THESIS

EFFICACY OF ANTIMICROBIAL TREATMENTS AGAINST *SALMONELLA ENTERICA* ON PORK AND *CAMPYLOBACTER JEJUNI* ON POULTRY

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ABSTRACT

EFFICACY OF ANTIMICROBIAL TREATMENTS AGAINST *SALMONELLA ENTERICA* ON PORK AND *CAMPYLOBACTER JEJUNI* ON POULTRY

Two studies were conducted to evaluate efficacy of antimicrobial treatments against *Salmonella enterica* on pork and *Campylobacter jejuni* on poultry. The first study was conducted to (i) evaluate decontamination efficacy of six chemical treatments when applied to pork jowls inoculated with *Salmonella enterica* and (ii) determine the antimicrobial efficacy of the test solutions against a high and low inoculum level of *Salmonella*. Chilled pork jowls were cut into 10 × 5 × 1 cm portions and were surface-inoculated on the skin side with a mixture of six *S. enterica* serotype strains of swine origin. The inoculation levels targeted were 6 to 7 log CFU/cm^2 (high) and 3 to 4 log CFU/cm^2 (low). Following inoculation, samples were left untreated (control) or were treated by spray application (10 s, 18 to 19 psi, 1.0 gpm flow rate) with water, a proprietary blend of sulfuric acid and sodium sulfate (SSS, pH 1.2), formic acid (1.5%), peroxyacetic acid (PAA, 400 ppm), PAA (400 ppm) acidified with acetic acid (1.5%), PAA (400 ppm) acidified with formic acid (1.5%), or PAA (400 ppm) acidified with SSS (pH 1.2). Samples were analyzed for inoculated *Salmonella* counts immediately after treatment application (0 h) and after 24 h of refrigerated (4°C) storage. Overall, all seven spray treatments were effective ($P < 0.05$) at reducing the high and low *Salmonella* inoculation levels. At the high inoculum level (6.2 log CFU/cm^2), pathogen counts ranged from 5.4 (water; 0.8 log CFU/cm^2 reduction) to 4.3 (PAA acidified with SSS; 1.9 log CFU/cm^2 reduction) log CFU/cm^2 for

samples analyzed immediately after spray treatment. *Salmonella* counts obtained at the 0-h sampling time for treated samples inoculated at the low inoculum level (3.5 log CFU/cm^2) ranged from 2.8 (water; 0.7 log CFU/cm^2 reduction) to 1.8 (PAA acidified with SSS; 1.7 log CFU/cm^2 reduction) log CFU/cm^2. Thus, regardless of inoculum concentration, similar reductions of *Salmonella* populations were obtained immediately following treatment application (0 h). For the high inoculation level, *Salmonella* counts of samples analyzed after 24 h of refrigerated storage were, in general, similar ($P \geq 0.05$) to the counts of the corresponding treatment at 0 h. However, for the low inoculation level, pathogen counts of jowls treated with SSS, formic acid, or PAA acidified with formic acid, and held at 4°C for 24 h, were 0.6 log CFU/cm^2 lower ($P < 0.05$) than the 0-h counts of the corresponding treatment. Regardless of inoculation level and sampling time, no ($P \geq 0.05$) differences in efficacy were obtained between PAA on its own and any of the acidified PAA treatments evaluated.

The second study was conducted to (i) evaluate decontamination efficacy of five chemical treatments when applied to chicken wings inoculated with *Campylobacter jejuni* and (ii) determine antimicrobial efficacy of the treatments as a result of applying test solutions by immersion or spraying. Skin-on chicken wings were surface-inoculated with a six-strain mixture of *C. jejuni* of poultry origin. The target inoculation level was 3 to 4 log CFU/mL of wing rinsate. Following inoculation, samples were left untreated (control) or were treated by immersion (500 mL solution per wing; 5 s) or spray application (10 to 12 psi; 4 s) with water, SSS (pH 1.2), formic acid (1.5%), PAA (550 ppm), PAA (550 ppm) acidified with SSS (pH 1.2), or PAA (550 ppm) acidified with formic acid (1.5%). Samples were analyzed for *C. jejuni* counts immediately after treatment application (0 h) and following 24 h of storage (4°C). All five acid treatments evaluated in this study were effective ($P < 0.05$) at reducing the initial inoculated (3.9

log CFU/mL) *C. jejuni* populations on chicken wings, regardless of the antimicrobial treatment application method. Pathogen counts for samples spray-treated with one of the chemical solutions and analyzed immediately (0 h) after treatment ranged from 3.4 (SSS; 0.5 log CFU/mL reduction) to 2.7 (PAA acidified with formic acid; 1.2 log CFU/mL reduction) log CFU/mL. When the chemical treatments were applied by immersion, *C. jejuni* counts of 2.2 (SSS; 1.7 log CFU/mL reduction) to 1.7 (PAA, and PAA acidified with SSS; 2.2 log CFU/mL reduction) log CFU/mL were obtained for wings analyzed at the 0-h sampling time. The PAA and acidified PAA treatments were equally ($P \geq 0.05$) effective at reducing initial *C. jejuni* populations, regardless of treatment application method. However, following refrigerated storage, samples treated with SSS- or formic acid-acidified PAA had lower ($P < 0.05$) pathogen counts than those that had been treated with the non-acidified PAA treatment.

Overall, findings of the two studies should be useful to the pork and poultry industries as they consider new interventions against *Salmonella* and *Campylobacter* contamination on pork and chicken parts, respectively.

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CHAPTER 1: LITERATURE REVIEW

Foodborne Illnesses

Foodborne illness, also known as food poisoning, is an infection of the gastrointestinal tract that involves the ingestion of food that is contaminated with viruses, parasites, or pathogenic bacteria (CDC, 2019a). Usually, foodborne diseases resolve on their own without the need for medical treatment; however, in some cases, infected individuals experiencing severe symptoms, or those with underlying health conditions, and/or suppressed immune systems, need to be hospitalized (CDC, 2019a). The Centers for Disease Control and Prevention (CDC) estimates that each year 48 million people in the United States experience a foodborne disease, and 3,000 die from it; therefore, foodborne illnesses represent a major health problem (Scallan et al., 2011; CDC, 2019a).

In 1995, the CDC, in collaboration with 10 state health departments, the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS), and the U.S. Food and Drug Administration (FDA) established the Foodborne Diseases Active Surveillance Network (FoodNet) that tracks cases of laboratory-diagnosed infection caused by eight pathogens normally transmitted through the ingestion of contaminated food (CDC, 2019b; Tack, 2019). The eight tracked pathogens are *Salmonella*, *Campylobacter*, *Listeria*, O157 and non-O157 Shiga toxin-producing *Escherichia coli* (STEC), *Shigella*, *Vibrio*, *Yersinia*, and *Cyclospora* (CDC, 2019b). FoodNet conducts active, population-based surveillance for laboratory-diagnosed infections in 10 sites, determined by the 10 state health departments previously mentioned, that represent 15% of the U.S. population (CDC, 2019b). During 2018, FoodNet identified 25,606 infections, 5,893 hospitalizations, and 120 deaths (Tack, 2019). In general, incidence of

foodborne infections (per 100,000 population) is reportedly increasing, mainly due to those caused by *Campylobacter* and *Salmonella* (Tack, 2019). In 2018, compared to 2015 to 2017 data, incidence of *Campylobacter* and *Salmonella* infections increased by 12% and 9%, respectively (Tack, 2019). This increase in incidence might, in part, be due to more and better use of culture-independent diagnostic tests (Tack, 2019).

