.82 ± 0.06^{x}

[¥]Mean ± Standard deviation

n=9 carcasses per treatment

x and y superscripts indicate significant differences ($P \leq 0.05$) between application methods within the same treatment

ND = Not Detectable

DBDMH = 1,3-Dibromo-5, 5-Dimethylhydantoin

SH = Sodium Hypochlorite

PAA = Peracetic Acid

ppm = parts per million

CHAPTER 5: FUTURE RESEARCH

The evolution of the regulatory landscape necessitates the use of antimicrobials in poultry processing. More importantly, the application of novel antimicrobials at various points throughout poultry processing can be useful to aid in meeting regulatory requirements.

While the results obtained in the present study are useful and relevant, further research regarding the application of 1,3-Dibromo-5, 5-Dimethylhydantoin (DBDMH) in the poultry industry is still necessary. In the same manner as it was applied in this study *Campylobacter*, DBDMH should be applied to both *Salmonella* and *Escherichia coli* on fresh poultry carcasses. *Salmonella*, like *Campylobacter* is an organism which colonizes the avian gut and is more stringently regulated by the United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS). Although not regulated by USDA-FSIS *Escherichia coli* is an organism indicative of fecal contamination throughout many food processing environments.

The present study can be expanded upon by applying DBDMH on fresh poultry in a commercial poultry production facility. This would provide insight as to whether or not DBDMH is able to help processors consistently achieve regulatory requirements for *Campylobacter* and other poultryborne organisms set forth by the USDA-FSIS. This research would also shed light as to whether or not a dose-response can be observed as higher concentrations of DBDMH are used on fresh poultry.

Another important topic regarding antimicrobials applied to fresh poultry products is the yield effects associated with these products. Sometimes, antimicrobials can aid in the uptake and absorption of water during the chilling of fresh poultry. This would be helpful for processors to know if DBMDH can consistently achieve equal or better yields to what is obtained using other commercially available antimicrobials. Although no reports of negative organoleptic effects of DBDMH have been reported, this is a topic which should be explored to ensure that the sensory properties of DBDMH are the same or better when compared to other commercially available antimicrobials.

Furthermore, additional studies should evaluate the use of DBDMH in other fields of research and applied not only on poultry products. Data for the application of DBDMH on other livestock intended for human consumption are also of equal relevance for the further development of this product. All of these things will help form a more complete representation DBDMH's ability to compete with other antimicrobials used for poultry processing.

CHAPTER 6: CONCLUSIONS

The microbiological safety of fresh poultry products is an issue which continues to plague both consumers and the poultry industry. Studying the capabilities of chemical antimicrobials, such as 1,3-Dibromo-5, 5-Dimethylhydantoin (DBDMH), is of importance because they are the primary means to combat this issue. Knowing which steps (i.e. pre-chill or post-chill), which application method (i.e. spray or immersion), and the duration of application are all important factors to consider when investigating antimicrobial treatments.

Strategies other than chemical decontamination have been studied (i.e. thermal and irradiation treatments) but overall applicability of these treatments to the commercial poultry industry does not fit as well as chemical decontamination strategies. Chemical decontamination strategies fit so well because immersion chilling is already widely used in the United States and these chemical treatments can be applied to poultry carcasses during this time. Furthermore, studying the properties of these antimicrobials pertaining to both *Campylobacter* and *Salmonella* are most important to the commercial poultry industry due to recent regulatory standards associated with these pathogens.

Poultry carcasses may become contaminated at the processing plant for many reasons, however contact with contaminated surfaces during processing and potential contact with contaminated carcasses during chilling are prominent reasons for cross-contamination. This, in turn, can lead to illness in humans if proper handling practices are

not followed. Although cooking meat products to an internal temperature of 165°F is the primary method of eliminating foodborne pathogens, improper preparation and mishandling can contribute to cross-contamination. Thus, at home food safety practices should be followed since there has yet to be a method to completely eliminate foodborne pathogens on fresh poultry.

Although relevant, *in vitro* studies only show the ability of chemical antimicrobials to reduce survival of planktonic bacterial cells. *In vivo* studies demonstrating the ability of antimicrobials to reduce the survival of bacterial cells which have been allowed to attach to a matrix such as chicken skin is more important because it more closely mimics their real-world application. Numerous studies regarding chemical antimicrobials have been carried out; however with the recent implementation of *Campylobacter* performance standards by the United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) more findings regarding the ability to reduce survival of *Campylobacter* are warranted. Furthermore, DBDMH should be studied in a real-world scenario to further corroborate the findings from the present study.