Determining the Antibacterial Effects of *Trans*-Cinnamaldehyde, the Major Component of Cinnamon Essential Oil, on *Salmonella* Heidelberg in Commercial Broiler Chickens

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Abstract

Foodborne illness is a major cause of gastrointestinal diseases in humans, despite many efforts to control foodborne pathogens. In the United States, non-typhoidal *Salmonella* is one of the major pathogens that causes the most foodborne illnesses per year. Of these pathogens, *Salmonella enterica* serovar Heidelberg has become one of the most important pathogens implicated in *Salmonella* outbreaks, especially in poultry. *Salmonella enterica* serovar Heidelberg is known to be resistant to some common antibiotics such as gentamicin, streptomycin, tetracycline, and ampicillin. Poultry and poultry products are a significant source of *Salmonella* for humans. This is because poultry are a natural reservoir for *Salmonella*, as it colonizes in the gastrointestinal tract of the bird, and can be spread easily through feces, infecting the flock.

Alternatives that are safe and environmentally sustainable are needed as antibiotics are being phased out of the agriculture industry due to the potential for development of antibiotic resistance in bacteria. Essential oils have received attention as alternatives because of their many functional benefits, including antimicrobial properties. One essential oil, *trans*-cinnamaldehyde, has been investigated for its use against *Salmonella* in both pre-harvest and post-harvest interventions. In this thesis project, we investigated the use of *trans*-cinnamaldehyde for both pre- and post-harvest control of the pathogens in poultry. We hypothesized that TC would be effective against the multidrug resistant *S.*

Heidelberg in broiler chickens and on poultry products. Two objectives were used to test this hypothesis: 1) to determine the effect of *trans*-cinnamaldehyde on *S.* Heidelberg when used prophylactically and therapeutically, in broiler chickens and 2: to determine the effect of *trans*-cinnamaldehyde on *S.* Heidelberg on broiler meat and drumstick during the scalding step in poultry processing.

In the first objective, we determined the effect of *trans*-cinnamaldehyde on *S.* Heidelberg when delivered through feed and water in broiler chickens. Broiler chicks (5-week study – 3 broilers/group, two experiments; 7-week study – 3 broilers/group, two experiments) were divided into 4 groups: Negative control (NC), Positive Control (PC), Antibiotic Control (AB) and *trans*-cinnamaldehyde group (TC). In the 5-week study, the broilers in NC and PC groups were fed a standard diet from day 1 to week 5. The AB group received standard diet containing 50g/ton of bacitracin (BMD) for 5 weeks. The TC group was fed with a standard diet containing 0.5% TC for 4 weeks through feed and then after the pathogen challenge, 0.03% TC was supplemented through drinking water for the remaining 7 days. All groups except NC were challenged with *S*. Heidelberg (3.8 log10 CFU/bird) on week 4. The broilers were euthanized 7-days after the challenge. Cecum and cecal contents were collected for *S.* Heidelberg recovery. In the 7-week study, the NC or PC groups and AB group were fed with standard and bacitracin diets, respectively, through 7 weeks of age. The pathogen challenge (8.2 log10 CFU/bird) was conducted on week 6. *Trans*-cinnamaldehyde (0.03%) was provided as therapeutic supplementation to broilers in TC group through

drinking water for 7 days after *S*. Heidelberg inoculation. *Salmonella* recovery was done 7 days after inoculation. The supplementation of 0.5% TC through feed and 0.03% TC in water resulted in ~2.0 log10 CFU/g reduction of *S.* Heidelberg from the cecum of 5-weeks old broilers whereas bacitracin reduced the pathogen to non-detectable levels (~3.7 log10 CFU/g reduction) ($P<0.05$). Conversely, bacitracin did not show significant reduction in *S.* Heidelberg colonization in 7-week old broilers. However, TC through water resulted in >90% reduction (~1.2 log10 CFU/g reduction) in *S.* Heidelberg colonization ($P<0.05$) without affecting body weights of broilers.

In the second objective, we determined the effect of *trans*-cinnamaldehyde on *S.* Heidelberg during a simulated scalding treatment, and analyzed the effects on contaminated drumsticks during chilling and storage after the scalding treatment. Broiler chicken drumsticks were inoculated with either low (~3.0 log10 CFU/g) or high (~4.5 log10 CFU/g) concentrations of *S.* Heidelberg and were immersed in treatment water containing 0.5% TC, 1% TC, 0.05% peracetic acid (PAA), 0.5% TC + 0.05% PAA or 1% TC + 0.05% PAA at 54°C for 2 min (USDA-recommended time-temperature combination for scalding). The drumsticks inoculated with *S.* Heidelberg and without any antimicrobial treatment in scalding water served as the positive control (PC). After scalding, the drumsticks were homogenized in 350 mL Phosphate Buffered Saline for 30 s to obtain surviving *S.* Heidelberg populations. Also, the populations of *S.* Heidelberg on drumsticks and in the scalding water were enumerated by surface plating and enrichment

methods. We also determined the efficacy of the scalding treatments against *S.* Heidelberg survival on drumsticks during chilled storage. For this study, the drumsticks were inoculated with 4.5 log10 CFU/g *S.* Heidelberg and immersed in scalding water containing 1% TC, 0.05% PAA or 1% TC + 0.05% PAA. After scalding, the drumsticks were immersed in chilling water without any antimicrobials for 30 min, packed and stored at 4◦C for 48 h. The surviving *S.* Heidelberg populations were determined immediately after chilling and after 48 h of storage. Additionally, the effect of the treatments on the surface color of drumsticks was evaluated. All experiments were repeated six times and data were analyzed. Results revealed that PAA and its combination with TC (0.5 or 1%) resulted in the significant reduction of *S.* Heidelberg for low (1.7 to 2.4 log10 CFU/g reduction) and high inoculum (2.1 to 3.1 log10 CFU/g reduction) levels of *S.* Heidelberg ($P<0.05$). Moreover, the same treatments inactivated *S.* Heidelberg to non-detectable levels from the scalding water whereas 2.0- (low inoculum study) and 4.0- (high inoculum study) log10 CFU/ml *S.* Heidelberg survived in PC ($P<0.05$). The scalding treatments were also effective in inhibiting *S.* Heidelberg on the drumsticks compared to the respective controls during chilled storage. The treatments did not affect the surface color of the drumsticks ($P>0.05$). Both of these studies revealed that TC could be used as an effective alternative to antibiotics for *S.* Heidelberg control in the poultry industry.

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Literature Review

1.1 Foodborne Illnesses

Foodborne illnesses are caused by harmful microorganisms found in contaminated foods. There are over 250 identified foodborne illnesses caused by bacteria, viruses, or parasites (CDC, 2018). The Centers for Disease Control and Prevention (CDC) estimates that 48 million people are affected by foodborne illnesses every year, leading to 128,000 hospitalizations and 3,000 deaths in the United States (CDC, 2018). Among the several agents that cause foodborne infections in humans, *Salmonella* plays a very significant role in causing critical public health issues and economic burden in the country. While the average, healthy adult can typically get through salmonellosis without any medical treatment, foodborne diseases can sometimes be severe, especially for the elderly, young children, pregnant women, or immunocompromised individuals (CDC, 2019). Therefore, it is of particular importance that strategies to prevent *Salmonella* entering the food chain are implemented.

1.2 *Salmonella*

Salmonella are Gram-negative, rod-shaped bacteria that cause foodborne illness in humans. *Salmonella* was first documented in 1880 in typhoid patients by Karl Eberth, and was grown independently in culture by Georg Theodor Gaffky. However, it was not given a genus status until Theobald Smith of the USDA declared it. The bacteria were named by Smith's supervisor, Daniel E Salmon (FDA, 2013). *Salmonella* has two species, *Salmonella enterica*, and *Salmonella bongori*. *Salmonella enterica* has six subspecies: *S. enterica* subsp. *enterica*, *S.*

enterica subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *indica*, and *S. enterica* subsp. *houtenae.* (Porwollik, 2004). Although *Salmonella* has over 2,500 recognized serotypes, the most common disease-causing *Salmonella* is *Salmonella enterica* serotype Enteritidis (*S*. Enteritidis). Additionally, *Salmonella enterica* serotype Typhi is associated with Enteric fever or more commonly known, Typhoid fever. Beyond these, many serotypes have been associated with major outbreaks and typically have some differentiating virulence factors, causing a need to further investigate the specific serotypes (CDC, 2019b)

Salmonella enterica is known to cause the most bacterial foodborne illnesses (non-typhoidal) in the United States with 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths occurring every year (CDC, 2019a). Usually, people infected with *Salmonella*, or salmonellosis, start showing symptoms from 12 to 72 hours after consuming the infected product. Then, the illness typically lasts for 4 to 7 days, with most people recovering without any treatment. Similar to other gastrointestinal illnesses, general symptoms of salmonellosis in humans include diarrhea, fever, and abdominal cramping (Mayo Clinic, 2018; CDC, 2019c).

In humans, poultry meat, poultry byproducts, and raw or undercooked eggs are the leading cause of death caused by foodborne illness in the United States (Painter, et al. 2013). *Salmonella* causes large incidence of illness and associated costs. In 2016, the USDA Food Safety Inspection Service (FSIS) estimated that of

all diagnosed salmonellosis, 360,000 were associated with poultry or poultry products (USDA FSIS, 2016). Additionally, the FSIS found that 15% of raw meat, including broilers, turkeys, and ground beef, were contaminated with *Salmonella*. Of this 15%, the highest incidence rate of *Salmonella* was found in broilers. In addition to broilers, layers could be contributing to the problem of human salmonellosis. While the incidence due to egg-associated *Salmonella* is lower, a modeling study estimated that if the laying hen is infected, there is an 8.62% chance that the *Salmonella* will be present within the egg itself (Bichler et al, 1996).

1.3 Poultry-borne *Salmonella* and Transmission

Poultry are natural reservoir hosts for bacteria such as *Salmonella*. Through multiple sources of infection, the birds are continually infected with the bacteria, with the primary colonization sites being the cecum and crop (Bryan, et al. 1995). The high colonization within birds allows for the horizontal transmission from one bird to another, transmission from feces to eggs, contamination from one carcass to another during the slaughter and processing of poultry, and vertical transmission from the layers to the egg (Timoney, et al. 1989; Shivaprasad, 1990; Miyamoto, et al. 1997; Okamura, et al. 2001; Heyndrickx, et al. 2002; Liljebbelke, et al. 2006; Trampel, et al. 2014). According to the USDA National Agriculture Statistics Survey, there were 9,037,100,000 broiler chickens and 391,300,000 layer hens in the United States in 2018 (USDA NASS, 2018). Due to the number and proximity of these birds within a farm, the transmission of *Salmonella* throughout the flock is frequent.

Within a flock, horizontal transmission leads to the spread of *Salmonella* from one bird to another through various on-farm sources. This can be through contaminated feed, litter, or water, and spread through rodents or insects. Additionally, the bacteria can spread quickly through contaminated equipment, during transportation to and from the facility, feed trucks, egg flats, and even service personnel can promote the transmission (Heyndrickx, et al. 2002; Trampel, et al. 2014). Transmission can also occur from parent to offspring at or during laying eggs. *Salmonella* can travel via the trans-ovarian route through the fallopian tubes to the egg. This can cause one generation of layer hens to pass on *Salmonella* to the next (Timoney, et al. 1989; Shivaprasad, 1990). This is especially important because the spread of bacteria can occur even without the parent flock showing any signs of clinical infection. Finally, *Salmonella* can potentially be spread within a facility through airborne droplets of the bacteria and dust particles (Bailey, et al. 1992).

While the main focus of reducing *Salmonella* within poultry is to protect the human population from foodborne illness, poultry can also be significantly affected by the bacteria (Davison. 2019). When one bird within the flock is infected, the pathogen can spread throughout the flock rapidly. Despite there being many serotypes of *Salmonella* and the variation of virulence characteristics between them, most isolates can colonize the intestinal tract of the bird, as well as invade the spleen and liver, increasing the gravity of infection in the birds. *Salmonella* can be invasive in both young and adult birds, and there are many signs of illness if the

bacteria causes a severe infection. Affected birds can have symptoms such as little movement around the barn, dehydration and anorexia, reduction in growth, diarrhea, huddling in groups, and even depression (Davison. 2019). These symptoms are more common in chicks than in adult birds (Davison. 2019). Adult birds are more likely to be asymptomatic carriers of the pathogen, which can shed both continuously or periodically to the birds' surroundings (Davison. 2019). Despite the likeliness of asymptomatic carrying in adult birds, birds infected with a high dosage of *Salmonella* see a high rate of mortality after developing clinical salmonellosis (Davison. 2019). Infected laying hens can result in the invasion of the reproductive organs which leads to the pathogen being spread to the eggs or offspring.

1.4 Antibiotic Resistance

Antibiotics have played an integral role in the history of human and animal disease control and treatment. While this is a very effective strategy, there is recent concern about overusing antibiotics in agriculture leading to potential development of antibiotic resistance in bacteria. It has been suggested that bacteria developing resistance to drugs in an agriculture setting can have adverse effects in humans because of the limited number of adequate antibiotics to treat resistant bacterial infections effectively (Bell, et al. 2014; CDC, 2013). Although a definite connection between the use of antibiotics in animal agriculture and human infections with drug resistant pathogens has not been proven, some studies have shown that the non-therapeutic use of antibiotics in the agriculture industry could outweigh the amount

used in humans (Sneering, et al. 2015; Mellon, et al. 2001). There have been multiple environmental sources that have been shown to harbor drug-resistant bacteria, including farms, retail meat products, and different environmental samples (Jorgensen, et al. 2002; Chen, et al. 2004).

Another aspect of the agriculture system that could promote drug-resistant pathogens is the farm environment itself (Kelly, et al. 1998; Chen, et al. 2014). Feces from swine, cattle, and poultry could contain such pathogens that are found within the animal itself, leading to the contamination of the farm environment and water. Feces in water runoff can spread resistant bacteria to municipal drainage systems, and potentially to humans (Khachatourians, 1998; Sayah, et al. 2005). Additionally, contamination of waterways on a farm can lead to the spread of resistant bacteria to fresh produce if the farm cultivates fresh produce, or if the farm is upstream from a produce farm (Khachatourians, 1998).