Estimated costs of foodborne illnesses, determined by USDA's Economic Research Service, is more than $15.6 billion per year (USDA-ERS, 2019). Fourteen foodborne pathogens account for $14.1 billion (2009 dollars) in costs of illness, and more than 90% of this health load is caused by five pathogens, two of which are *Salmonella* and *Campylobacter* (Morris et al., 2011). The top three pathogens associated with the cost of illness are *Salmonella* spp. ($3.3 billion), *Toxoplasma gondii* ($2.9 billion), and *Campylobacter* spp. ($1.7 billion) (Morris et al., 2011). Within the group of 14 foodborne pathogens, 50 pathogen-food combinations are estimated to be responsible for more than 90% of the foodborne illness cases in the United States, and the top 10 account for $8 billion in costs of illness annually (Morris et al., 2011). These top 10 pathogen-food combinations are ranked by their cost of illness, and the first four are meat and poultry product combinations, specifically, *Campylobacter*-poultry ($1.3 billion), *Toxoplasma*-pork ($1.2 billion), *Listeria*-deli meats ($1.1 billion), and *Salmonella*-poultry ($0.7 billion) (Morris et al., 2011).

Salmonella enterica

In 1884, Theobald Smith, a veterinarian, isolated bacteria from the intestine of a pig that succumbed to the disease known as hog cholera (Smith, 1894; Evangelopoulou et al., 2010). At that time, Dr. Smith was supervised by Dr. Daniel Salmon, a veterinary pathologist working in the U.S. Department of Agriculture's Bureau of Animal Industry (Evangelopoulou et al., 2010).

The isolate was initially named *Bacillus choleraesuis*, but in 1900, it was renamed as *Salmonella choleraesuis* (currently known as *Salmonella enterica* serovar Choleraesuis) in honor of Dr. Salmon (Cockerham, 2016; Ryan et al., 2017).

The genus *Salmonella* is a group of highly adapted bacteria, described as Gram-negative, non-spore-forming, rod-shaped, motile, facultative anaerobes (Yan et al., 2004; Jay et al., 2005). Moreover, *Salmonella* is considered one of the most pathogenic bacteria within the *Enterobacteriaceae* family (Evangelopoulou et al., 2010). The majority of *Salmonella* serotypes can grow at temperatures ranging from 5 to 47°C, with an optimum temperature of 35 to 37°C, and over a wide pH range of 4 to 9, with an optimum of between 6.5 and 7.5 pH units (Baer et al., 2013; Dodd et al., 2017). *Salmonella* are sensitive to heat, and temperatures above 70°C usually kill them (Dodd et al., 2017).

Salmonella nomenclature is complex, and scientists use several systems when referring to this genus (Dodd et al., 2017). Currently, two species of *Salmonella* are recognized, *S. bongori* and *S. enterica*, with the latter being associated with human foodborne illness (Yan et al., 2004; Coburn et al., 2007). *Salmonella enterica* is further subdivided into six subspecies: *S. enterica* (I), *S. salamae* (II), *S. arizonae* (IIIa), *S. diarizonae* (IIIb), *S. houtenae* (IV), and *S. indica* (VI) (Yan et al., 2004; Dodd et al., 2017). These subspecies are further divided into serotypes (or serovars), that separate strains based on three antigenic sites; their somatic (O), capsular (K, if present), and flagellar (H) structure (Yan et al., 2004; Jay et al., 2005; Coburn et al., 2007). With more than 2,600 *Salmonella* serotypes, over 99% of these belong to the species *S. enterica* (Guibourdenche et al., 2010). Furthermore, 60% of serotypes within species *S. enterica* are grouped into subspecies *enterica* (subspecies I), and 99% of *S. enterica* subspecies *enterica* serotypes are responsible for disease in humans and warm-blooded animals (Brenner et al., 2000;

Chan et al., 2003; Yan et al., 2004; Coburn et al., 2007). Serotype names of *S. enterica* subspecies *enterica* are written in nonitalicized Roman letters with the first letter capitalized. Therefore, an example of the complete, formal designation of a *Salmonella* serotype is *Salmonella enterica* subspecies *enterica* serotype Montevideo, or *Salmonella* Montevideo for short (Brenner et al., 2000; CDC, 2011; Dodd et al., 2017).

Several phenotypes contribute to *Salmonella* virulence, and these have been mapped to pathogenicity islands (SPIs; Dodd et al., 2017). Currently, there are at least 21 SPIs identified in *Salmonella*, with SPI-1 and SPI-2 critical for invasion and replication, respectively, of nonphagocytic cells (Dodd et al., 2017). Pathogenicity island SPI-1 encodes a type III secretion system that carries bacterial proteins to the cytosol of host cells, resulting in cytoskeletal changes that facilitate uptake of a *Salmonella* cell into a membrane-bound vesicle (Dodd et al., 2017). The primary function of SPI-1 is proposed to be mediating the invasion of intestinal epithelial cells during the infection process (Dodd et al., 2017). Pathogenicity island SPI-2 encodes a second type III secretion system that is needed for intracellular maintenance of *Salmonella* cells inside a specific membranous compartment (Dodd et al., 2017).

Salmonella enterica subspecies *enterica* can cause two types of illness: enteric or typhoid fever, and nontyphoidal salmonellosis (Coburn et al., 2007). Infections with *Salmonella enterica* subspecies I serotypes typically result in nontyphoidal salmonellosis (NTS). However, infection with *Salmonella* Typhi or *Salmonella* Paratyphi can cause typhoid fever, which is more severe and involves spread via blood and lymphatic channels to other organs (D'Aoust, 1991; Altier, 2005). With either type of disease, the invasion of *Salmonella* is usually initiated with the ingestion of contaminated food or water by the host, and the passage of bacterial cells to the intestine (Velge et al., 2012). In the small intestine, bacteria induce cytoskeletal changes to the

membrane of epithelial cells (Velge et al., 2012; Dodd et al., 2017). In the case of NTS, bacterial cells remain in the gut mucosa and induce a robust inflammatory response from the host's immune system (Chopra et al., 1999; Coburn et al., 2007; Velge et al., 2012). This immune reaction from the host limits replication and propagation of pathogens (Dodd et al., 2017). However, *Salmonella*, during its evolutionary process, has developed specific mechanisms that let it avoid the host immune response and survive in the inflamed gut mucosa (Santos et al., 2009; Winter et al., 2010).

NTS is usually self-limiting and requires a relatively high infection dose of more than 50,000 cells of *Salmonella* for a healthy individual to become symptomatic (Chan et al., 2003; Coburn et al., 2007). The illness is characterized by enterocolitis after 6 to 72 h of bacterial colonization, and the onset of symptoms typically includes abdominal pain, nausea, and diarrhea with or without blood (FDA, 2012a; Coburn et al., 2007). These symptoms usually last 4 to 7 days, with acute symptoms in the first two days. However, illness in children, the elderly, and immunocompromised individuals are marked by increased duration and severity of the infection, bloody diarrhea, and risk of complications (Coburn et al., 2007). Complications of NTS include dehydration and electrolyte imbalance, and infections outside of the gastrointestinal tract, such as septicemia and bacteremia (Coburn et al., 2007; FDA, 2012a).