In poultry, there have been reports of incidents with antibiotic-resistant bacteria recovered from poultry carcasses, poultry by-products, and poultry farms (Lilijebjelke, et al. 2017; Mederios, et al. 2011; Dutil, et al. 2010; Duffy, et al. 1999; White, et al. 2001). *Salmonella* Heidelberg is an example of a serovar of *Salmonella* that has developed resistance to antibiotics. Other serovars, *S.* Enteritidis, *S.* Typhimurium, and *S.* Newport, also have clonal groups that are resistant to various antibiotics. According to the Laboratory-based Enteric Disease Surveillance (LEDS) system, Of the 369,254 samples that were tested, the

serovars mentioned above accounted for 52% of positive samples (CDC. 2018a). Data collected from both LEDS and the National Antimicrobial Resistance Monitoring System (NARMS), it was determined that from 2004 to 2012, 6,200 samples collected had bacteria that were resistant to at least one antibiotic (NARMS, 2012). In 2002, a study from England determined the prevalence of resistant bacteria, where 241 whole, raw chickens were sampled. Of these carcasses, 25% were positive for *Salmonella*, and of the positive samples, 73% were resistant to at least one antibiotic (Jorgensen, et al. 2002). Another study found that of 133 *Salmonella* isolates recovered from retail meats purchased in the United States and China, 82% were resistant to at least one antibiotic (Chen, et al. 2004). These studies show that the poultry products could harbor antibiotic resistant *Salmonella*.

1.5 *Salmonella enterica* serovar Heidelberg

Salmonella enterica serovar Heidelberg (*S*. Heidelberg) is a serotype of *Salmonella* of particular importance to humans, and food animals such as swine, cattle, and poultry (USDA APHIS, 2018b; CDC, 2017; Foley, et al. 2008). This serovar is important because of its high virulence and resistance to multiple antibiotics. *S*. Heidelberg has been estimated to cause 84,000 enteric illnesses in the United States (Gast et al., 2004; CDC, 2011, 2013; Foley et al., 2011; Young et al., 2012; Amand et al., 2013; Foley, et al. 2011; Gieraltowski, et al. 2016). Reports suggest that *S*. Heidelberg strains are developing resistance to third generation cephalosporins like ceftiofur and ceftriaxone which were previously

used to treat *S*. Heidelberg in pregnant women and young children. (Dutil, et al. 2010; Zhao, et al. 2008).

In the past, there have been significant number of outbreaks associated with *Salmonella* Heidelberg in multiple food animal species. *S.* Heidelberg had been commonly associated with poultry as they were frequently isolated from different poultry products (Snoeyenbos et al., 1969; CDC, 1986; Bokanyi et al., 1990; Schoeni et al., 1995; Layton et al., 1997; Gast et al. 2004; Hennessy et al., 2004; Scharff, 2012, Jackson et al., 2013). More recently, two significant outbreaks with *S.* Heidelberg occurred through poultry products such as ground turkey and mechanically separated chicken (Gieraltowski, et al. 2016). In the 2011 ground turkey outbreak, a total of 136 illnesses with 37 hospitalizations and one death was reported (CDC, 2011). Additionally, from an outbreak occurred in 2014 associated with Foster Farm's brand chicken, 634 people were affected, but no deaths occurred (CDC, 2013). Apart from the outbreaks it caused in poultry, recently, in 2018, *S.* Heidelberg has been associated with high mortality in Wisconsin dairy calves (USDA APHIS, 2018; CDC, 2017). Due to tremendous resistance potential in humans and the number of outbreaks in food animals, both sporadic and nationwide, it is very important to implement strategies to reduce the number of illnesses due to *S.* Heidelberg.

1.6 Pre-harvest interventions against *Salmonella*

Antibiotics were previously used in food animal agriculture as growth promoters (Rabsch et al., 2001). However, the issue of development of antibiotic resistance in foodborne bacteria has led to the exploration of alternative antimicrobials for control of foodborne pathogens, including *Salmonella* (Kollanoor Johny et al., 2009; 2010a, b; 2012 a, b; 2013; Nair and Kollanoor-Johny, 2016; Nair et al., 2016; 2018). As previously stated in the literature review, *Salmonella* colonization of the ceca and crop could result in the horizontal transmission of the pathogen, contamination of eggshells with feces and carcass contamination during slaughter. Therefore, reducing the *Salmonella* numbers harboring poultry would potentially reduce contamination of poultry products. Some of the previously studied pre-harvest approaches to control *Salmonella* in general include probiotics, prebiotics, phytobiotics, antibiotics, and vaccination (Fernandez et al., 2000; Spring et al., 2000; Byrd et al., 2001; Stern et al., 2001; Fernandez et al., 2002; Chadfield and Hinton, 2003; Heres et al., 2004; Fiorentin et al., 2005; Higgins et al., 2007; Methner et al., 1995; Dueger et al., 2001; Khan et al., 2003; Inoue et al., 2008). In this thesis, a Generally Recognized as Safe phytochemical, *trans*-cinnamaldehyde, derived from cinnamon essential oil, is investigated as a preharvest control strategy against *S*. Heidelberg.

1.7 Phytobiotics

Phytobiotics have received renewed attention due to their potential to be an effective alternative to antibiotics (Burt. 2004; Nair, et al. 2014, 2015; Kollanoor-Johny, et al. 2008; Surendran Nair, et al. 2016, 2017; Venkitanarayanan. 2016).

Phytobiotics are divided into four categories: essential oils, botanicals, herbs, and oleoresins. Essential oils are volatile and lipophilic compounds obtained from plants through hydrodistillation, Botanicals are the entire or processed parts of plants including the bark, roots, and leaves. Herbs are flowering, non-woody and non-persistent plants, and Oleoresins are the extracts obtained using non-aqueous solvents (Nair, et al. 2019).

Historically, phytobiotics have been used as flavoring agents and as natural remedies to simple ailments. Most typically, these have been used in Southeast Asia and India. Today, phytobiotics, specifically essential oils, have gained popularity in the western world for many benefits (Fernandez et al., 2000; Spring et al., 2000; Byrd et al., 2001; Stern et al., 2001; Fernandez et al., 2002; Chadfield and Hinton, 2003; Heres et al., 2004; Fiorentin et al., 2005; Higgins et al., 2007; Methner et al., 1995; Dueger et al., 2001; Khan et al., 2003; Inoue et al., 2008). In addition to their direct use in the cosmetics and human supplements industries, essential oils are receiving attention as alternatives to antibiotics in the agriculture industry, including the commercial and alternative systems. Phytobiotics are an excellent fit for organic farmers because plants that produce essential oils can be organically farmed, as well as their inherent quality to be environmentally friendly and potential to be branded natural if it originates from plant sources. This is ideal, as consumers are concerned about the environment, and prefer natural compounds being used in the products intended for consumption (Winter, et al. 2006; Pokorny, et al. 1991; Smid, et al. 1999).

1.8 Essential Oils

Essential oils, in particular, have acquired significant consideration in the agriculture industry. Many essential oils have been used as insect repellent for crops with pest issues, antifungals, as well as antibacterials. Essential oils are effective, as they contain various components which are volatile, lipophilic, hydrophobic and aromatic, and during hydrodistillation, these compounds are concentrated. However, because of the hydrodistillation, these compounds do not fit the exact definition of oil, but, due to the low solubility in water, they are still commonly classified within that group (Calo et al., 2015). While there has been extensive research on the potential uses of essential oils, the mechanisms of action still need more exploration.

1.9 Potential Mechanisms of Essential Oils

With the number of different components in essential oils, it is expected that there is more than one potential mechanism of action on the target bacteria. The structure of each essential oil can determine its effectiveness as an antibacterial. For example, having a phenolic compound, or the positioning of a hydroxyl group on the phenyl group can affect the potential antimicrobial activity (Burt. 2004). Essential oils are also hydrophobic, allowing them to target the cytoplasmic membrane of bacteria. The essential oils can alter the membrane structure by reducing the unsaturated fatty acids and increasing the long chain fatty acids within the cell membrane. Finally, essential oils can destroy the cell membrane and deplete intracellular ATP, causing cell death. Due to the multiple components in

essential oils and their many mechanisms of action, bacterial resistance to essential oils becomes significantly reduced (Burt. 2004; Burt and Reinders. 2003; Kollanoor Johny, et al. 2017b; Lv, et al. 2011; Surendran-Nair, et al. 2017; Venkitanarayanan, et al. 2013).

1.10 *Trans*-Cinnamaldehyde

A major essential oil that has undergone significant studies outlining its mechanism is a plant compound called *trans*-cinnamaldehyde (TC). In a review, Friedman (2017), makes it clear that although many people have investigated the compound, the exact mechanisms are not defined. Gill and Holley (2004, 2006a, 2006b) show that when cinnamaldehyde interacts with the cell membrane of *L. monocytogenes* and *E. coli,* an interruption of proton motive forces occurs, resulting in leakage of small ions. This is associated with the inhibition of ATP generation and ATPase activity, without leakage of ATP. Two studies showed that within the cell membrane, cinnamaldehyde significantly altered the fatty acid composition without provoking the collapse of the membrane (Helander, et al. 1998; Di Pasqua, et al. 2007). Another study showed that cinnamaldehyde could significantly reduce the transcription of virulence factors in *L. monocytogenes*, therefore affecting the bacterial virulence genes. Finally, studies on model membranes showed that cinnamaldehyde can alter the structure of the lipid monolayer by creating aggregates of cinnamaldehyde and lipids (Nowotarska, et al. 2014). Based on all of these findings, it is clear that there are multiple pathways by which cinnamaldehyde acts, and it may vary depending on the bacteria.

Trans-cinnamaldehyde is a pale, yellow compound that is extracted from the bark of the cinnamon tree. This is a Generally Recognized as Safe (GRAS) status compound (FDA 21 CFR Part 82. 2018). This status was determined based on multiple toxicity studies, as indicated in the U.S. Flavoring Extract Manufacturers' Association. Studies in 2009 and 2010 showed that TC did not have any cytotoxic effect on human epithelial or urinary tract cell lines *in vitro*. During an *in vivo* study in piglets, oral supplementation of TC showed no toxic effects at a supplementation level of 13 mg/kg. While synthetic TC does exist in the market, it is more commonly attained through steam distillation of natural cinnamon. This is a volatile compound that is most stable at refrigeration temperatures. *Trans*-cinnamaldehyde has been used frequently as a flavorant in chewing gum and other confectionaries, and in Alzheimer's and obesity research. The increased interest of TC has come from its antibacterial activity against both Gram-positive and negative bacteria, specifically on foodborne pathogens.

The uses of TC have been widely explored in the food industry. Studies have shown promising results for the use of TC both in animal and produce agriculture. There have been multiple studies that showed health benefits in pigs. First, TC did not affect the overall performance of pigs while reducing noxious ammonia and hydrogen sulfide gas levels and harmful *E. coli* in the feces when 1g TC/kg feed was supplemented (Yan and Kim. 2012). Another study using encapsulated TC at 1g/kg improved immune response to *Ascaris suum*, a parasitic

nematode found in the gastrointestinal tract of pigs (Stensland, et al. 2015). Finally, a study showed that the supplementation of TC to sows increased consumption and growth of the sow's piglets after weaning (Blavi, et al. 2016). In dairy cows, 17% cinnamaldehyde in combination with 28% eugenol, another component derived from cinnamon oil, could increase both dry matter intake and milk production by approximately 8% when added to the feed. The addition of TC and eugenol also did not affect the milk composition or milk urea nitrogen (Wall, et al. 2014).

Trans-cinnamaldehyde has also been investigated for its antibacterial efficacy on fresh produce and ready to eat foods. Studies have shown that using TC as a wash significantly reduced *E. coli* O157:H7 on apples, *E. coli* and *Penicillium digitatum* on blueberries, and yeast and mold growth on fresh-cut papayas, resulting in a more extended storage period while maintaining freshness (Baskaran, et al. 2013; Sun, et al. 2008; Albertini, et al. 2016). In ready to eat meats, a 0.75% TC solution dip treatment at 65^{o}C for 30 s on frankfurters completely reduced *L. monocytogenes* (6.0 log10 CFU/frankfurter reduction) for 70 days (Upadhyay, et al. 2013). Additionally, fumigation of shiitake mushrooms with TC increased sensory qualities throughout the storage period (Jiang et al. 2015). All of these studies show the potential for the use of TC throughout the entire agriculture industry.

1.11 TC in Poultry

1.11.1 *In vitro* Studies

Many *in vitro* studies have also indicated that TC may have the potential to reduce pathogen colonization within the gastrointestinal tract of birds. Important pathogens such as *Salmonella* Enteritidis and *Campylobacter jejuni* have been found to be inhibited by TC in laboratory scale studies (Kollanoor-Johny, et al. 2010a). A concentration as low as 25mM of TC eliminated *Salmonella* Enteritidis in chicken cecal contents within 8 hours of incubation. The same concentration of TC reduced *C. jejuni* to 1 log CFU/mL after 8 and 24 hours after incubation.

Another study that determined the effects of TC on reducing *S.* Enteritidis in chicken cecal contents found that concentrations as low as 10mM reduced *S*. Enteritidis by 6.0 log CFU/mL after 8 hours, and 8.0 log CFU/mL of the pathogen after 24 hours (Kollanoor-Johny, et al. 2010b). All concentrations, 10mM, 15mM, and 25mM TC, had a large, significant effect on *Salmonella*. The highest concentration, 25mM TC completely inhibited the growth of *Salmonella* after 8 hours (Kollanoor-Johny, et al. 2010b). Another study investigated the possibility of using TC in poultry drinking water with and without some of the most typical organic material contamination found in the barn setting. In water containing 1% feces, 0.06% TC completely reduced *S.* Enteritidis within 24 hours. Additionally, in water containing 1% feed, 0.06% TC completely reduced *S.* Enteritidis after 3 days (Kollanoor-Johny, et al. 2008).

Trans-cinnamaldehyde has been investigated for its potential to inhibit mold growth in the feed that can affect the birds adversely (Yin, et al. 2015). The common molds present in feed ingredients such as *Aspergillus flavus* and *Aspergillus parasiticus*, produce toxic metabolites in the poultry feed on storage under high humid conditions (Yin, et al. 2015). Aflatoxins are of significant concern to the industry because of the economic losses that are caused by poor bird performance, reduced egg production, and low hatchability. Yin et al. (2015) investigated the efficacy of TC on *A. flavus* and *A. parasiticus* found that it could reduce the growth and down-regulate key genes in the molds responsible for toxin production *in vitro*. Additionally, the role of TC to reduce mold growth in feed during storage at room temperature was also investigated.

Additionally, 0.38mM of TC showed a significant reduction of *C. difficil*e toxin production and cytotoxicity after 24 hours of incubation (Mooyotu, et al. 2014). *C. difficile* is an anaerobic pathogen in humans causing *Clostridium difficile* associated diarrhea (CDAD) that has been also isolated from poultry meat sporadically. This study is especially important as TC did not inhibit the growth of beneficial bacteria (*Lactobacillus*) that could be present in the gastrointestinal tract of the chicken that were tested in the same study (Mooyotu, et al. 2014).

1.11.2. In *vivo* Studies in Poultry

With *Salmonella* being a significant problem affecting the industry, many studies have been done on determining the efficacy of TC as a pre or post-harvest

strategy in poultry for food safety. A hand full of studies have investigated the potential as a pre-harvest intervention strategy to control, *S*. Enteritidis, one of the most important serotypes affecting humans for which poultry serves as contamination vehicles.

Trans-cinnamaldehyde was tested against five strains of *S.* Enteritidis colonization in studies involving 20-day-old broiler chicks (Kollanoor-Johny, et al. 2012a). The plant compound at 0.5% and 0.75% reduced the colonization of *Salmonella* by more than 3.5 log CFU/g of cecal contents in the chickens without adversely affecting the body weight (Kollanoor-Johny, et al. 2012a). This was followed by testing the efficacy of TC as a therapeutic intervention in commercial broiler chicken at market age. In this study, TC was supplemented only for 5 days prior to being slaughtered for meat. Similar to the chick study, TC at 1% level reduced *S.* Enteritidis colonization without affecting body weights, although the magnitude of reduction of the pathogen was reduced to 1.5 log CFU/g of cecal contents (Kollanoor-Johny, et al. 2012b).

Trans-cinnamaldehyde was tested in layers to reduce eggborne transmission of the pathogen as well. An in-feed supplementation study showed a significant reduction in *S.* Enteritidis populations when supplemented 1 or 1.5% TC for 66 days (Upadhyaya, et al. 2015a). The reduction in *S*. Enteritidis was seen in a lowered bacterial count on both the egg surface and in yolk when compared to the control eggs. This study also showed promising results regarding consumer

consumption. During a sensory study, consumers found no difference in eggs treated with TC when compared to the control eggs (Upadhyaya, et al. 2015a). Additionally, this study looked at the effect TC had on virulence factors including macrophage survival and oviduct colonization, and revealed a reduction in adhesion and invasion of *S*. Enteritidis in oviducts as well as reduced *S*. Enteritidis survival in macrophages (Upadhyaya, et al. 2015a).

A study done in 2015 used an in-feed supplementation to reduce the amount of aflatoxin production to, therefore, make the feed safe for consumption by birds. Concentrations of 0.005%, 0.01%, and 0.05% TC lowered the aflatoxin by at least 60%. Additionally, TC down-regulated genes associated with aflatoxin production (Yin, et al. 2014).

1.11.2 Studies on Shelled and Embryonated Chicken Eggs

Trans-cinnamaldehyde has been tested as a fumigation treatment against *S*. Enteritidis on the surface of embryonated eggs (Upadhyaya, et al. 2015b). Fumigation using 1% TC in 0.04% of ethanol significantly reduced *Salmonella* populations on the surface of table eggs when compared to the control 18 days after inoculation. On untreated eggs, *Salmonella* was recovered at approximately 6.0 log CFU/egg (Upadhyaya, et al. 2015b). Another study on shelled eggs determined the efficacy of TC as a dip treatment against a cocktail of *S*. Enteritidis strains. Eggs inoculated with 8.0 log CFU/mL *S.* Enteritidis were dipped into sterile deionized water containing TC at 0, 0.25, 0.5, and 0.75% TC. The control eggs

had about 4.0 log CFU/mL remaining on the shells, while the 0.75% egg dip completely inactivated the *Salmonella* within 30 seconds (Upadhyaya, et al. 2013).

1.12 Poultry Processing

Processing of poultry is a highly regulated and essential operation especially from the food safety perspective. The United States Department of Agriculture (USDA) has guidelines when it comes to the recommendations of how the processing should be carried out (FSIS 2015). The processing guidelines are widely followed, although many steps have the potential to spread bacteria from one bird or carcass to another. Carefully following these steps is essential to determine where bacterial contamination occurs, as then, it will be possible to both minimize contamination and add interventions to both reduce bacteria on the carcasses and minimize the potential for cross-contamination during processing.

When birds are ready for processing, they are transferred from the grow-out houses to a processing facility where they will be hung on shackles. Cross contamination is a possibility during transportation, as the transportation crates have close contact with the birds. Pathogens can survive on the feathers, skin, and cloaca of the birds, making it easy to spread bacteria through physical contact with one another, or contact with feces. After the hanging, the birds will be rendered unconscious by electrical stunning or modified atmosphere (CO2) as a practice to dismiss pain while being bled. After bleeding, carcasses proceed to the scalding step. Scalding is a process where the fully feathered carcasses are dipped into a

hot water tank to prepare for defeathering. The heat weakens the proteins that hold feathers in the follicles. All carcasses are dipped in a water bath, where there will be a high chance for cross-contamination. In order to reduce bacteria and cross-contamination, water flows against the carcasses to move contaminated water away from the carcasses. Additionally, using multiple scalding tanks to dip the carcasses can dilute the amount of bacteria exposure to the carcasses. However, cross-contamination of bacteria continues to be a problem. Table 1 shows the standard scalding times and temperatures used in the poultry industry in the United States (FSIS. 2015).

When scalding is completed, the feathers are most commonly removed using a mechanical picker. Then, during evisceration, the internal organs like the crop, intestines, heart, liver, and gizzard are removed either mechanically or manually. Any outstanding imperfections can also be removed at this step. Since the gastrointestinal tract is removed at this step, there is potential for cross-contamination. Pathogens such as *Salmonella* and *Campylobacter* could be present in the GI tract of the bird, specifically within the ceca. Any breakage of the intestinal tract can lead to contamination of the carcass or surrounding carcasses. After the evisceration, the carcasses are placed in cooling tanks to drop the internal temperature of the meat to 4^{o}C. The carcasses can be placed in this tank for hours in order to reach the refrigeration temperature (approximately, 1 hour per lb. of meat; FSIS, 2015). This is another important step where cross-contamination can occur, as the removal of the internal organs occurs just before this step. While the

temperature will prevent the growth of bacteria, there is still a high chance of contamination. This step is where facilities typically introduce antimicrobials such as organic or inorganic acids. Finally, the chilled carcasses will be placed under chilling temperature and remain until they are ready to be packaged and shipped (FSIS, 2015).

1.13 Industry Antimicrobials

In this study, we compared TC to an industry standard antimicrobial called peracetic acid (PAA). Peracetic acid is an organic acid that is commonly used in the chilling tanks of industrial poultry processing facilities. It is often sold in a combination solution containing 1% PAA and 3% hydrogen peroxide. Currently, the FSIS Directive states that PAA is approved for concentrations between 50 - 2000 parts per million (ppm) (USDA AMS. 2016). Peracetic acid has been proven to be effective against *Salmonella*, *E. coli*, *Listeria*, and *Staphylococcus* on food matrices (Bauermeister, et al. 2008 a, b, Brines, et al. 2005). Due to its antimicrobial effects, PAA has become a standard organic acid in chilling tanks, and as dip treatment for further processed poultry parts.

While PAA's antimicrobial activity has been proven, some major disadvantages qualify the need to search for an alternative. Like some of the organic and inorganic acids, PAA has been reported to cause negative color and flavor changes to meat and has the potential to cause corrosion of pipe lines (Blankenship. 1990; Stewart-Wade. 2011). Additionally, PAA is considered a

respiratory irritant, which could be irritating to processing facility workers (USDA AMS, 2016). While these reasons alone are enough to start the search for an alternative, the most critical disadvantage of PAA is that it can be less effective in the presence of organic matter (Dominguez, et al. 2018).

Despite a multitude of methods employed in the poultry industry, sporadic and multistate outbreaks occur in the United States where poultry and poultry products are commonly implicated (CDC 2013, 2016). It is obvious that more effective means of preventing *Salmonella* outbreaks through poultry products are required underscoring the continued attempts to find stronger, and safer antimicrobials by the industry. In this MS thesis, the use of TC for pre- and post-harvest applications is explored. Pre-harvest strategy with TC could be promising because preventing the pathogen from entering the processing facility by reducing *Salmonella* populations in poultry, the major source, would be ideal and may contribute to improving the microbiological safety of the products. On the other hand, using antimicrobial interventions in the processing facilities, especially during scalding and chilling conditions, would increase the safety of the poultry products before they are shipped into stores. Therefore, the objectives of the study narrated in this thesis are:

1) To determine the effects of long-term prophylactic supplementation of TC on multidrug-resistant *S*. Heidelberg in 5-week-old broiler chickens

2) To determine the effects of therapeutic supplementation of TC though water against multidrug-resistant *S*. Heidelberg in 7-week-old broiler chickens

3) To determine the effect of TC on *S*. Heidelberg on breast meat and drumsticks in simulated scalding, chilling, and during storage, and

4) To determine the effect of TC on the surface color of drumsticks after scalding.

Chapter 2

Effect of *Trans*-Cinnamaldehyde to Reduce *Salmonella* Heidelberg Colonization in Broiler Chickens – A Pre-Harvest Approach

Synopsis

Poultry serve as the reservoir host for non-typhoidal *Salmonella*, a major foodborne pathogen causing large numbers of foodborne illnesses. *Salmonella* colonizes the cecum of chickens and contaminates poultry carcasses. We determined the effect of *trans*-cinnamaldehyde (TC) on cecal *S.* Heidelberg populations in two different studies. The first study determined the effects when TC was fed through feed during the growth phase (day 1 to 4 weeks) and through water during challenge phase (5^{th} week), in broiler chickens. The second study determined the effects of TC when supplemented through water only during the challenge phase on the 7^{th} week. In each study, broiler chicks (3 broilers/treatment group, two experiments) were divided into 4 groups: Negative control (NC), Positive Control (PC), Antibiotic Control (AB) and TC. In the first study, the broilers in NC and PC groups were fed a standard diet from day 1 to week 5. The AB group received standard diet containing 50g/ton of bacitracin (BMD) for 5 weeks. The TC group was fed with a standard diet containing 0.5% TC for 4 weeks through feed and then after pathogen challenge, 0.03% TC was supplemented through drinking water for 7 days. All groups except NC were challenged with *S*. Heidelberg (3.8 log10 CFU/bird) on week 5. The broilers were euthanized 7-days after the challenge. Cecum and cecal contents were collected for *S.* Heidelberg recovery. In the second study, the NC or PC groups and AB group were fed with standard and bacitracin diets, respectively, through 7 weeks of age. The pathogen challenge (8.2 log10 CFU/bird) was conducted at the beginning of week 7. *Trans*-cinnamaldehyde (0.03%) was provided as therapeutic supplementation to broilers

in TC group through drinking water for 7 days after *S*. Heidelberg inoculation. *Salmonella* recovery was done 7 days after inoculation. Results of the first study indicated that supplementation of TC through feed and water resulted in ~2.0 log10 CFU/g reduction of *S.* Heidelberg from the cecum of 5-week-old broilers whereas bacitracin reduced the pathogen to non-detectable levels (~3.7 log10 CFU/g reduction) ($P<0.05$). In the second study, TC through water for only 7 days after challenge resulted in >90% reduction (~1.2 log10 CFU/g reduction) in *S.* Heidelberg colonization ($P<0.05$) without affecting body weights of broilers. Unlike the first study, bacitracin did not show significant reduction in *S.* Heidelberg colonization. Results of the study indicate that TC could be an effective anti-*S*. Heidelberg strategy when supplemented throughout the growth phase, and moderately effective at the low concentration tested through water post-challenge.

2.1. Introduction

Salmonella has consistently been identified as a major etiological agent causing foodborne illness in the United States (CDC. 2019a). *Salmonella* is commonly associated with the poultry industry, as the pathogen can colonize within the gastrointestinal tract of birds (Bryan, et al. 1995). *Salmonella* Heidelberg is one of the top five common serovars of *Salmonella* that colonizes poultry and frequently isolated from poultry products (Gast et al., 2004; CDC, 2011, 2013; Foley et al., 2011; Young et al., 2012; Amand et al., 2013; Foley, et al. 2011; Gieraltowski, et al. 2016). Unlike other serovars, *S.* Heidelberg is highly invasive in humans and is often resistant to multiple drugs commonly used to treat salmonellosis Dutil,et al. 2010; Zhao, et al. 2008).

The primary site of colonization of *S.* Heidelberg are the ceca, which are two blind sacs located in the junction of large and small intestines of the gastrointestinal tract. Due to high colonization ability, *Salmonella* is easily spread to other birds via the feces of the infected birds resulting in the contamination of barn, and most importantly, litter, feeders, and waterers (Jorgensen, et al. 2002; Chen, et al. 2004). Vertical transmission occurs when the bacteria reach the ovaries through blood and get deposited in the yolk during egg formation (Miyamoto, et al.1997; Okamura, et al. 2001).

Due to the potential for horizontal and vertical transmission, it is very difficult to control *Salmonella* within a flock. Therefore, identification and application of

strategies reducing *salmonella* in birds thereby reducing the transmission between the birds in the farm are warranted. Previously, antibiotics were used to reduce the pathogen populations in chickens (Rabsch et al., 2001). However, with the concern over antibiotic resistance in bacteria, development of alternative strategies to control *Salmonella* in poultry is highly critical (Snoeyenbos et al., 1969; CDC, 1986; Bokanyi et al., 1990; Schoeni et al., 1995; Layton et al., 1997; Gast et al. 2004; Hennessy et al., 2004; Scharff, 2012, Jackson et al., 2013).

Essential oils have received attention as alternatives to control *Salmonella* because of their antimicrobial activity and popularity as natural options in food as antioxidants, antibacterials, immunomodulators, and gut function augmenters (Nair et al., 2019). *Trans*-cinnamaldehyde is an essential oil ingredient that has been explored for its potential use in poultry. It is the active ingredient derived from cinnamon oil, and already has use in the food industry as a natural flavoring agent (FDA 21 CFR Part 82. 2018). In addition to the *in vitro* investigations, a handful of research has tested its potential for use in poultry. Previous studies have shown that TC, at concentrations 0.75 and 1%, reduced *S.* Enteritidis within chicken cecum (Kollanoor-Johny, et al. 2010). Additionally, TC has been tested in layer hens to reduce egg-borne transmission of *Salmonella* at up to 1.5% (Upadhyaya, et al. 2015 b). However, none of these studies explored long-term supplementation of TC in broiler chickens against *S.* Heidelberg, or its supplementation through water. Therefore, the objective of the current study was to investigate the potential of TC against *Salmonella* Heidelberg colonization in broiler chickens when

supplemented throughout the growth phase via feed and water at lower concentrations, and therapeutically through water alone.

2.2. Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota.

2.2.1. Experimental Birds and Management

One-day old commercial non-vaccinated Cornish cross (Ross 708) broiler chicks were procured from a commercial hatchery in Minnesota. Birds were allocated into floor pens in the Poultry Teaching and Research Facility (PTRF) at the University of Minnesota. Age appropriate temperature, light and humidity were provided during the study period. Feed for each group was formulated as per the requirements and manufactured in the feed mill at the Southern Research and Outreach Center, Waseca, MN. The birds had access to *ad libitum* feed and water.