Livestock, such as poultry and swine, are primary reservoirs for *Salmonella*; therefore, it is not surprising that meat products are a common source of human infection (Jay et al., 2005). Poultry products, such as eggs and chicken, are the top causes of salmonellosis, followed by pork (Laufer et al., 2015). According to FoodNet data, *Salmonella* was the second leading bacterial cause of foodborne illness in 2018, and was responsible for 9,084 cases of foodborne illness, 2,416 hospitalizations, and 36 deaths (Tack, 2019). From 2009 to 2017, there were a total of

1,988 *Salmonella* outbreaks in the United States, and 62% (1,232) were food-related (CDC, 2018). Within the same time period, 149 and 71 of the *Salmonella* outbreaks were linked to poultry and pork consumption, respectively (CDC, 2018). From 2015 to 2017, 20 *Salmonella* pork-related outbreaks occurred, resulting in a total of 793 illnesses, 95 hospitalizations, and two deaths (CDC, 2018). In August 2015, Kapowsin Meats issued a recall for more than 500,000 pounds of raw pork products that were potentially contaminated with *Salmonella enterica* serotype I 4,[5],12:i:- (CDC, 2015; USDA-FSIS, 2017). These products were linked to 192 cases of illness and 30 hospitalizations over five U.S. states (CDC, 2015; USDA-FSIS, 2017).

Campylobacter jejuni

Campylobacter was first isolated by John McFadyean in 1906 from samples taken from aborting ewes (Skirrow, 2006). However, it was not until the 1970s when the role of *Campylobacter* as an enteric pathogen was defined (Skirrow, 2006).

The genus *Campylobacter*, a member of the family *Campylobacteraceae*, is described as Gram-negative, non-spore-forming, spiral-shaped, measuring 0.5 to 5 μm in length and 0.2 to 0.8 μm in width, and with a single polar flagellum at one or both ends (Dodd et al., 2017). Most *Campylobacter* spp. strains are microaerophilic, requiring small amounts of oxygen (5 to 10%) and carbon dioxide (5 to 10%) for optimal growth. When oxygen concentrations exceed 21%, growth of *Campylobacter* is inhibited (Jay et al., 2005; Dodd et al., 2017). The optimum temperature for growth is usually between 37 to 42°C, and at a temperature of 40°C, they can grow within a pH range of 5.5 to 8.0. *Campylobacter* spp. are oxidase- and catalase-positive, and growth is inhibited in 3.5% of NaCl or at 25°C (Dodd et al., 2017).

Currently, there are 26 species of *Campylobacter* and nine subspecies (Dodd et al., 2017). The primary *Campylobacter* spp. associated with human foodborne illness are *C. jejuni* and *C. coli* (Moore et al., 2005; Dodd et al., 2017). *C. jejuni* is responsible for 80 to 85% of enteric *Campylobacter* infections, and *C. coli* 10 to 15% (Moore et al., 2005; Dodd et al., 2017). Usually, infection with *C. jejuni* leads to severe gastroenteritis, characterized by inflammation, diarrhea, fever, and abdominal cramps (Robinson, 1981; Black et al., 1988; Acheson and Allos, 2001; Young et al., 2007). The fever can be low-grade or above 40°C and persists for up to 1 week, and by that time, the disease has typically resolved, even without specific antibiotic treatment (Acheson and Allos, 2001). The infectious dose can be as low as 500 to 800 *Campylobacter* cells (Black et al., 1988).

Enteritis caused by *C. jejuni* and *C. coli* is usually self-limiting and rarely results in fatality (Acheson and Allos, 2001; Dodd et al., 2017). However, complications such as bacteremia, hepatitis, and pancreatitis may occur and are most likely to occur in immunocompromised patients (Skirrow et al., 1993; Dodd et al., 2017). Occasionally, long-term severe autoimmune conditions occur after campylobacteriosis (Dodd et al., 2017). The most important, but rare, complication from *C. jejuni* infection is Guillain-Barré syndrome, which consists of a rapid-onset muscle weakness leading to paralysis due to damage to the peripheral nervous system (Guarino et al., 1998; Acheson and Allos, 2001; Dodd et al., 2017). Other postinfectious complications include Miller Fisher syndrome, reactive arthritis, irritable bowel syndrome, and inflammatory bowel disease (Dodd et al., 2017).

Infection of the host by *Campylobacter* includes colonization of the mucosa, attachment to the epithelial cells, invasion, and toxin production (Dodd et al., 2017). First, *Campylobacter* cells penetrate the mucus layer of intestinal epithelial cells and locate deep within intestinal

crypts to replicate (Dodd et al., 2017). For successful host colonization, motility, and chemotaxis, which is a mechanism that bacteria use to sense and migrate towards a nutrient-rich environment, are essential. *C. jejuni* and *C. coli* are very motile, and between the flagella and the helical (spiral) cell shape, they generate a corkscrew movement that provides the capability to penetrate the mucosal layers (Ketley, 1997; Dodd et al., 2017). Several proteins have been identified as adhesin-proteins that allows bacteria to bind to specific host cell components (Young et al., 2007). When bacterial cells are at the host epithelial cell surface, these binding-proteins act and the inflammation processes occur (Young et al., 2007; Dodd et al., 2017). Once colonized, bacterial cells release a cytolethal distending toxin (CDT) that has been determined to kill immune response intestinal cells. The exact mechanism of this toxin in the pathogenesis of *Campylobacter* spp. is not entirely understood (Jay et al., 2005; Young et al., 2007; Dodd et al., 2017).

Foods of animal origin, and especially poultry, have been recognized as primary sources of *Campylobacter* (Moore et al., 2005). According to 2018 FoodNet data, *Campylobacter* is the leading cause of bacterial-mediated foodborne illness in the United States, with 9,723 illnesses, 1,811 hospitalizations, and 30 deaths (Tack, 2019). From 2009 to 2017, there were a total of 552 *Campylobacter* outbreaks, and 50% (278) were food-related (CDC, 2018). Within the same time period, 47 *Campylobacter* outbreaks, 392 illnesses, and 26 hospitalizations were linked to poultry consumption (CDC, 2018). From 2015 to 2017, 23 chicken-related *Campylobacter* outbreaks occurred, which resulted in 168 illnesses and 11 hospitalizations (CDC, 2018). In 2012, an outbreak of *C. jejuni* infection was linked to raw or lightly cooked chicken liver that originated from the same poultry establishment in Vermont (CDC, 2013). Six persons were identified as affected, and two of them were hospitalized (CDC, 2013). *Campylobacter* outbreaks

are not usually reported, considering how often people get infected from this bacterium (CDC, 2019c). This could be because patients usually recover without treatment (Acheson and Allos, 2001). It is interesting that such a fragile and environmentally sensitive microorganism is the leading cause of foodborne illness (Jay et al., 2005).

Pathogen Contamination of Pork

Pathogenic bacteria, like *Salmonella enterica* and *Campylobacter*, are common inhabitants of swine intestines. As a result, there is a high prevalence of these pathogens on the farm that can then potentially be carried to the slaughter process (Baer et al., 2013). An analysis of the risk of *Salmonella* illness per kilogram or serving consumed of four meat commodities (poultry, beef, pork, and lamb) suggests that pork has the second-highest per unit risk, with poultry having the highest (Hsi et al., 2015).

Incoming pigs to the slaughter process, carrying pathogens in their intestinal tract, increase the risk of contamination of pork carcasses and products (Rostagno and Callaway, 2012). In addition, since *Salmonella* is capable of surviving outside the host, cross-contamination of carcasses from processing equipment or the processing environment is of concern (Baer et al., 2013; De Busser et al., 2013). Different processing steps during slaughter can result in pork carcass contamination or cross-contamination (Baer et al., 2013). The typical pork slaughter process is comprised of the following steps: stunning and exsanguination, scalding, dehairing, singeing and polishing, head removal, evisceration, carcass splitting, final wash and chilling (Buncic and Sofos, 2012; Baer et al., 2013). Since *Salmonella* is primarily harbored in the intestinal tract, contaminated feces on the carcass or those released during processing, are a high risk for *Salmonella* contamination over the same carcass, the subsequent carcasses, and plant equipment and environment (Baer et al., 2013; De Busser et al., 2013). Certain processing steps,

like scalding and singeing, are known to decrease the prevalence of *Salmonella* on pork carcasses (Pearce et al., 2004). However, these steps are performed prior to evisceration, which is a critical control point since it provides an opportunity for spillage of intestinal contents onto the carcass surface or slaughter equipment (Berends et al., 1998).