2.2.2 Bacterial Strain & Dosing

S. Heidelberg 1904 (2014 Tennessee correctional facility outbreak strain) was used for inoculating birds. The strain was cultured in 10 mL tryptic soy broth (TSB) supplemented with 50 µg/ml of nalidixic acid (NA) incubated at 37°C for 24 h (TSB, Hardly Diagnostics; NA, Alfa Aesar). After three subcultures, 1 mL was transferred to 100 mL TSB and incubated overnight at 37°C. The culture was centrifuged (3600 X *g* for 15 minutes at 4C) and the pellet was resuspended in 100

mL phosphate buffered saline (PBS). Bacterial populations in the diluted culture was confirmed by plating appropriate dilutions of the culture on the Xylose Lysine Desoxycholate Agar (XLD, Hardly Diagnostics) plates, and incubated at 37°C for 24 h.

2.2.3 Experimental Design:

Experiment 1 (5-week study):

Day-old broiler chicks were weighed and randomly assigned to 4 groups of 3 birds each. Treatment groups included negative and positive control groups (basal diet), antibiotic group [basal diet + Bacitracin Methyl Disalicylate (BMD) at 50g/ton of basal diet], and TC group (0.5% TC in the basal diet). Feed consumption and body weight were measured on a weekly basis. These treatments were fed for the entire 5-week study period at PTRF and when the birds were transferred to the BSL2 isolation facility. The birds in the TC group were switched from the 0.5% TC + basal diet to basal diet alone while in the PTRF.

Experiment 2 (7-week study):

All management practices and nutrition program were the same as Experiment 1, except the TC group was fed the basal diet alone. All birds received their respective feeds all 7 weeks in the PTRF and in the BSL2 isolation facility.

2.2.4 Challenge Studies:

At the beginning of the 5th week, birds were transferred to the BSL2 isolation facility and were fed with age-appropriate diets. At the end of the 5th week, birds in the PC and TC groups were challenged with 5 mL inoculum containing 3.69 log10 CFU of *S.* Heidelberg by crop gavage. Negative Control birds did not receive a challenge. Positive control birds were taken from the group that received basal diet alone. The treatment groups were negative control (-*S.* Heidelberg, -TC, -BMD), positive control (+*S.* Heidelberg, -TC, -BMD), antibiotic group (-*S.* Heidelberg, -TC, +BMD), and TC group (-*S.* Heidelberg, + 0.03% TC in water, -BMD). Two replicates for each treatment group were included. Seven days after challenge, birds from each treatment group were euthanized by carbon dioxide asphyxiation for sample collection. Necropsy and sample collection were performed at the Veterinary Diagnostic Laboratory at the University of Minnesota.

For the 7-week study, similar protocols were followed, except that the birds were brought in to the BSL2 facility at the beginning of 7th week and given the treatments for 7 days. Samples were collected during necropsy.

2.2.5 Determination of *S.* Heidelberg in Cecum

Samples from all experiments were collected in 10 mL PBS during necropsy. Cecal samples were weighed, homogenized and serially diluted in PBS. From the homogenized sample, 200µl of appropriate dilutions were surface plated on XLD + NA plates. Bacterial colonies were enumerated after incubating the plates at 37ºC for 48 h. When the colonies were not detected by direct plating, the

samples were tested for surviving cells by enrichment in 10 mL of selenite cysteine broth (SCB) at 37°C for 8 h followed by streaking on XLD + NA plates and incubation at 37ºC for 24 h. Liver and spleen samples were enriched in 10 mL SCB after collection. After incubation at 37ºC for 12 h, cultures from enrichment broth was streaked on XLD + NA plates. Plates were incubated at 37ºC for 24 h to detect the presence of *S.* Heidelberg.

2.2.6 Statistical Analysis

A completely randomized design was used to determine the effect of TC on *S.* Heidelberg in broiler chickens. Each pen was considered an experimental unit and each treatment had 3 birds. Factors included 4 treatments (NC, PC, AB, and TC) and three organ samples. Colonies were counted and appropriate dilution factor and cecal weights were applied to obtain bacterial populations per g of cecum. The numbers of *Salmonella* colonies were logarithmically transformed before analysis. A P value < 0.05 was considered statistically significant.

2.3. Results

The effect of supplementation of TC through feed during pre-challenge phase and through water during post-challenge phase on *S*. Heidelberg populations in the cecum are summarized in Figure 1. As expected, *S.* Heidelberg survived in the cecum of broiler chickens at the end of 5-week study (3.5 log10 CFU/g). In addition, NC groups did not yield any bacteria, indicating that there was no cross-contamination across the pens during the study.

The supplementation of TC resulted in a significant reduction of *S*. Heidelberg in broiler chickens (Figure 1). Compared to PC, TC groups resulted in 1.8 log10 CFU/g reduction of *S*. Heidelberg. Additionally, AB resulted in almost 4 log reduction compared to PC. It was also observed that the AB groups maintained body weights comparable to NC, whereas the other challenge groups, PC and TC, showed significantly reduced body weights at the end of the study (Figure 2).

In the second study, we observed that supplementation of TC at a much lower concentration (0.03%) only during the post-challenge phase maintained a significant reduction in the *S*. Heidelberg populations at the end of the 7th week (~1 log reduction; Figure 3) without adversely affecting the body weights (Figure 4). However, in this study, AB group did not show any reduction of the pathogen compared to PC, but displayed a significantly higher pathogen load than TC supplemented group (Figure 3).

2.4. Discussion

Previous research on the use of TC in poultry production has indicated that the compound is effective against one of the major poultry-borne *Salmonella*, *S*. Enteritidis, in broiler and layer chickens (Kollanoor-Johny, et al. 2012; Upadhyaya, et al. 2015). In most studies, TC was supplemented for a shorter duration of time for the intended purpose. For example, Kollanoor-Johny et al. (2012) used TC at 1% continuously for 20 days as a prophylactic method to control *S*. Enteritidis in

chicks. It was found that a reduction of ~4 logs of the pathogen was possible when TC was used prophylactically. However, when TC was used at 1% as a therapeutic dose through feed for 7 days before the market age, the reduction observed was reduced by 1.5 logs (Johny et al., 2012b). Similarly, Upadhyaya et al. (2013, 2015) also used TC to control eggborne transmission of *S*. Enteritidis, but at a high concentration of 1 and 1.5%. Our study was designed to determine two additional factors: 1) if TC could be more beneficial if supplemented throughout market age (5 weeks) at a relatively lower concentration (0.5%), and 2) if TC would be effective when supplemented through water.

Results from our first study (5-week-old birds) indicated that TC when supplemented at 0.5% for longer duration through feed, and further through water at 0.03% after *S*. Heidelberg challenge, resulted in 1.7 log reduction of the pathogen compared to the controls (Figure 1). This strategy could be comparable to what Johny et al. (2012a, b) found with regards to pathogen reduction, and only half the concentration of TC (0.5% vs 1%) was used in this study. In addition, we also found that 0.03% TC, around 50-fold reduced concentration of TC, applied through water over 7 days was able to retain the efficacy of TC post challenge (Figure 1). Although the strategy to add antibacterial agents through water is relatively new, the possibility of reducing the concentration of essential to significantly lower levels when applied through water could be more profitable for the commercial poultry production sector.

Result from the first study led us to ponder more on the therapeutic application of TC through water alone in the second study (7-week-old birds). We observed that TC at 0.03% when applied through water resulted in a log reduction of *S*. Heidelberg compared with controls (Figure 3). Although this reduction was lower than what could be obtained when a combined approach (prophylactic feed and therapeutic water application), the concentration of TC could be reduced to around 50-fold and applied only through water. Additional studies determining the potential of TC at 0.05 and 0.075% against *S.* Heidelberg through water will be pursued.

We also found that BMD was able to reduce *S*. Heidelberg populations to >3.5 log10 CFU/g in the 5-week study (Figure 1), although it failed to maintain the reduction in the 7-week study (Figure 3). Studies determining the efficacy of BMD on Gram negative organisms, such as *Salmonella*, are scantily available (Hofacre, et al. 2007). This is because its intended purpose is for use against Gram positive pathogens (Johnson, et al. 1945).

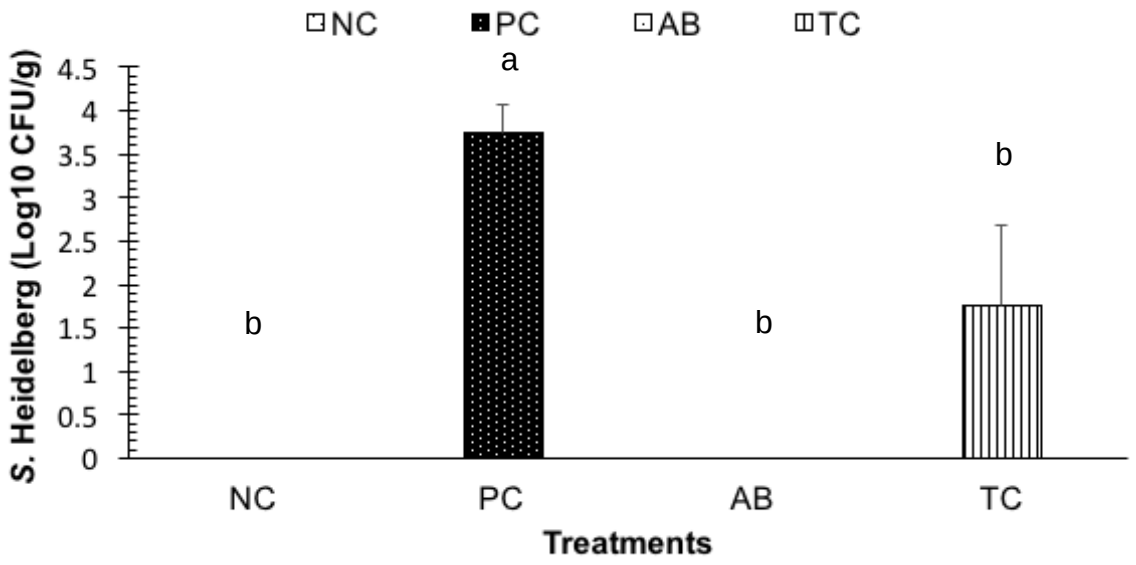
Furthermore, challenge with *Salmonella* caused reduction in the body weights in PC and TC groups in the first study, however, this effect was no observed in the 7-week study (Figures 2 and 4) as the reduction of pathogen populations observed with TC was without any adverse effects on the body weights. This effect is not thoroughly understood from our study due to an insufficient number of birds to determine a growth performance parameter.

However, we speculate that there could be alternations in the microbiota in response to BMD at these age groups. We are currently exploring this possibility using a microbiomics approach.

Overall, our study found that TC was effective against another major serotype of *Salmonella* colonizing broilers, *S*. Heidelberg. Additionally, we observed that a reduced concentration of TC (0.5%) if supplemented throughout the life time of the birds could result in a consistent reduction of the pathogen in broiler chickens. Moreover, water supplementation could be a better strategy for essential oils but should be explored further to validate a working concentration post challenge.

Figure 1

Effect of long-term prophylactic supplementation TC at 0.05% through feed and therapeutic supplementation at 0.03% through water on *S*. Heidelberg in 5-week-old commercial broiler chickens

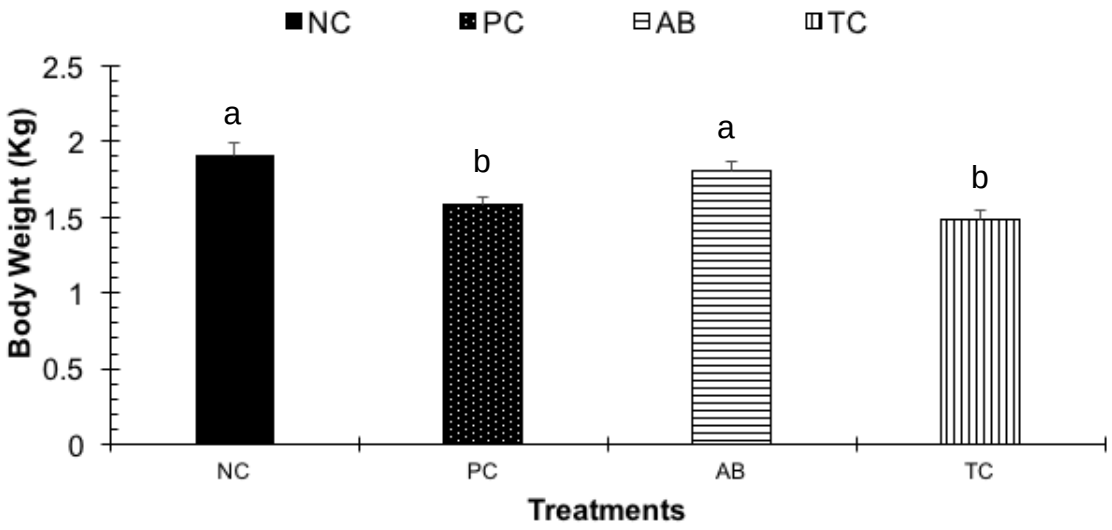


NC - Negative Control; PC - Positive Control; AB - Bacitracin; TC - *Trans*-cinnamaldehyde

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 2

Effect of long-term prophylactic supplementation TC at 0.05% through feed and therapeutic supplementation at 0.03% through water on body weights of 5-week-old commercial broiler chickens

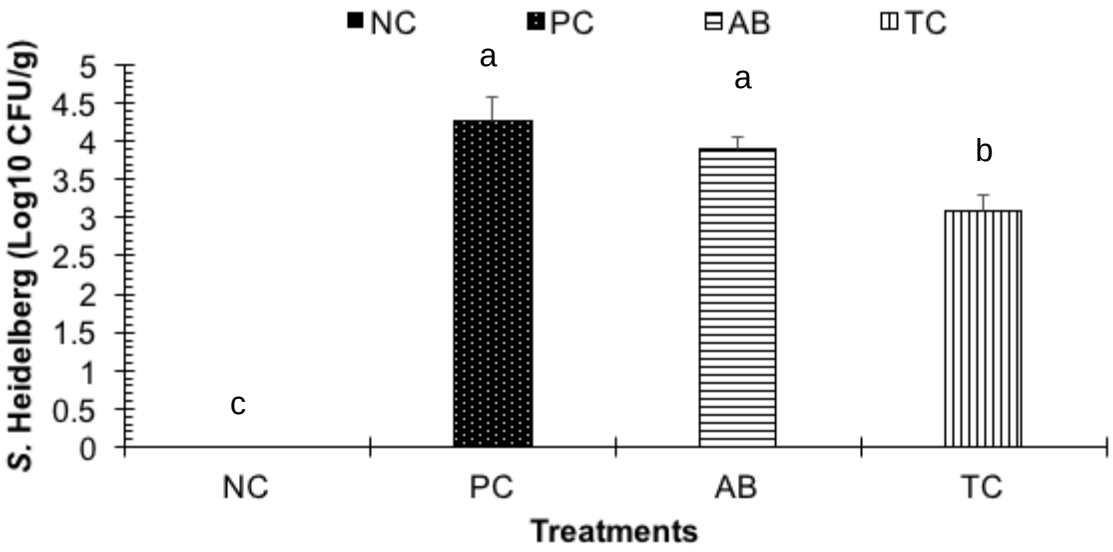


NC - Negative Control; PC - Positive Control; AB - Bacitracin; TC - *Trans*-cinnamaldehyde

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 3

Effect of therapeutic TC (0.03%) through water on *S*. Heidelberg in 7-week-old commercial broiler chickens

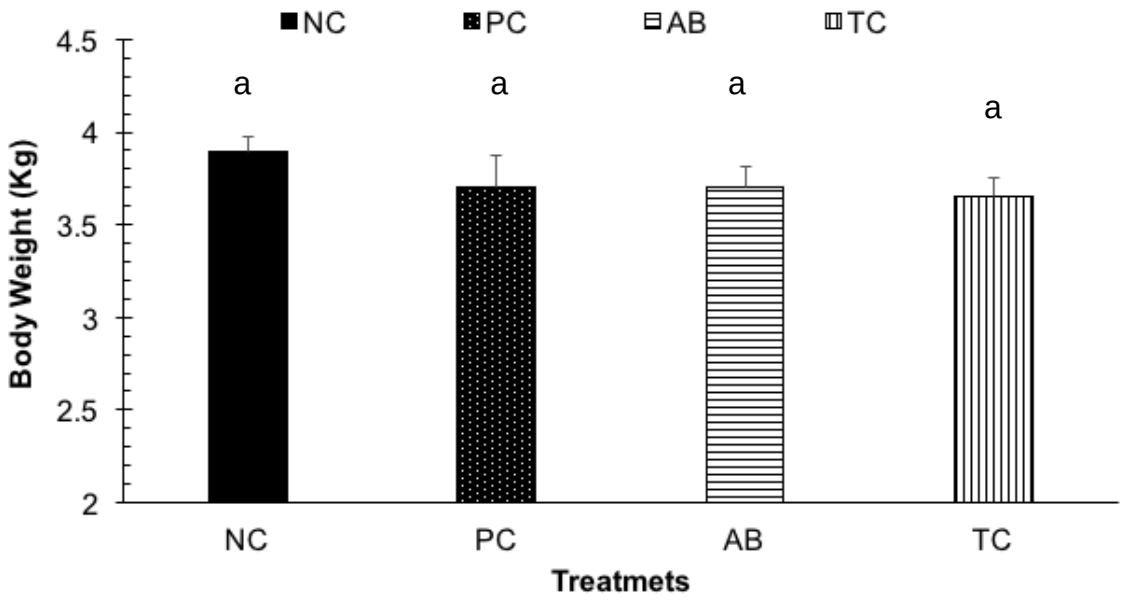


NC - Negative Control; PC - Positive Control; AB - Bacitracin; TC - *Trans*-cinnamaldehyde

[a,b,c] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 4

Effect of therapeutic TC (0.03%) through water on body weights in 7-week-old commercial broiler chickens



NC - Negative Control; PC - Positive Control; AB - Bacitracin; TC - *Trans*-cinnamaldehyde

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Chapter 3

Effect of *Trans*-Cinnamaldehyde as a Scalding Antibacterial against *Salmonella* Heidelberg on Broiler Chicken Meat and Drumsticks – A Post-Harvest Approach

Synopsis

Salmonella is a major foodborne pathogen that causes gastrointestinal illness in humans worldwide. Contaminated poultry products play a significant role in the transmission of the pathogen to humans. In this study, we determined the antibacterial effect of *trans*-cinnamaldehyde (TC), the major constituent of cinnamon essential oil, against *Salmonella* Heidelberg on broiler drumsticks at scalding conditions intended for poultry processing. Broiler chicken drumsticks were inoculated with either low (~3.0 log10 CFU/g) or high (~4.5 log10 CFU/g) concentrations of *S.* Heidelberg and were immersed in treatment water containing 0.5% TC, 1% TC, 0.05% peracetic acid (PAA), 0.5% TC + 0.05% PAA or 1% TC + 0.05% PAA at 54^{o}C for 2 min (USDA-recommended time-temperature combination for scalding). The drumsticks inoculated with *S.* Heidelberg and without any antimicrobial treatment in scalding water served as the positive control (PC). After scalding, the drumsticks were homogenized in 350 mL Phosphate Buffered Saline for 30 s to obtain surviving *S.* Heidelberg populations. Also, the populations of *S.* Heidelberg on drumsticks and in the scalding water were enumerated by surface plating and enrichment methods. We also determined the efficacy of the scalding treatments against *S.* Heidelberg survival on drumsticks during chilled storage. For this study, the drumsticks were inoculated with 4.5 log10 CFU/g *S.* Heidelberg and immersed in scalding water containing 1% TC, 0.05% PAA or 1% TC + 0.05% PAA. After scalding, the drumsticks were immersed in chilling water without any antimicrobials for 30 min, packed and stored at 4^{o}C for 48 h. The surviving *S.* Heidelberg populations were determined immediately after

chilling and after 48 h of storage. Additionally, the effect of the treatments on the surface color of drumsticks was evaluated. All the experiments were repeated six times and data were analyzed. Results revealed that PAA and its combination with TC (0.5 or 1%) resulted in the significant reduction of *S.* Heidelberg for low (1.7 to 2.4 log10 CFU/g reduction) and high inoculum (2.1 to 3.1 log10 CFU/g reduction) levels of *S.* Heidelberg ($P<0.05$). Moreover, the same treatments inactivated *S.* Heidelberg to non-detectable levels from the scalding water whereas 2.0- (low inoculum study) and 4.0- (high inoculum study) log10 CFU/ml *S.* Heidelberg survived in PC ($P<0.05$). The scalding treatments were also effective in inhibiting *S.* Heidelberg on the drumsticks compared to the respective controls during chilled storage. The treatments did not affect the surface color of the drumsticks ($P>0.05$). This study reveals that TC could be an alternative antimicrobial for *S.* Heidelberg control in poultry processing

3.1. Introduction

Salmonella enterica serovar Heidelberg (*S*. Heidelberg) is one of the top five serovars of zoonotic *Salmonella* that colonizes poultry and causes severe disease in humans (Snoeyenbos et al., 1969; CDC, 1986; Bokanyi et al., 1990; Schoeni et al., 1995; Layton et al., 1997; Gast et al. 2004; Hennessy et al., 2004; Scharff, 2012, Jackson et al., 2013). This serotype of *salmonella* is important to human and veterinary public health because it is one of the most invasive *Salmonella* species and is also likely to be resistant to multiple antibiotics (Dutil,et al. 2010; Zhao, et al. 2008). *Salmonella* Heidelberg is commonly associated with poultry during the last 3 to 4 decades, and has caused recent outbreaks in 2011, 2013, and 2014 through poultry products (CDC, 2011; Gieraltowski, et al. 2016). Contaminated ground turkey was the cause of a multistate outbreak of *Salmonella* Heidelberg in 2011 (CDC. 2011). Mechanically separated poultry products from a correctional facility in Tennessee and rotisserie chicken produced from a California plant resulted in the outbreaks of 2013, and 2014, respectively (Gieraltowski, et al. 2016).

Salmonella is able to colonize within the cecum of chickens, and the spread of the pathogen could occur while processing. Feces from the infected carcasses can contaminate other carcasses during the evisceration step, or feces attached to carcass skin can be transferred to other carcasses during scalding and chilling steps. Scalding is the step where entire chicken carcasses are immersed into 50 to 54^{o}C for 90 to 120 seconds. This step will loosen feather follicles before the

feathers are picked up by a picker. However, since a large number of carcasses are immersed into the same tank of water at a given time, this step is considered a critical control point where bacteria cross-contaminate the carcasses. On the other hand, chilling is the final step of processing where the carcasses are immersed in cold water to bring the core temperature of meat to 4^{o}C. This is another critical control point, and the cold conditions cause the skin to swell which can allow bacteria to attach more frequently (USDA FSIS. 2015).

Usually, antimicrobials are not added to the scalding tanks due to the inefficacy of most of the antimicrobials to act on the pathogen in a contaminated, highly organic rich water. However, in the frequent washes that follow, and particularly in the chilling step, processing industry uses an organic acid called peracetic acid (PAA). It has been proven to be effective against *Salmonella*, *E. coli, Listeria*, and *Staphylococcus* on different food matrices (Bell, et al. 2001; Brinez, et al. 2006). Due to its antimicrobial effects, PAA has become a standard organic acid in chilling tanks, and in dip treatments for further processed poultry parts.

While PAA's antimicrobial activity has been proven, some major disadvantages qualify the need to search for an alternative in scalding and chilling tanks. Like most of the water acidifiers, PAA has been reported to cause negative color and flavor changes to meat (Blankenship; 1990). Additionally, PAA is considered a respiratory irritant, which is an annoyance to processing facility

workers (USDA AMS. 2016). While these reasons alone are enough to start the search for an alternative, the most critical disadvantage of PAA is that it is less effective in the presence of high levels of bacteria and in the presence of organic matter (Dominguez, et al. 2018).

Currently, the use of essential oils as antimicrobials has received renewed interest in the agriculture industry as an alternative to synthetic chemicals for pathogen control. Essential oils have been shown to be effective against *Salmonella* and other important foodborne pathogens (Nair et al., 2019). *Trans*-cinnamaldehyde (TC), an essential oil derived from cinnamon oil, is a GRAS compound approved by the FDA as a food additive (FDA 21 CFR Part 82. 2018). Studies have shown the effectiveness of TC *in vitro*, as well as in water with and without contaminants such as feces, litter, and feed (Kollanoor-Johny, et al. 2008). While the previous experiments applied TC in drinking water, the same principle could be applied for the use of TC in scalding and cooling tanks. In this regard, the current study evaluated the efficacy of TC against *S.* Heidelberg, a major serovar in poultry, in poultry processing conditions, in both scalding tanks and cooling tanks.

Therefore, the objective of the current study was to determine the effects of TC on *S.* Heidelberg during (1) scalding using breast meat, (2) scalding using broiler drumsticks at low inoculation, (3) scalding using broiler drumsticks at high

inoculation, (4) chilling using broiler drumsticks, (5) storage using broiler drumsticks, and (6) the color of broiler drumsticks after scalding treatments.

3.2. Materials and Methods

3.2.1 Bacterial Strain and Growth Conditions

S. Heidelberg 1904 and 466 (2014 Tennessee correctional facility outbreak strains) were used in the study. These strains were independently cultured in 10 mL tryptic soy broth (TSB) supplemented with 50 µg/ml of nalidixic acid (NA) incubated at 37°C for 24 h. After three subcultures, 1 mL was transferred to 100 mL TSB and incubated overnight at 37°C. The cultures were centrifuged separately (3600 X*g* for 15 minutes at 4^{o}C) and the pellets were resuspended in 10 mL PBS. Thereafter, the cultures were mixed and used as inoculum. The bacterial count in the diluted culture was determined by surface plating appropriate dilutions on Xylose Lysine Desoxycholate Agar (XLD) + NA plates incubated at 37°C for 24 h.

3.2.2 Broiler Meat and Drumstick Preparation

All meat samples used in this study were purchased from a local retail store. Either chicken breast meat (25g) or broiler drumsticks (150g) were used for the studies. All meat samples were exposed to UV light for 5 minutes to kill the background flora present before *S*. Heidelberg inoculation. In the following experiments, 6 replications (n=6) were conducted: (1) scalding using breast meat, (2) scalding using broiler drumsticks at low inoculation, (3) scalding using broiler

drumsticks at high inoculation, (4) chilling using broiler drumsticks, and (5) storage using broiler drumsticks.

3.2.3 Inoculation of *S*. Heidelberg on Broiler Meat and Drumsticks

Meat and drumstick samples were inoculated with either 2.8 log10 CFU/g *S*. Heidelberg (low inoculation) or 4.5 log10 CFU/g of *S*. Heidelberg (high inoculation) using the spot inoculation technique. Bacteria was allowed to attach for 20 min. After the attachment time, each meat piece or drumstick time was allocated to its respective treatment.

3.2.4 Scalding

After inoculation, the meat piece or drumstick was placed into tap water at 54ºC for 2 minutes. Then, the samples were homogenized for 30 s via stomaching procedure. The samples were then diluted and plated on XLD + NA plates.

3.2.5 Chilling

After the scalding step described above, the drumsticks were placed in tap water at 4ºC for 30 minutes. Samples were removed and homogenized for 30 seconds before dilution and surface plating.

3.2.6 Storage

After the scalding and chilling steps described above, the drumsticks were packaged aerobically and stored at 4°C for 48 hours. After the storage period, the samples were homogenized for 30 s to retrieve surviving bacterial populations.

3.2.7 Microbiological Analysis

After surface plating, 200 µl of appropriate dilutions of the samples and the treatment waters were plated on XLD + NA plates. Bacterial colonies were enumerated after incubating the plates at 37°C for 48 h. All samples were enriched in 10 mL sodium selenite cysteine (SCB) broth. After incubation at 37°C for 12 h, cultures from SCB was streaked on XLD + NA plates. Plates were incubated at 37°C for 24 h to detect the presence of *S.* Heidelberg.

3.2.8 Color Analysis

The scalding experiment on drumsticks was repeated as stated above with the same treatments but without any bacterial inoculation. After the 2-min scalding step, drumsticks were analyzed for color using a Hunter handheld colorimeter (Hunter Lab. MiniScan EZ 4500S Spectrophotometer. Reston, VA). Six color readings were taken of each drumstick in the same order, at the wide end, middle end, and small end of each side of the drumstick.

3.2.9 Statistical Analysis

A completely randomized design was used to analyze the effect of TC on *S.* Heidelberg in scalding and chilling conditions on either chicken breast meat or

drumsticks. The factors included 5 treatments (0.5% TC, 1% TC, 0.5% TC + 0.05% PAA, 1% TC + 0.05% PAA, and 0.05% PAA alone), time (0, 24, 48 hours wherever appropriate), and level of inoculation (low and high inoculum wherever appropriate). Colonies were counted and appropriate dilution factor and sample weights were applied to obtain bacterial populations per g of meat. The numbers of *Salmonella* colonies were logarithmically transformed before analysis. All data were analyzed using the PROC MIXED procedure of SAS. A P-value < 0.05 was considered statistically significant.