Duggan et al. (2010) conducted a study in which pigs from 13 herds were tracked through four Irish slaughter plants and were sampled for *Salmonella* at different stages of slaughter in order to identify critical points of *Salmonella* contamination. In this study, 11 of the 36 *Salmonella*-positive carcasses or cut pork samples had the same *Salmonella* serotype as that found in the cecal or rectal contents, indicating that the animal's intestinal contents were the source of contamination. For the remaining 25 *Salmonella*-positive carcasses (69%), cross-contamination within the slaughter plant environment was suggested as the likely source of the contamination (Duggan et al., 2010). Therefore, decontamination of knives, splitting saw, and other equipment between carcasses is essential (Duggan et al., 2010; Buncic and Sofos, 2012; Baer et al., 2013).

Overall, the slaughter process decreases *Salmonella* contamination levels on pork (Dickson et al., 2002). Schmidt et al. (2012) conducted a study where samples from pork carcasses were taken at three points along the slaughter process; pre-scald (post-exsanguination), pre-evisceration (post-scald, singe, and polish), and the chilled final carcass, at two commercial U.S. facilities. Samples were analyzed for *Salmonella*, and the results for prevalence were reported (Schmidt et al., 2012). *Salmonella* was isolated from 1,386 of the 1,520 pre-scald carcass samples resulting in a *Salmonella* prevalence of 91.2% (Schmidt et al., 2012). Following scalding, singing, and polishing, prevalence of *Salmonella* decreased to 19.1%, and after the final wash and carcass chilling, the prevalence of *Salmonella* was found to be 3.7% (Schmidt et al.,

2012). However, in a study conducted by USDA-FSIS to determine the prevalence of *Salmonella* in a variety of raw pork products (intact cuts, nonintact cuts, and comminuted products) collected from 285 federally-inspected slaughter and processing establishments, 13.6% out of the 4,014 samples analyzed were found positive for this pathogen (Scott et al., 2020). *Salmonella* prevalence was the highest in comminuted products (21.2%), followed by intact (8.3%) and nonintact (6.5%) cuts (Scott et al., 2020). Even when the best hygiene practices are applied, complete prevention of carcass contamination is unachievable under commercial conditions; therefore, application of decontamination interventions may be necessary and are recommended in order to reduce contamination (Buncic and Sofos, 2012).

Pathogen Contamination of Poultry

Poultry meat and products are considered a significant source of *Salmonella* and *Campylobacter* (Jones et al., 1991; Bolder, 1997; Yang et al., 2001; Oyarzabal, 2005; NidaUllah et al., 2016). Live birds are usually contaminated with these pathogens at the farm from different vehicles, including the hatching and housing environment (Jones et al., 1991; Mead, 2000). Studies have shown that pathogens need to be controlled pre- and post-harvest to minimize contamination in final poultry products (Bolder, 1997; Wideman et al., 2016). Contaminated birds are a hazard to further contaminating carcasses during the slaughter process. Therefore, pre- and post-harvest interventions are utilized in the industry (Bolder, 1997; Mead, 2000; Wideman et al., 2016).

Commercial poultry slaughter consists of different stages that attempt to decontaminate carcasses; however, studies have shown that often, these same processing steps may be a source of cross-contamination (Bolder, 1997; Geornaras and von Holy, 2000; Hinton et al., 2004). These steps include scalding, defeathering, evisceration, and chilling (Hinton et al., 2004).

Scalding and defeathering processes, because they remove the epidermis of the skin, new surfaces are exposed for bacteria to inhabit (Hinton et al., 2004). Evisceration can lead to further contamination of carcasses with intestinal tract content if this is ruptured during the removal of viscera, which could increase the prevalence of *Salmonella* and *Campylobacter* if the bird was infected (Georgnaras and von Holy, 2000; Hinton et al., 2004; Peyrat et al., 2008; Guerin et al., 2010). During processing, cross-contamination between *Campylobacter*-positive and -negative broilers can increase the overall carcass contamination (Normand et al., 2008).

Prevalence of *Salmonella* and *Campylobacter* at each step of poultry processing has been well documented (Corry et al., 2002; Oyarzabal, 2005; McCrea et al., 2006; Peyrat et al., 2008; Guerin et al., 2010). Son et al. (2007) conducted a study to determine the prevalence of *Campylobacter* on broiler carcasses at three sampling sites along the processing line (pre-scald, pre-chilled, and post-chill) of a commercial U.S. poultry processing plant. Pre-scald and pre-chill carcasses had the highest *Campylobacter* prevalence (92% and 100%, respectively) (Son et al., 2007). After chilling, the proportion of *Campylobacter*-positive carcasses decreased to 52% (Son et al., 2007). Moreover, it was reported that prevalence of *Campylobacter* on carcasses varied significantly by sampling day and site (Son et al., 2007).

Contamination of poultry products with *Salmonella* and *C. jejuni* are biological hazards to consumers that unintentionally undercook poultry products (Acheson and Allos, 2001; Morris et al., 2011). Thus, the poultry industry is constantly evaluating antimicrobial interventions for their ability to reduce pathogen contamination on carcasses and parts (Chen et al., 2014). Post-chill application of antimicrobials consists of applying the treatment after the regular chilling process as part of the multiple hurdle intervention system (McKee, 2011). In comparison with pre-chill and regular immersion chill, the application of antimicrobials in a post-chill system

allows the use of higher concentration levels of the antimicrobial because of the shorter contact time with the product (Chen et al., 2014). These higher levels of the treatment lead to effective pathogen reductions on poultry carcasses and parts (Chen et al., 2014).

Regulations Relative to Pork and Poultry Production

In other to minimize pathogen contamination of raw meat and poultry products, in 1996, USDA-FSIS released the Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) System final rule. This rule would identify where in the processing system, unacceptable food safety risk could happen and design a monitoring system for these critical control points (USDA-FSIS, 1996). Also, this document shifted control from USDA-FSIS to each processing facility and required that all meat and poultry processing facilities implement a HACCP system, sanitation standard operating procedures (SSOPs), and microbial testing (USDA-FSIS, 1996). Microbiological testing is conducted on meat and poultry with a focus on the top four pathogens associated with these food products, namely, Shiga toxin-producing *Escherichia coli* (for raw beef and pork products), *Salmonella* spp. (for raw beef, pork, chicken, and turkey products, and ready-to-eat [RTE] meat and poultry products), *Campylobacter* spp. (for raw chicken and turkey), and *Listeria monocytogenes* (for RTE meat and poultry products) (Hulebak and Schlosser, 2002; USDA-FSIS, 2012).

In an attempt to further control pathogen contamination on poultry products, the USDA-FSIS implemented a testing program for the prevalence of *Salmonella* and *Campylobacter* in young chicken and turkey products (USDA-FSIS, 2011). This program is based on a preventive approach with a scientific risk assessment, and findings are reported quarterly (USDA-FSIS, 2011). Since more than 85% of poultry meat in the United States is sold as parts (Ramirez-Hernandez et al., 2018), in 2015, the FSIS included performance standards sampling for both

pathogens in the cut-up room to test poultry parts (USDA-FSIS, 2016a). The name of the testing program was updated to Performance Standards for *Salmonella* and *Campylobacter* in raw chicken parts and not-ready-to-eat comminuted chicken and turkey products, in 2016 (USDA-FSIS, 2016a). The current performance standards for the maximum acceptable *Campylobacter*-positives for chicken are 15.7%, 9.6%, and 7.7% of broiler carcasses, comminuted, and parts, respectively (USDA-FSIS, 2019a). Moreover, the maximum acceptable *Salmonella*-positives for chicken are 9.8%, 25%, and 15.4% of broiler carcasses, comminuted, and parts, respectively (USDA-FSIS, 2019a). Thus, the performance standard for *Campylobacter* in chicken parts is stricter than that for *Salmonella*.