3.3. Results

In the first experiment that tested the efficacy of TC against *S*. Heidelberg on breast meat, TC alone reduced the pathogen populations by 1.25 log10 CFU/g when compared to the positive control (2.75 log10 CFU/g) ($P<0.05$; Figure 5). On the other hand, PAA alone reduced *S*. Heidelberg populations by 1.6 log10 CFU/g when compared to the positive control ($P<0.05$). The combination of TC and PAA was comparable to the reductions observed with either of them alone (Figure 5). In scalding water, however, PAA alone or in combination with TC resulted in non-detectable levels of *S.* Heidelberg ($P<0.05$; Figure 6). Whereas, TC treatment was positive for *S*. Heidelberg (Figure 6).

In the second experiment, TC was tested against *S*. Heidelberg on drumstick at a low and high inoculum levels in scalding water. A similar trend followed for PAA, TC, and PAA + TC treatments at both inoculation levels (Figures 7 – 10). At both inoculation levels, the greatest reduction was seen with the PAA

treatment (Figure 7, 9). Peracetic acid alone reduced *S.* Heidelberg populations by 2.25 log10 CFU/g (low inoculum; Figure 7) and 3 log10 CFU/g (high inoculum; Figure 9) when compared to the positive control kept at 54^{o}C. On the other hand, the two concentrations of TC (0.5 and 1%) did not significantly differ in the efficacy to reduce *S*. Heidelberg populations and was in the range of 0.5 to 1 log10 CFU/drumstick (Figure 7, 9). Finally, the combination of PAA and TC reduced the *Salmonella* by 1.75 log10 CFU/drumstick (low inoculum) and 2.25 log10 CFU/g (high inoculum) when compared to the positive control maintained at 2.5- and 4.5 log10 CFU/drumstick with low inoculum and high inoculum, respectively (Figures 7, 9). Moreover, PAA and TC at 1% resulted in non-detectable levels of the pathogen in the scalding water. The combination, obviously, did not yield any *Salmonella* either (Figures 8, 10).

In the third experiment that included a chilling step after the scalding treatments were applied, none of the treatments resulted in a significant reduction of the pathogen (Figure 11), whereas the combination of TC at 1% and PAA, and PAA alone resulted in 2 log reduction of *S*. Heidelberg populations in scalding water (Figure 12). The combination of TC and PAA resulted in 1.5 log reduction of *Salmonella* compared to the controls after storing the treated samples for 48 hours at 4^{o}C (Figure 13). The treatments alone resulted in approximately a log reduction of *Salmonella*.

The color analysis indicated no statistical difference between the treatments applied alone or in combination compared to the controls (Figure 14).

3.4. Discussion

Despite multitude of efforts to control foodborne pathogens in poultry processing, *Salmonella* results in cross-contamination of carcasses and cut-up parts, resulting in sporadic and multistate outbreaks in the U.S. (CDC, 2012, 2013, 2015, 2018b, 2019 a, b). To counteract the pathogen, the industry has employed several antibacterial treatment steps in processing (USDA FSIS. 2015). Most antimicrobials are used at the chilling step due to its economic and food safety benefits. One of the major industry antimicrobials used in processing is PAA due to its direct action on foodborne pathogens however, the effects are generally not effective at scalding temperature (54^{o}C) due to evaporative loss and interaction with high organic load in the tanks (Dominguez, et al. 2018; Bell, et al. 2001; Brinez, et al. 2006).In this paper, we explored the potential of TC as a natural option to control *S.* Heidelberg in the scalding step, and evaluate its effects on the pathogen immediately after the treatment, after chilling, and refrigerated storage.

Mostly, our results revealed that PAA was the most effective treatment across different experiments when applied alone (Figure 5, 7, 9, 12, 13). This is supportive of the current industry strategy where PAA is used in various steps during poultry processing. Although little information is available on PAA's mechanism of action, it is reported to cause problems to bacterial cell wall, affect

integral bonds in proteins, thereby potentially affecting the activity of enzymes and metabolites (Sattar, et al. 1998; Middleton, et al. 1997). It is reported that PAA could inactivate both Gram positive and negative organisms, fungi, and yeasts in less than 5 min at <100 ppm and in the presence of organic matter at 500 ppm (Block 2001). In the current study, we observed that PAA at 500 ppm was effective on breast meat and drumsticks, (Figures 5, 7, 9), although the efficacy was reduced after a post-scalding chilling step was applied (Figures 11 and 13). This could be due to the loss of PAA during a chilling treatment and 48 h of storage. However, it was highly effective and reduced the pathogen to non-detectable levels in the scalding water immediately after the application (Figures 6, 8, 10).

Given the caveats in using PAA as an antimicrobial agent, including the potential evaporative loss during chilled storage and multiple wash steps, and the pulmonary irritant property, we explored the potential of a natural essential oil ingredient obtained from cinnamon oil, TC, to tackle the aforementioned negative effects of PAA (USDA AMS. 2016). We found that 1% TC was comparable to 500 ppm of PAA with regards to its rapid activity against *S*. Heidelberg on breast meat inactivating the pathogens in 2 min of scalding and after a 2-min chilling step on drumsticks (Figures 5, 11). But TC was less effective than PAA on rapid inactivation of *S*. Heidelberg populations on broiler drumsticks at both inoculum levels (Figures 7, 9, and 12). However, TC was highly effective as a scalding water disinfectant on *S.* Heidelberg (Figures 6, 8, 10). TC has been previously reported to be highly effective against another major *Salmonella* serovar, *S*. Enteritidis, in

poultry drinking water alone or in the presence of organic matter such as litter, droppings, and chicken feed, and has been found to be effective at low concentrations in chicken cecal contents *in vitro* (Kollanoor-Johny, et al. 2008, 2010). Similar to PAA, little investigation has been carried out on the mechanism of action of TC in applied environments; we are currently exploring this possibility.

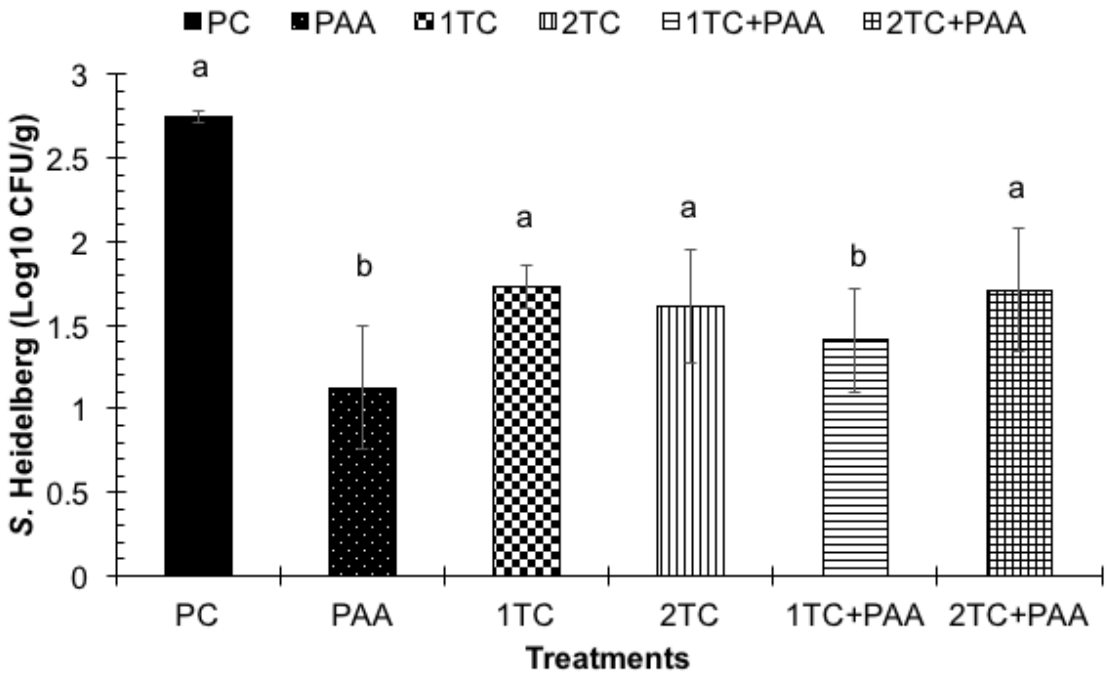
It is reported that TC causes rapid inhibition of energy metabolism (Gill, and Holley, 2004, 2006 a, b), by inhibition of membrane bound ATPase activity. It can also change the composition of fatty acids in the membrane affecting the overall structure and strength of the microbe (Di Pasqua, et al. 2007). In addition, investigators have reported the effect of TC on amino acid, carbohydrate and lipid metabolism in *Cronobacter sakazakii*, a pediatric Gram-negative pathogen, by proteomic analysis (Amalaradjou, et al. 2014), and on virulence mechanisms, including motility, invasion, type 3 secretion systems in *S*. Enteritidis using DNA microarray analysis (Kollanoor-Johny, et al. 2017).

The key finding from this study is that TC was almost equally effective as PAA in controlling the pathogen in scalding tanks. This finding is highly important since cross-contamination of pathogens in scalding and chilling tanks is enhanced by water; our finding will support the continued industry use of PAA, and devise alternative water disinfection methods using essential oil ingredients. Although PAA, TC, and their combinations were found to be bacteriostatic or bactericidal on *S*. Heidelberg, their activity could be enhanced with other hurdle approaches. We

also find the necessity of scale-up investigations to determine the efficacy of TC and PAA against *S*. Heidelberg on chicken carcasses in the presence of organic matter such as feathers and feces in scalding tanks.

Figure 5

Effect of TC, PAA, and their combination on *Salmonella* Heidelberg in scalding conditions on broiler breast meat

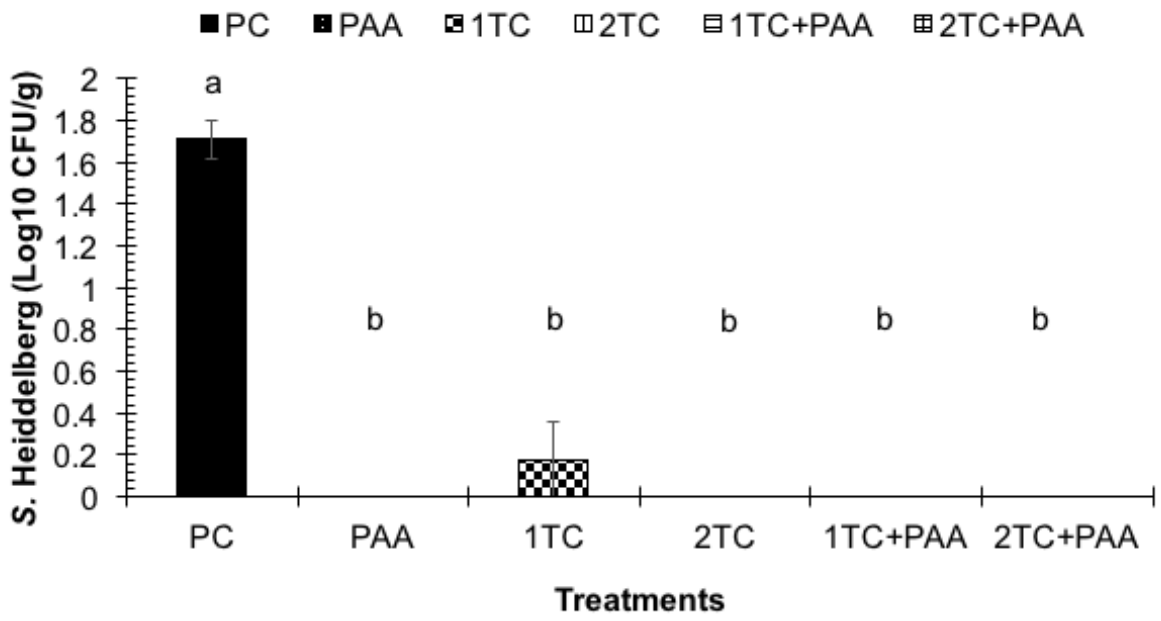


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 2 TC - 2% TC; 1TC + PAA - 1% TC + PAA; 2TC + PAA - 2% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 6

Effect of TC, PAA, and their combination on *Salmonella* Heidelberg in scalding water after treatment on breast meat

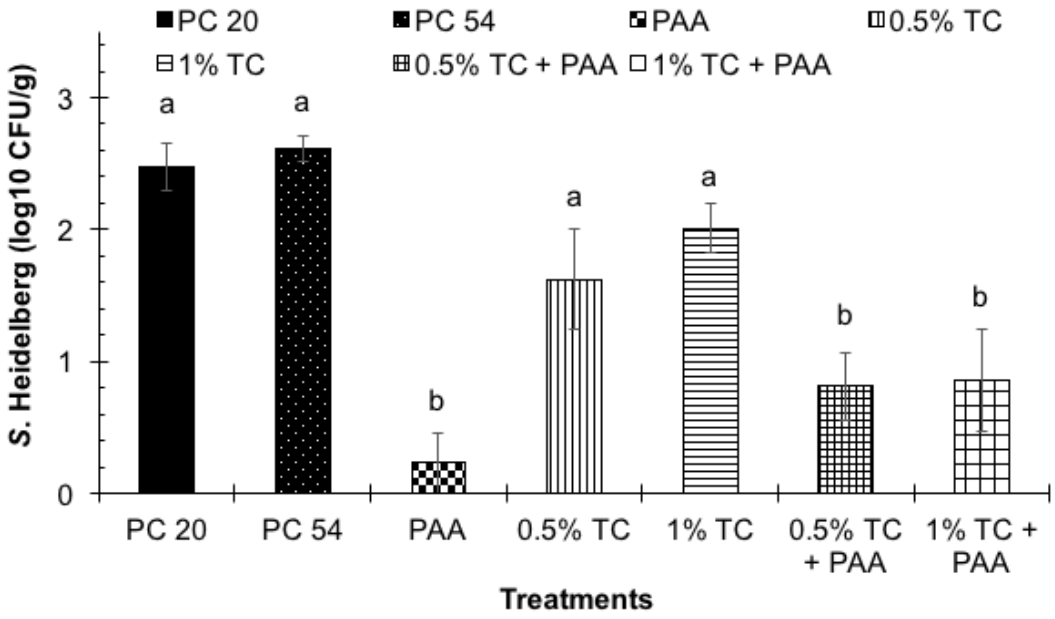


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 2 TC - 2% TC; 1TC + PAA - 1% TC + PAA; 2TC + PAA - 2% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 7

Effect of TC, PAA, and their combination on *Salmonella* Heidelberg applied at low-inoculation level on broiler drumsticks under scalding conditions