Also, in recent years there has been concern over the possible carryover of residues of chemical decontamination treatments used during poultry processing and their effect on detection of *Salmonella* and *Campylobacter* in poultry rinses collected for testing by USDA-FSIS (Williams et al., 2018). Presence of chemical residues in the sample could potentially lead to false-negative results (Gamble et al., 2016; Williams et al., 2018). To address this concern, the USDA's Agricultural Research Service and FSIS developed a new buffered peptone water (BPW) formulation with additional neutralizing properties (Gamble et al., 2017). In 2016, FSIS replaced BPW with neutralizing BPW for all poultry rinse samples collected for testing (USDA-FSIS, 2016b).

With regards to pork production, in October of 2019, FSIS released a Final Rule for modernization of swine slaughter inspection (USDA-FSIS, 2019b). This rule amended the Federal meat inspection regulations to establish an optional New Swine Slaughter Inspection System (NSIS) for market swine slaughter facilities (USDA-FSIS, 2019b). Hog slaughter establishments that decide not to operate under the NSIS may continue to operate under their

existing inspection system (USDA-FSIS, 2019b). The new rule has two mandatory components; FSIS requires swine slaughter facilities to develop, implement, and maintain as part of their HACCP systems, written protocols to ensure that no visible fecal material, ingesta, or milk is present at the point of FSIS post-mortem inspection of pork carcasses (USDA-FSIS, 2019b). The second mandatory requirement states that each establishment will be responsible for developing and implementing its own microbiological sampling plan (USDA-FSIS, 2019b). Every facility, except for the low volume establishments (less than 10 employees or annual sales less than $2.5 million), is required to include carcass sampling at pre-evisceration and post-chill, starting March 30, 2020 (USDA-FSIS, 2019b).

An essential component of the NSIS is that it revokes maximum line speeds and authorizes plants to determine their own line speeds that allow them to maintain process control for preventing fecal contamination and meeting microbial performance during the slaughter process (USDA-FSIS, 2019b). This allows establishments flexibility to reconfigure evisceration process lines, generating potential for improving the efficiency of the whole process in plants that decide to follow this new rule (USDA-FSIS, 2019b).

Chemical and Physical Decontamination of Pork

Since pork production involves different physical decontamination treatments as part of the normal slaughtering process, only a few physical and chemical methods have been investigated for the specific decontamination of pork carcasses (Loretz et al., 2011). For example, spray washes with organic acids or hot water have been evaluated for their effect on reducing pathogen contamination, and especially *Salmonella* prevalence, on pork (Loretz et al., 2011; Baer et al., 2013). If the spray wash consists of organic acids, these need to be generally recognized as safe (GRAS), as determined by the FDA, to use in food production. The most used

interventions in pork production are carcass washes with hot water, lactic acid, and peroxyacetic acid (Loretz et al., 2011; Baer et al., 2013).

Hot water carcass washes are usually applied at 80°C for 14 to 16 s (De Busser et al., 2013). The temporary increase of temperature on the meat surface might lead to slight changes in color after treatment; however, these changes usually disappear after chilling the carcass (Goldbach and Alban, 2006; De Busser et al., 2013). Studies (Alban and Sørensen, 2010; Hamilton et al., 2010) have shown that hot water decontamination effectively reduces *Salmonella* prevalence on pork carcasses.

Lactic acid is an organic acid often used in the meat industry since it is a natural compound produced during postmortem glycolysis (Pipek et al., 2006). Lactic acid is approved by USDA-FSIS as an antimicrobial treatment of pork products with the following parameters: i) on carcasses before fabrication (pre- and post-chilling), variety meats, and offal at a concentration up to 5.0% acid solution, and, ii) on subprimal cuts and trimmings at a concentration of 2.0% to 5.0% acid solution and at a solution temperature of up to 55°C (USDA-FSIS, 2019c). Several studies have reported on the antimicrobial efficacy of lactic acid against pathogens, including *Salmonella*, in pork products (Epling et al., 1993; Larsen et al., 2003; Pipek et al., 2006; Carpenter et al., 2011).

Carpenter et al. (2011) reported that counts of *Salmonella*-inoculated pork belly samples that were spray-treated with 2.0% lactic acid (20 s, 20 psi, 55.4°C) and stored for 24 h at 4°C were 1.35 log CFU/cm^2 lower than the counts of the control (no-wash) treatment. In another study (Fabrizio and Cutter, 2004), pork bellies inoculated with *Salmonella* Typhimurium were sprayed with lactic acid (2%, 15 s) and counts were recovered immediately following treatment, after two days aerobic storage at 4°C, and after five days of vacuum-packaged storage at 4°C.

Inoculated *Salmonella* Typhimurium counts on treated pork bellies were 1.79 log CFU/cm^2 (immediately after treatment), 1.46 log CFU/cm^2 (following two days of aerobic storage), and 1.76 log CFU/cm^2 (after five days of vacuum-packaged storage) lower ($P < 0.05$) than the counts recovered from untreated pork belly samples stored under the same conditions (Fabrizio and Cutter, 2004).

Another commonly used antimicrobial intervention treatment in the meat industry is peroxyacetic acid (PAA), also known as peracetic acid. Peracetic acid is formed from the reaction of acetic acid and hydrogen peroxide; therefore, it has a strong vinegar-like odor with a low pH (Kitis, 2004). This combination reduces the negative changes in color and flavor that might occur with the use of organic acids (Bauermeister et al., 2008). PAA is considered GRAS by the USDA-FSIS and is approved for use on meat at concentrations of up to 400 ppm; a higher concentration (2,000 ppm) is permitted on poultry (USDA-FSIS, 2019c). Even though it is known that PAA is commonly used in the meat industry, including pork production, published data on the efficacy of PAA against *Salmonella* contamination on pork products are limited. However, different application parameters for PAA have been evaluated against pathogens, including *Salmonella*, on beef products (Ransom et al., 2003; Ellebracht et al., 2005; King et al., 2005; Geornaras et al., 2012b; Mohan and Pohlman, 2016).

Other interventions that have been assessed for use in pork processing are, acetic acid, citric acid, potassium sorbate, trisodium phosphate, and steam; all have been reported as possibly suitable treatments for pork production multiple intervention systems (Morris et al., 1997; Kang et al., 2003; Latha et al., 2009). Additionally, studies (Eggenberger-Solorzano et al., 2002; Pipek et al., 2006) have tested the antimicrobial effects of a combination of a physical treatment with an organic acid, such as steam and lactic acid or hot water and acetic acid.

Chemical Decontamination of Poultry

The poultry industry has been investing in a "multi-hurdle" intervention approach to enhance the safety of its products by reducing pathogen contamination (Nagel et al., 2013). There are several physical and chemical interventions used in the poultry industry aimed at reducing pathogen levels on carcasses and parts (Nagel et al., 2013; Ramirez-Hernandez et al., 2018). Organic acids, used individually or as blends, and oxidizing agents are among the most commonly tested interventions, and they are used at various steps of the processing chain (Loretz et al., 2010; Ramirez-Hernandez et al., 2018). Chlorine was considered the standard antimicrobial intervention for poultry since it was demonstrated to be an effective intervention for the reduction of pathogen contamination on poultry (Bolder, 1997; Bauermeister et al., 2008). But, recent studies reported variability in the efficacy of this antimicrobial treatment, and results indicate that reductions obtained are not significantly different from those obtained with water washes (Chen et al., 2014). Nowadays, chlorine is more often used for decontamination of processing equipment instead of as a decontamination treatment of poultry products (Mixon, 2020). Therefore, other chemicals have been evaluated and are more commonly used in recent times. Some of these chemical treatments include PAA, a blend of sulfuric acid and sodium sulfate, and lactic acid.