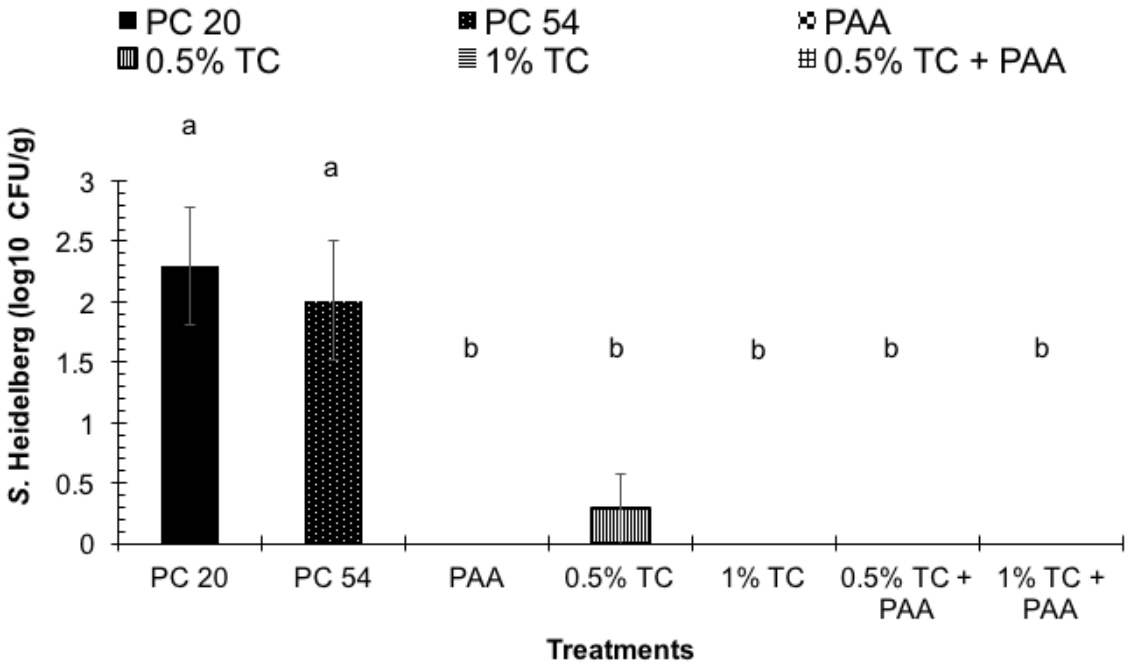


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 2 TC - 2% TC; 1TC + PAA - 1% TC + PAA; 2TC + PAA - 2% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 8

Effect of TC, PAA, and their combination on *Salmonella* Heidelberg in scalding water after application of low-inoculation level on broiler drumsticks under scalding conditions

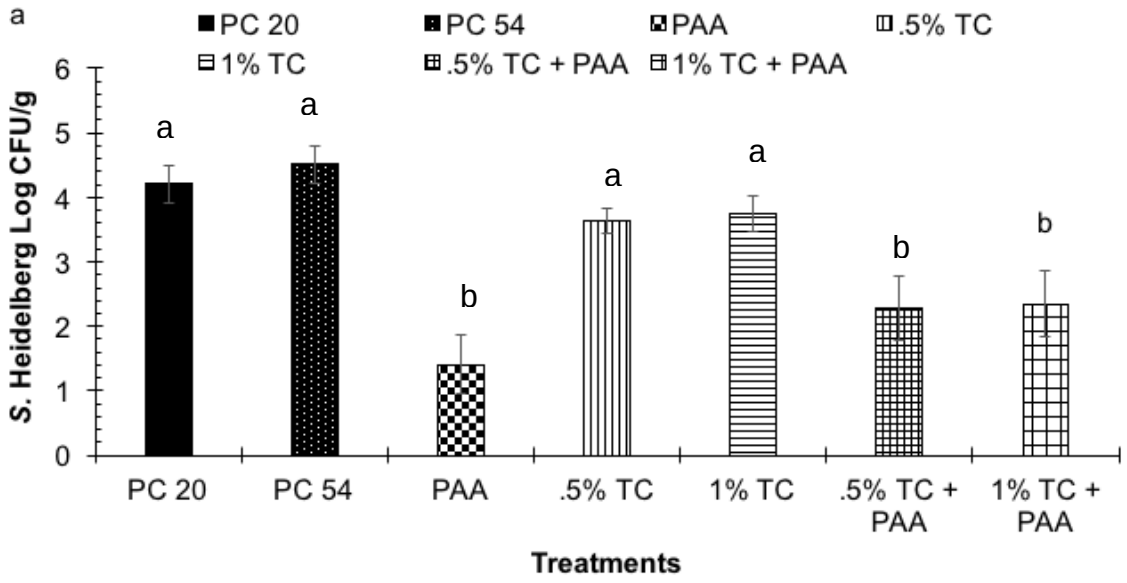


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 2 TC - 2% TC; 1TC + PAA - 1% TC + PAA; 2TC + PAA - 2% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 9

Effect of TC, PAA, and their combination on *Salmonella* Heidelberg applied at high-inoculation level on broiler drumsticks under scalding conditions

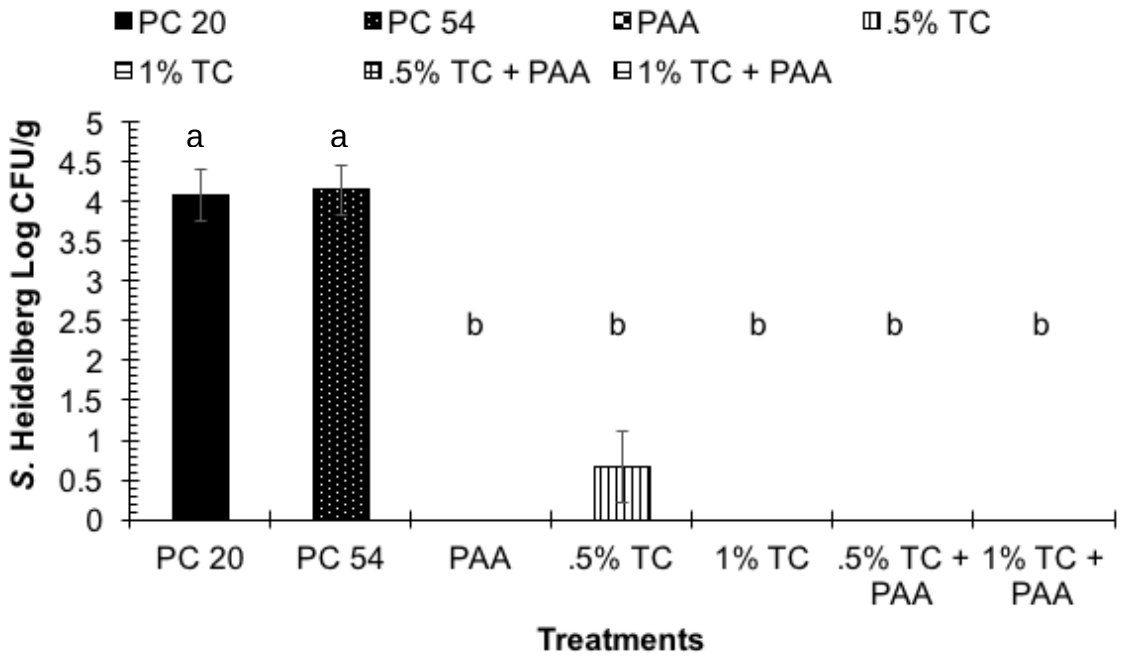


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 2 TC - 2% TC; 1TC + PAA - 1% TC + PAA; 2TC + PAA - 2% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 10

Effect of TC, PAA, and their combination on *Salmonella* Heidelberg in scalding water after application of high-inoculation level on broiler drumsticks under scalding conditions

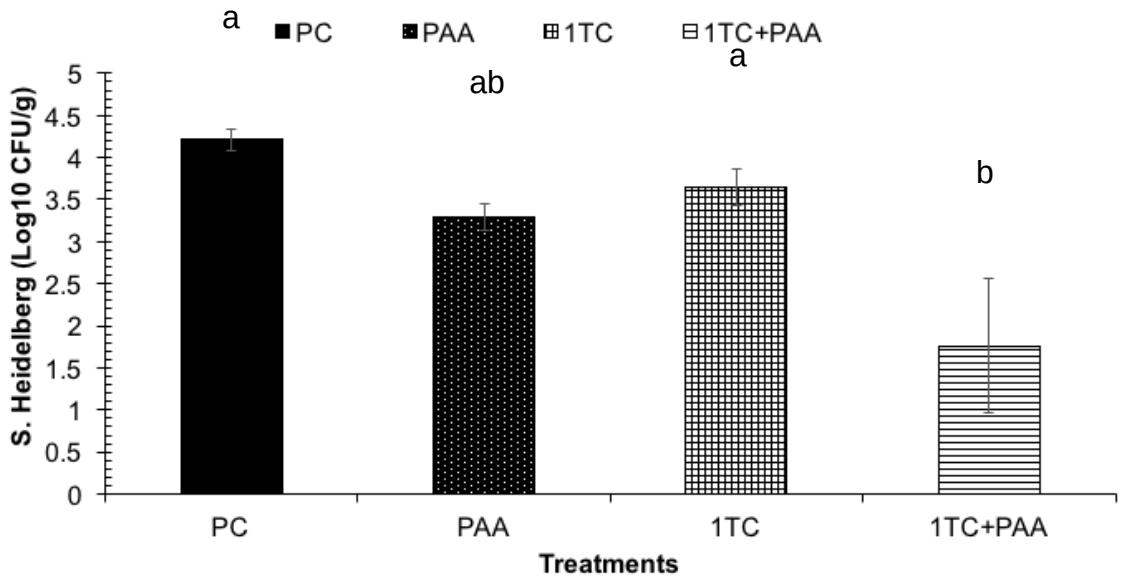


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 2 TC - 2% TC; 1TC + PAA - 1% TC + PAA; 2TC + PAA - 2% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 11

Effect of TC, PAA, and their combination on *Salmonella* Heidelberg after scalding with different treatments and chilling on broiler drumsticks

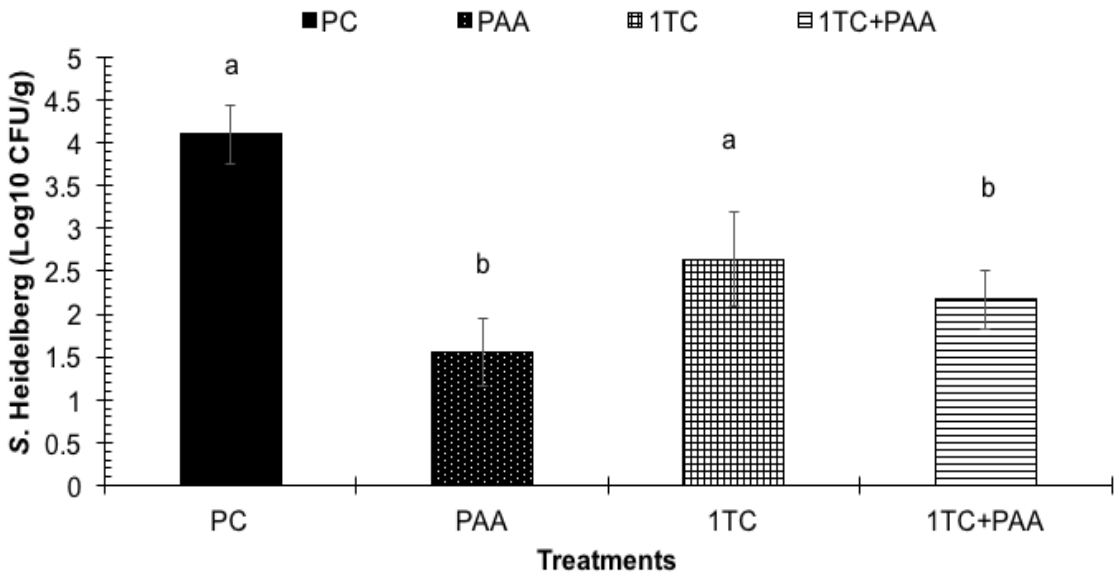


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 1TC + PAA - 1% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 12

Effect of TC, PAA, and combination on *Salmonella* Heidelberg in simulated chilling water after simulated scalding using broiler drumsticks

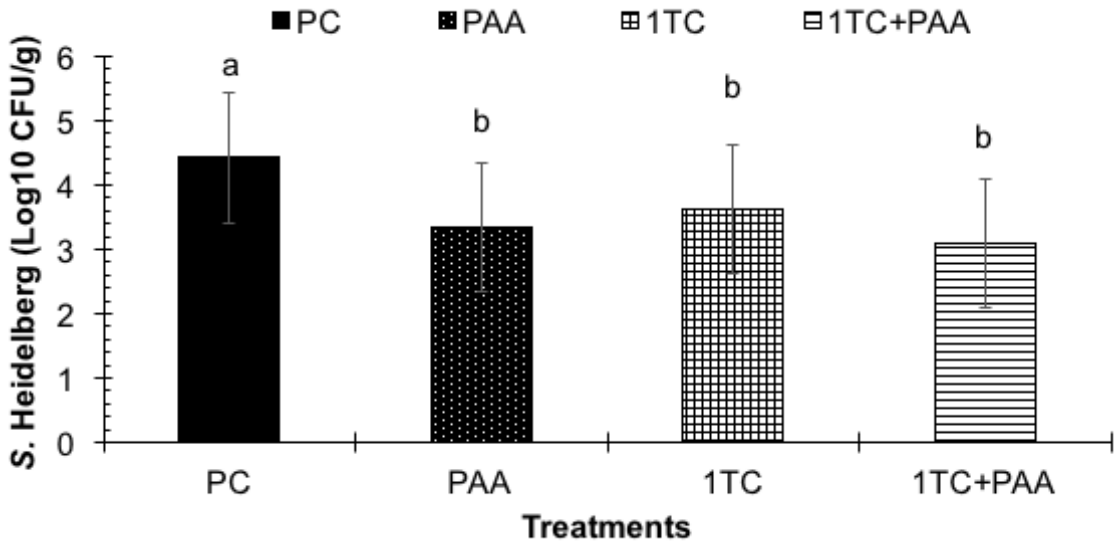


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 1TC + PAA - 1% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 13

Effect of TC, PAA, and their combination on *Salmonella* Heidelberg after the application of scalding treatments and chilled storage on broiler drum

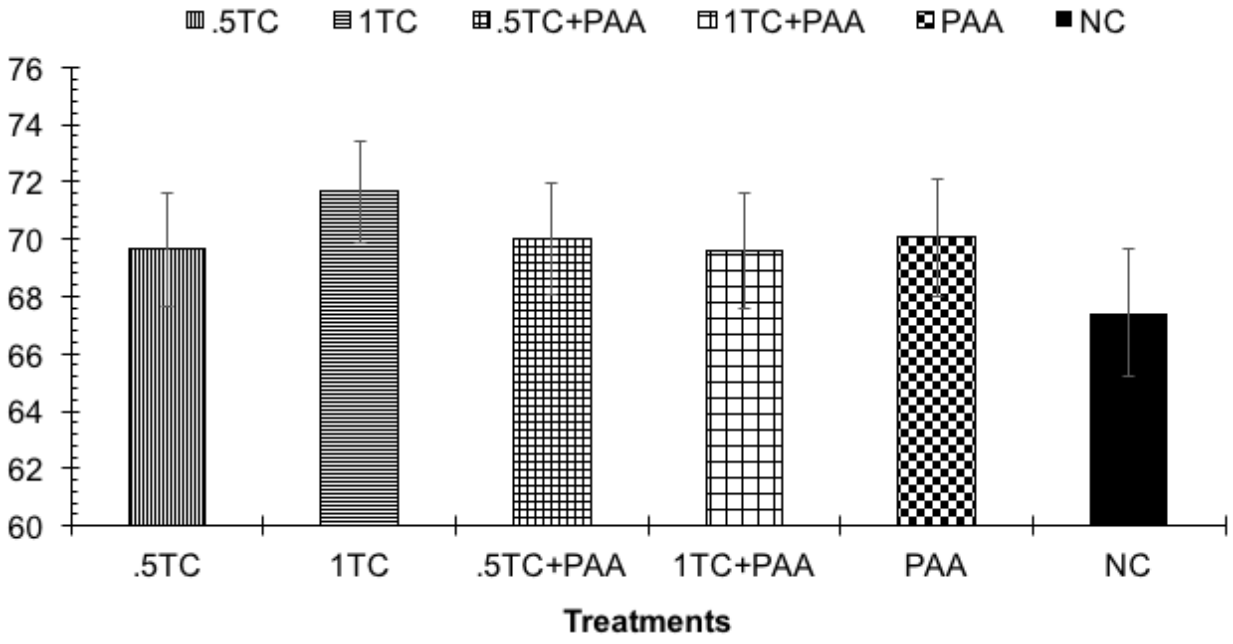


PC - Positive Control; PAA - 0.05% Peracetic Acid; 1TC - 1% TC; 1TC + PAA - 1% TC + PAA

[a,b] – Bars with different superscripts are significantly different from each other at $P<0.05$

Figure 14

Effect of TC, PAA, and combination on on L* after simulated scalding conditions+

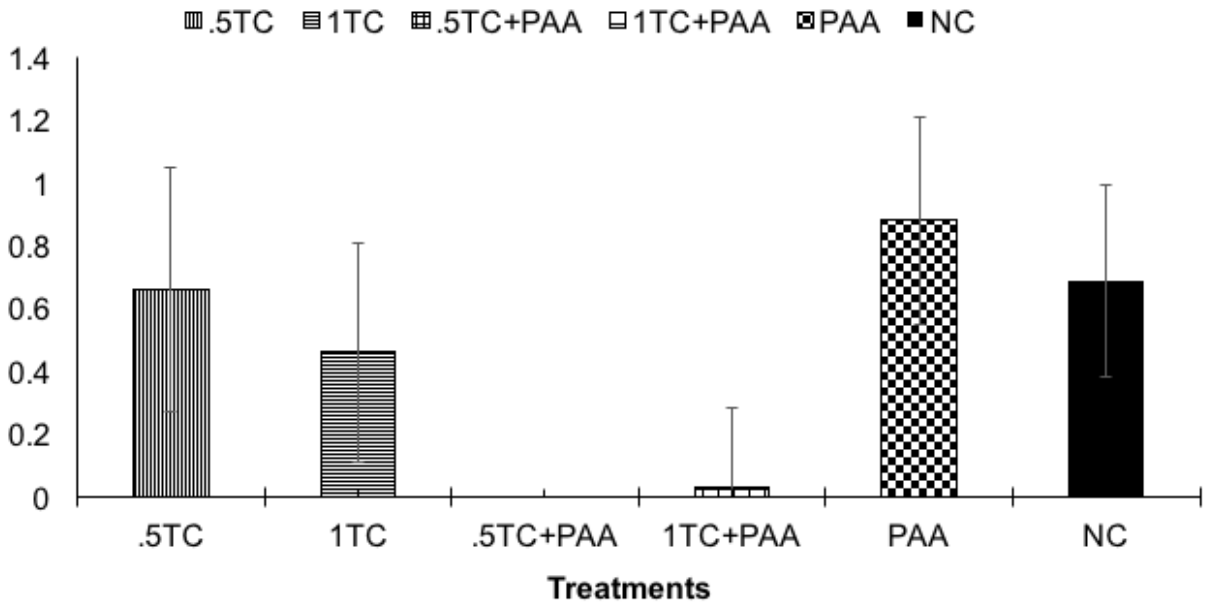


NC - Negative Control; PAA - 0.05% Peracetic Acid; 0.5TC – 0.5% TC; 1TC - 1% TC; 0.5TC + PAA – 0.5% TC + PAA; 1TC + PAA - 1% TC + PAA

+ None of the treatments were significantly different from each other at P<0.05

Figure 15

Effect of TC, PAA, and combination on a* after simulated scalding conditions+

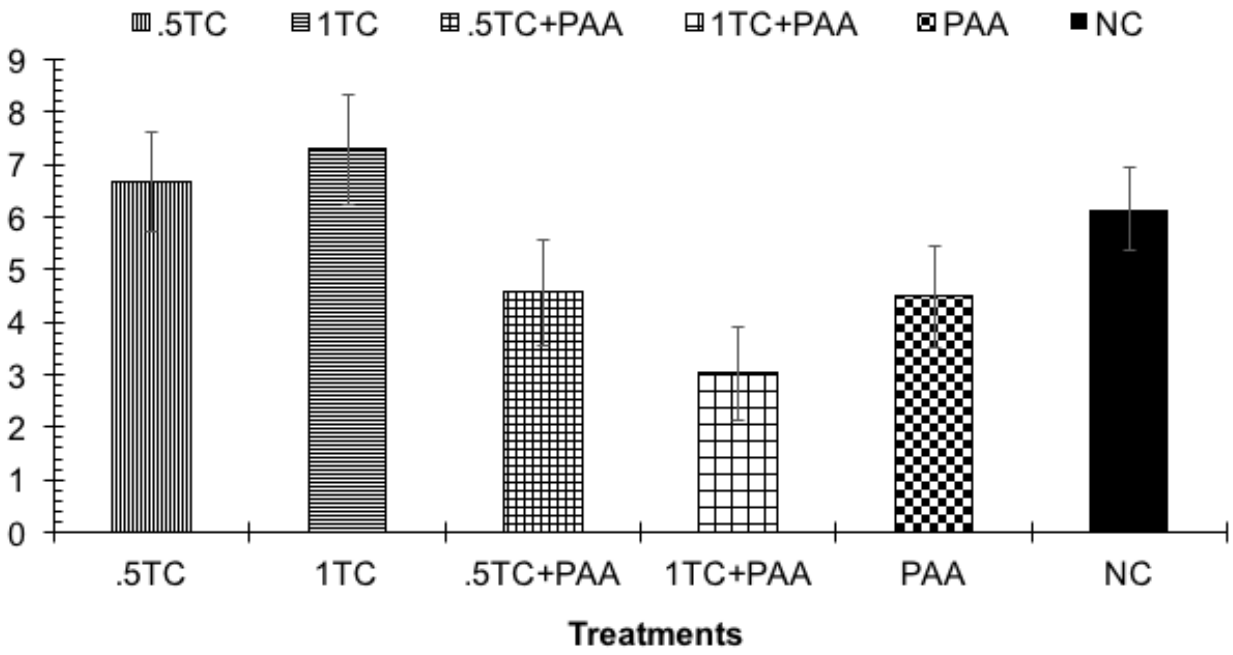


NC - Negative Control; PAA - 0.05% Peracetic Acid; 0.5TC – 0.5% TC; 1TC - 1% TC; 0.5TC + PAA – 0.5% TC + PAA; 1TC + PAA - 1% TC + PAA

+ None of the treatments were significantly different from each other at $P<0.05$

Figure 16

Effect of TC, PAA, and combination on on b* after simulated scalding conditions+



NC - Negative Control; PAA - 0.05% Peracetic Acid; 0.5TC – 0.5% TC; 1TC - 1% TC; 0.5TC + PAA – 0.5% TC + PAA; 1TC + PAA - 1% TC + PAA

+ None of the treatments were significantly different from each other at $P<0.05$

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Appendix A

Common Scalding Times and Temperature for Various Classes of Poultry (USDA FSIS. 2015)		
Broiler (hard scald)	30-75 seconds	138.2-147.2 ℉ (59-64℃)
Broiler (soft scald)	90-120 seconds	123.8-129.2℉ (51-54℃)
Turkeys	50-125 seconds	138.2-145.4℉ (59-63℃)
Quail	30 seconds	127.4℉ (53℃)
Waterfowl	30-60 seconds	154.4-179.6℉ (68-82℃)

Appendix B

Treatments	Log 10 CFU/g	SE
NC	0	0
PC	4.262	0.31
AB	3.9	0.15
TC	3.08	0.21

Figure 1: Effect of long-term prophylactic supplementation TC at 0.05% through feed and therapeutic supplementation at 0.03% through water on *S*. Heidelberg in 5-week-old commercial broiler chickens

Treatments	Body Weight (Kg)	SE
PC	1.91	1.512
TC	1.585	1.482
NC	1.8	1.91
AB	1.482	1.8

Figure 2: Effect of long-term prophylactic supplementation TC at 0.05% through feed and therapeutic supplementation at 0.03% through water on body weights of 5-week-old commercial broiler chickens

Treatments	Log 10 CFU/g	SE
NC	0	0
PC	3.74	0.33
AB	0	0
TC	1.77	0.91

Figure 3: Effect of therapeutic TC (0.03%) through water on *S*. Heidelberg in 7-week-old commercial broiler chickens

Treatments	Body Weight (Kg)	SE
NC	3.895	0.076
PC	3.705	0.169

AB	3.7	0.117
TC	3.662	1

Figure 4: Effect of therapeutic TC (0.03%) through water on body weights in 7-week-old commercial broiler chickens

Treatments	**Log 10 CFU/g**	**SE**
PC	2.75	0.0338
PAA	1.126	0.371
1TC	1.73	0.126
2TC	1.614	0.334
1TC+PAA	1.41	0.313
2TC+PAA	1.71	0.37

Figure 5: Effect of TC, PAA, and their combination on *Salmonella* Heidelberg in scalding conditions on broiler breast meat

Treatments	**Log 10 CFU/g**	**SE**
PC	1.71	0.093
PAA	0	0
1TC	0.177	0.177
2TC	0	0
1TC+PAA	0	0
2TC+PAA	0	0

Figure 6: Effect of TC, PAA, and their combination on *Salmonella* Heidelberg in scalding water after treatment on breast meat

Treatments	**Log 10 CFU/g**	**SE**
PC 20	2.48	0.18
PC 54	2.61	0.1
PAA	0.23	0.23
.5% TC	1.62	0.38
1% TC	2.01	0.19

.5% TC + PAA	0.81	0.26
1% TC + PAA	0.86	0.39

Figure 7: Effect of TC, PAA, and their combination on *Salmonella* Heidelberg applied at low-inoculation level on broiler drumsticks under scalding conditions

Treatments	**Log 10 CFU/g**	**SE**
PC 20	2.3	0.49
PC 54	2.01	0.49
PAA	0	0
0.5% TC	0.29	0.29
1% TC	0	0
0.5% TC + PAA	0	0
1% TC + PAA	0	0

Figure 8: Effect of TC, PAA, and combination on *Salmonella* Heidelberg in scalding water after application of low-inoculation level on broiler drumsticks under scalding conditions

Treatments	**Log 10 CFU/g**	**SE**
PC 20	4.21	0.29
PC 54	4.51	0.29
PAA	1.41	0.448
.5% TC	3.64	0.2
1% TC	3.75	0.277
.5% TC + PAA	2.29	0.497
1% TC + PAA	2.35	0.5

Figure 9: Effect of TC, PAA, and combination on *Salmonella* Heidelberg applied at high inoculation level on broiler drumsticks under scalding condition

Treatments	**Log 10 CFU/g**	**SE**
PC	4.21	0.136
PAA	3.29	0.1556
1TC	3.65	0.216

1TC+PAA	1.76	0.794

Figure 10: Effect of TC, PAA, and combination on *Salmonella* Heidelberg in scalding water after application of high inoculation level on broiler drumsticks under scalding condition

Treatments	**Log 10 CFU/g**	**SE**
PC	4.1	0.344
PAA	1.56	0.4
1TC	2.64	0.55
1TC+PAA	2.17	0.34

Figure 11: Effect of TC, PAA, and combination on *Salmonella* Heidelberg after scalding with different treatments and chilling on broiler drumsticks

Treatments	**Log 10 CFU/g**	**SE**
PC	4.42	0.117
PAA	3.34	0.148
1TC	3.61	0.16
1TC+PAA	3.09	0.136

Figure 12: Effect of TC, PAA, and combination on *Salmonella* Heidelberg in simulated chilling water after the scalding treatments on broiler drumsticks

Meat	**Log 10 CFU/g**	**SE**
PC	2.75	0.0338
PAA	1.126	0.371
1TC	1.73	0.126
2TC	1.614	0.334
1TC+PAA	1.41	0.313
2TC+PAA	1.71	0.37

Figure 13: Effect of TC, PAA, and their combination on *Salmonella* Heidelberg after the application of scalding treatments and chilled storage on broiler drumsticks

Treatments	L*	SE
.5TC	69.64	1.977
1TC	71.65	1.76
.5TC+PAA	70.02	1.94
1TC+PAA	69.59	2.017
PAA	70.065	2.05
NC	67.435	2.211

Figure 14: Effect of TC, PAA, and combination on L* after simulated scalding conditions

Treatments	a*	SE
.5TC	0.66	0.39
1TC	0.46	0.35
.5TC+PAA	-0.386	0.316
1TC+PAA	0.034	0.251
PAA	0.88	0.33
NC	0.69	0.305

Figure 15: Effect of TC, PAA, and combination on a* after simulated scalding conditions

Treatments	b*	SE
.5TC	6.68	0.95
1TC	7.29	1.05
.5TC+PAA	4.56	1.005
1TC+PAA	3.03	0.88
PAA	4.49	0.966
NC	6.144	0.794

Figure 16: Effect of TC, PAA, and combination on b* after simulated scalding conditions