Use of PAA is extensive in the poultry industry and is approved for use on poultry carcasses, parts, and organs, to be treated by spray or immersion, at a concentration of up to 2,000 ppm (USDA-FSIS, 2019c). Several research studies have reported on the antimicrobial effects of PAA in poultry products (Bauermeister et al., 2008; Nagel et al., 2013; Chen et al., 2014; Purnell et al., 2014; Ramirez-Hernandez et al., 2018). For example, Purnell et al. (2014) sprayed broiler carcasses with 400 ppm of PAA for 30 s and reported that the treatment reduced

naturally occurring *Campylobacter* populations on neck and breast skin samples by 0.97 and 1.15 log CFU/g, respectively. In another study (Chen et al., 2014), boneless chicken breasts and thighs inoculated with *Salmonella* Typhimurium and *C. jejuni* were treated by immersion in 0.1% PAA for 23 s before being ground. Pathogen counts of ground chicken samples previously immersed in PAA were reported to be 1.5 (*Salmonella* Typhimurium) and 1.3 (*C. jejuni*) log CFU/g lower, respectively, than the pathogen counts obtained for untreated control samples (Chen et al., 2014). PAA (400 ppm and 1,000 ppm) was also evaluated as a post-chill treatment of broiler carcasses inoculated with *C. jejuni*, and reductions of 1.93 log CFU/mL (400 ppm) and 2.03 log CFU/mL (1,000 ppm) were reported (Nagel et al., 2013).

A proprietary blend of sulfuric acid and sodium sulfate (SSS; commercially available as Amplon [for poultry], Titon [for pork], Centron [for beef]), previously known as AFTEC 3000, is a combination of an inorganic acid with its natural conjugate salt (FDA, 2012b). The reaction produces a buffered solution as the salt serves as a buffering agent to the sulfuric acid (FDA, 2012b). This blend was developed for use as an acidifier or antimicrobial agent for meat and poultry to reduce pathogen contamination levels and to inhibit microbial growth (FDA, 2012b). SSS has GRAS status (GRAS Notice 000408; FDA, 2012b) and is approved for use as a spray, wash, or immersion treatment of meat and poultry at concentrations adequate to achieve a targeted pH range of 1.0 to 2.2 (USDA-FSIS, 2019c). Several published studies have reported on the antimicrobial efficacy of SSS in beef products (Geornaras et al., 2012a; Geornaras et al., 2012b; Schmidt et al., 2014; Acuff, 2017; Scott-Bullard et al., 2017; Yang et al., 2017; Muriana et al., 2019) and some in poultry products (Scott et al., 2015; Kim et al., 2017; Britton, 2018).

Scott et al. (2015) evaluated the antimicrobial effects of SSS (pH 1.1), applied as a 20-s immersion treatment, against *Salmonella* inoculated on chilled chicken wings. The treatment

effectively reduced inoculated *Salmonella* populations by 1.2 log CFU/mL (Scott et al., 2015). Moreover, it was reported that *Salmonella* counts of SSS-treated wings stored for 24 h at 4°C were 0.6 log CFU/mL lower than those obtained immediately after treatment (0 h) (Scott et al., 2015). In another study (Kim et al., 2017), the antimicrobial activity of SSS against *Campylobacter* on chicken carcasses was investigated in a pilot poultry processing plant. It was reported that spray treatment of defeathered carcasses with SSS (pH 1.3) reduced *Campylobacter* counts by 3.25 log CFU/mL (Kim et al., 2017).

Commercial poultry processing has several steps where cross-contamination can occur; thus, the poultry industry has invested in research to find cost-effective alternative antimicrobial treatments to add to the multiple hurdle intervention system (Chen et al., 2014). Other chemicals that have been evaluated for their antimicrobial effects against pathogenic bacteria in poultry include trisodium phosphate, cetylpyridinium chloride, lysozyme, and a lactic acid and acetic acid blend, among others (Oyarzabal, 2005; Scott et al., 2015; Ramirez-Hernandez et al., 2018).

Mechanisms of Action of Chemical Treatments

When working with different chemical decontamination treatments, it is important to understand which mechanism(s) they use to reduce microbial contamination. Also, when combination treatments are to be utilized, selection of compounds with two or more modes of action should be considered.

Organic acids

Organic acids (e.g., lactic, citric, acetic, formic, butyric, propionic, etc.) and their salts are classified as weak acids, which means that they do not completely dissociate in water; however, they do so in a pH-dependent way (Stratford et al., 2009; Mani-López et al., 2012). Therefore,

the antimicrobial properties of organic acids increase as the pH of the matrix of application is lowered to or below the pKa of the acid (e.g., 4.8 for acetic acid, 3.8 for formic acid) (Stratford et al., 2009; Mani-López et al., 2012). Organic acids, such as acetic and formic acid, are USDA-FSIS-approved as antimicrobials, up to a concentration of 2.5%, for use on meat and poultry products as part of a carcass wash applied before chilling (USDA-FSIS, 2019c).

Organic acids are thought to interfere with microbial activity via two primary modes of action: acidification of the cytoplasm, and, accumulation of dissociated acid anions to toxic levels (Mani-López et al., 2012). Both mechanisms of action start with the diffusion of undissociated acid molecules (lipophilic) over the neutral cell wall into the cytosol (Theron and Lues, 2007; Stratford et al., 2009). The cytoplasmic pH is close to neutral, and this difference of pH causes organic acid molecules to dissociate into anions and free protons (Stratford et al., 2009; Mani-López et al., 2012). Therefore, these charged molecules (lipophobic) accumulate in the cytoplasm (Stratford et al., 2009; Mani-López et al., 2012). Thus, cytoplasmic acidification occurs due to proton accumulation at levels that cause a decline in the intracellular pH, inhibiting cell metabolism (Krebs et al., 1983; Knarreborg et al., 2002). In order to maintain the internal pH, the cell activates specific transport mechanisms to efflux protons, which requires energy, since it is proposed that the cell membrane is impermeable to them (Hirshfield et al., 2003; Van Immerseel et al., 2006). Also, due to the high concentration of dissociated anions, a disruptive effect occurs on DNA synthesis (Cherrington et al., 1990). These two mechanisms have been proposed to have a bacteriostatic and bactericidal effect depending on the target microorganism, type of tissue, the acid used, and its concentration (Cherrington et al., 1990; Hirshfield et al., 2003; Mani-López et al., 2012).

Peroxyacetic acid

Peroxyacetic acid is a potent oxidizing and disinfectant agent that is the result of combining acetic acid and hydrogen peroxide (Kitis, 2004). The antimicrobial activity of PAA is considered to be higher than that of hydrogen peroxide in lower concentrations, and, against a wide range of microorganisms (Kitis, 2004; Ceragioli et al., 2010). Usually, its antimicrobial properties can be ranked, from most effective to least effective, as follows: bacteria, viruses, bacterial spores, and protozoan cysts (Wessels and Ingmer, 2013).

The precise mechanisms through which PAA oxidizes and kills microorganisms are not fully understood because of the complicated reaction pathway (Rokhina et al., 2010). However, it is speculated that its mode of action is comparable to other peroxides and oxidizing agents (Block, 2001), which is based on the release of reactive oxygen species (Luukkonen and Pehkonen, 2017). It is proposed that PAA disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane, and it is transported through the rupture of bacterial cell walls (Kitis, 2004). Once the PAA is inside the cell, it is proposed that it oxidizes essential enzymes, and this, in turn, disrupts or impairs vital biochemical pathways, active transport across membranes, and intracellular solute levels (Imlay, 2003; Kitis, 2004). Also, it seems that PAA can inactivate catalase, an enzyme that detoxifies free hydroxyl radicals (Block, 2001). The antimicrobial effect of PAA depends on the concentration, contact time, and target bacteria (Zoellner et al., 2018).

Sulfuric acid and sodium sulfate blend

The mode of action of SSS is similar to that of organic acids as it is a buffered acid, and ultimately, all of these are acidifiers. In general, the acid molecules enter the cell membrane in their undissociated form and lower the intracellular pH (Mani-López et al., 2012), due to the

disassociation of the molecules of the strong inorganic acid, sulfuric acid, into anions and protons (FDA, 2012b). Then, the cell spends its energy effluxing the accumulation of protons, to re-establish the cell pH (Stratford et al., 2009). Basically, the SSS makes the cell consume all its energy in order to maintain the intracellular pH and, therefore, preventing the organism from surviving. The sodium sulfate as a buffer minimizes any possible damage to the treated meat or to the persons handling the SSS treatment (FDA, 2012b).

Final Remarks

The CDC estimates that each year, 48 million people in the United States experience a foodborne disease, resulting in 128,000 hospitalizations and 3,000 deaths; therefore, foodborne illness is a major public health concern (Scallan et al., 2011; CDC, 2019a). *Campylobacter* and *Salmonella* rank first and second causes of bacterial-mediated foodborne illness, respectively, in the United States, with an estimate of 1.5 (*Campylobacter*) and 1.35 (*Salmonella*) million infections each year (CDC, 2020a). Food-related cases of campylobacteriosis are mainly attributed to poultry, while salmonellosis cases are mainly linked to poultry and pork consumption (Morris et al., 2011). Therefore, research on different strategies to reduce these pathogens on these foods is essential. Accordingly, the overriding goal of this thesis is to evaluate the antimicrobial efficacy of various chemical treatments against *Salmonella enterica* on pork and *Campylobacter jejuni* on chicken.

CHAPTER 2: ANTIMICROBIAL EFFECTS OF INDIVIDUAL AND COMBINED CHEMICAL TREATMENTS AGAINST TWO INOCULUM LEVELS OF *SALMONELLA ENTERICA* ON PORK JOWLS

Summary

A study was conducted to (i) evaluate decontamination efficacy of six chemical treatments when applied to pork jowls inoculated with *Salmonella enterica* and (ii) determine antimicrobial efficacy of the test solutions against a high and low inoculum level of *Salmonella*. Chilled pork jowls were cut into 10 × 5 × 1 cm portions and were surface-inoculated on the skin side with a mixture of six *S. enterica* serotype strains of swine origin. Inoculation levels targeted were 6 to 7 log CFU/cm^2 (high) and 3 to 4 log CFU/cm^2 (low). Following inoculation, samples were left untreated (control) or were treated by spray application (10 s, 18 to 19 psi, 1.0 gpm flow rate) with water, a proprietary blend of sulfuric acid and sodium sulfate (SSS, pH 1.2), formic acid (1.5%), peroxyacetic acid (PAA, 400 ppm), PAA (400 ppm) acidified with acetic acid (1.5%), PAA (400 ppm) acidified with formic acid (1.5%), or PAA (400 ppm) acidified with SSS (pH 1.2). Samples were analyzed for inoculated *Salmonella* counts immediately after treatment application (0 h) and after 24 h of refrigerated (4°C) storage. Overall, all seven spray treatments were effective ($P < 0.05$) at reducing the high and low *Salmonella* inoculation levels. At the high inoculum level (6.2 log CFU/cm^2), pathogen counts ranged from 5.4 (water; 0.8 log CFU/cm^2 reduction) to 4.3 (PAA acidified with SSS; 1.9 log CFU/cm^2 reduction) log CFU/cm^2 for samples analyzed immediately after spray treatment. *Salmonella* counts obtained at the 0-h sampling time for treated samples inoculated at the low inoculum level (3.5 log CFU/cm^2) ranged from 2.8 (water; 0.7 log CFU/cm^2 reduction) to 1.8 (PAA acidified with SSS; 1.7 log CFU/cm^2 reduction) log CFU/cm^2. Thus, regardless of inoculum concentration, similar

reductions of *Salmonella* populations were obtained immediately following treatment application (0 h). For the high inoculation level, *Salmonella* counts of samples analyzed after 24 h of refrigerated storage were, in general, similar ($P \geq 0.05$) to the counts of the corresponding treatment at 0 h. However, for the low inoculation level, pathogen counts of jowls treated with SSS, formic acid, or PAA acidified with formic acid, and held at 4°C for 24 h, were 0.6 log CFU/cm^2 lower ($P < 0.05$) than the 0-h counts of the corresponding treatment. Regardless of inoculation level and sampling time, no ($P \geq 0.05$) differences in efficacy were obtained between PAA on its own and any of the acidified PAA treatments evaluated. These results indicate that the treatment solutions tested are effective interventions for reducing high and low contamination levels of *Salmonella* on pork.

Introduction

The Centers for Disease Control and Prevention (CDC) estimates that *Salmonella* is responsible for more than 1.3 million foodborne infections in the United States every year, and is the leading cause of foodborne illness-related hospitalizations (26,500/year) and deaths (420/year) (Morris et al., 2011; Scallan et al., 2011; CDC, 2020a). Furthermore, in 2018, the Foodborne Diseases Active Surveillance Network reported a 9% increase in incidence of *Salmonella* infections compared to 2015-2017 data (Tack, 2019). According to the latest surveillance data compiled by the CDC, this bacterial pathogen was the confirmed etiologic agent of 125 food-related outbreaks in 2017 (CDC, 2018).

In the United States, pork is considered one of the riskiest per meat consumption unit with regards to *Salmonella* illness (Hsi et al., 2015). From 2009 to 2017, there were a total of 1,232 food-related *Salmonella* outbreaks in the United States, and 71 of these were linked to

pork consumption (CDC, 2018). From 2015 to 2017, 20 pork-related *Salmonella* outbreaks occurred, resulting in a total of 793 illnesses, 95 hospitalizations, and two deaths (CDC, 2018). In August 2015, Kapowsin Meats issued a recall for more than 500,000 pounds of raw pork products that were potentially contaminated with *Salmonella enterica* serotype I 4,[5],12:i:- (CDC, 2015; USDA-FSIS, 2017). These products were linked to 192 cases of illness and 30 hospitalizations over five U.S. states (CDC, 2015; USDA-FSIS, 2017).

Swine are usually asymptomatic carriers of *Salmonella*, primarily in their intestinal tract, and as such, there is a high prevalence of this pathogen at the farm that can then be carried to the slaughter process (Baer et al., 2013). Schmidt et al. (2012) reported that 1,386 of 1,520 (91.2%) pork carcasses sampled at the pre-scalding stage of slaughter, were positive for *Salmonella*. Subsequent processing steps, such as scalding and singing, decrease *Salmonella* contamination levels on carcasses, but do not completely eliminate its presence (Dickson et al., 2002). In the Schmidt et al. (2012) study, 19.1% *Salmonella*-positive carcasses were obtained after they were scalded, singed, and polished, and 3.7% were positive for the pathogen after the final wash and carcass chilling. However, in a recently published study (Scott et al., 2020), a 13.6% *Salmonella* prevalence was reported for raw pork products (intact cuts, nonintact cuts, and comminuted products) collected from federally-inspected slaughter and processing establishments in the United States.

Since the commercial slaughter process of pork typically involves several physical decontamination treatments as part of the normal slaughter process, only a few chemical intervention treatments have been investigated for the specific decontamination of pork carcasses (Loretz et al., 2011). For example, spray washes with organic acids have been evaluated for their effect on reducing pathogen contamination, and especially *Salmonella*, on pork (Epling et al.,

1993; Eggenberger-Solorzano et al., 2002; Kang et al., 2003; Larsen et al., 2003; Fabrizio and Cutter, 2004; Pipek et al., 2006; Carpenter et al., 2011; Loretz et al., 2011; Baer et al., 2013; Dan et al., 2017). Presently, lactic acid and peroxyacetic acid are two of the most commonly used antimicrobial interventions in commercial pork processing facilities (Loretz et al., 2011; Baer et al., 2013). Peroxyacetic acid (PAA) is a commercially available oxidizing agent, and its use as an antimicrobial treatment, applied at different application parameters, has been extensively evaluated against pathogens, including *Salmonella*, on beef and poultry products (Ellebracht et al., 2005; King et al., 2005; Geornaras et al., 2012b; Nagel et al., 2013; Scott et al., 2015; Mohan and Pohlman, 2016; Kim et al., 2017; Britton et al., 2018; Ramirez-Hernandez et al., 2018). In comparison, however, published data on the decontamination efficacy of PAA against *Salmonella* contamination on pork products are limited.

The pork industry continues to look for alternative chemical decontamination treatments that are cost-effective, and that can be used in commercial pork processing settings for effective reduction of pathogen contamination. Use of blends comprised of an organic acid and an oxidizing agent, which combine two antimicrobial mechanisms of action, is an example of such an alternative antimicrobial treatment. Therefore, the objectives of this study were to (i) evaluate the antimicrobial effects of formic acid, a proprietary blend of sulfuric acid and sodium sulfate (SSS), PAA, and PAA acidified with acetic acid, formic acid or SSS, when applied to pork jowls inoculated with *Salmonella* and (ii) determine the antimicrobial efficacy of the test solutions against two target inoculation levels (6 to 7 log CFU/cm^2 and 3 to 4 log CFU/cm^2) of *Salmonella*. Additionally, antimicrobial effects against inoculated populations were evaluated immediately after treatment application (0 h) and after 24 h of storage at 4°C.

Materials and Methods

Bacterial strains and inoculum preparation

The inoculum used in this study consisted of a mixture of six *S. enterica* serotype strains of swine origin (kindly provided by Dr. Tom Edrington, previously at U.S. Department of Agriculture, Agricultural Research Service, College Station, TX), including *Salmonella* Agona B E1-09, *Salmonella* Anatum E B1-03, *Salmonella* Derby B E1-13, *Salmonella* Montevideo C1 B2-51, *Salmonella* Schwartzengrund B B1-10, and *Salmonella* Tennessee C1 E3-10. All six strains were hydrogen sulfide producers, indicated by the growth of black-centered colonies on xylose lysine deoxycholate (XLD; Acumedia-Neogen, Lansing, MI) agar. Working cultures of the six strains were maintained at 4°C on XLD agar plates.

Inoculum preparation was initiated three days prior to sample inoculation and treatment application. The strains were individually activated by transferring a single colony from the XLD agar plate into 10 mL tryptic soy broth (TSB, Difco, Becton Dickinson and Company [BD], Sparks, MD) and incubated at 35°C (22 ± 1 h). Broth cultures were then subcultured by transferring a 0.1 mL aliquot of each culture into 10 mL of fresh TSB. After incubation (35°C, 22 h), cultures of the six *Salmonella* strains were combined and cells harvested by centrifugation (6,000 × g, 15 min, 4°C; Sorvall Legend X1R centrifuge, Thermo Scientific, Waltham, MA). After centrifugation, the supernatant was decanted, and the cell pellet washed with 10 mL of phosphate-buffered saline (PBS, pH 7.4; Sigma-Aldrich, St. Louis, MO). The cell suspension was centrifuged again (6,000 × g, 15 min, 4°C), the supernatant again decanted, and the washed cell pellet resuspended in 60 ml of PBS. This inoculum suspension was either left undiluted (8 to 9 log CFU/mL concentration) or was diluted 1000-fold (5 to 6 log CFU/mL concentration) in

PBS, and these suspensions were then used to inoculate pork jowls to the high (6 to 7 log CFU/cm^2) and low (3 to 4 log CFU/cm^2) target inoculation levels, respectively.

Inoculation of pork jowls

Pork jowls were collected from the harvest floor (prior to chilling) of a major pork processor and were shipped overnight, on dry ice, to the Department of Animal Sciences, Colorado State University (Fort Collins, CO). On arrival, jowls were held at 3°C and were used within one or two days. Two trials (repetitions) of the study, conducted on two separate days and with different production lots of jowls, were performed for each *Salmonella* inoculation level. The day before inoculation and treatment application, pork jowls were cut into 10 × 5 cm portions with an approximate thickness of 1 cm. Portioned samples were placed in a bag and were refrigerated (3°C) until the next day.

On the day of each experiment, pork jowl portions were randomly assigned to one of eight treatments. For inoculation, six samples per treatment were placed on trays lined with alcohol-sterilized aluminum foil, with the outer (skin side) surface facing up. Samples were inoculated under a biological safety cabinet. A 0.2-mL (200 µL) aliquot of the low or high concentration of the *Salmonella* inoculum was randomly deposited, with a micropipette (approximately 10 µL per drop), on the skin side of each jowl and then spread over the entire surface (50 cm^2) with a sterile disposable spreader. Samples remained undisturbed for 15 min to allow for bacterial cell attachment. The target inoculation level of samples inoculated with the 5 to 6 log CFU/mL or 8 to 9 log CFU/mL concentration of the inoculum mixture was 3 to 4 log CFU/cm^2 and 6 to 7 log CFU/cm^2, respectively.

Antimicrobial treatment of pork jowls

Pork jowl portions inoculated with the low or high *Salmonella* levels were left untreated, to serve as controls, or were subjected to one of the following treatments: water (room temperature), formic acid (1.5%; BASF Corporation, Florham Park, NJ), SSS (pH 1.2; Titon, Zoetis, Florham Park, NJ), PAA (400 ppm; Kroff, Pittsburgh, PA), PAA (400 ppm) acidified with acetic acid (1.5%; Fisher Scientific, Fair Lawn, NJ), PAA (400 ppm) acidified with formic acid (1.5%), and PAA (400 ppm) acidified with SSS (pH 1.2). Antimicrobial treatment solutions were prepared according to the manufacturers' instructions, and the pH of solutions was measured (Orion Star A200 Series pH meter and pH electrode, Thermo Scientific, Schaumburg, IL). Average pH values of the formic acid, SSS, and PAA solutions were 2.9, 1.2, and 3.4, respectively. For the acetic acid-, formic acid-, and SSS-acidified PAA solutions, average pH values were 2.6, 2.9, and 1.2, respectively. The PAA concentration was verified using a hydrogen peroxide and peracetic acid test kit (LaMotte Company, Chestertown, MD).

Water and chemical treatments were applied to the six inoculated samples per treatment using a custom-built spray cabinet (Birko/Chad Equipment, Olathe, KS) fitted with six 0.1-gpm (gallons per minute) floodjet spray nozzles (Spraying Systems Co., Glendale Heights, IL) positioned above the product belt. Treatments were applied at a flow rate of approximately 1.0 gpm at 18 to 19 psi, and a product contact time of 10 s per sample. Following the spray treatment, samples were placed on a sterile wire rack for 5 min to allow excess solution to drip off samples before microbial analysis or storage. Three of the six samples per treatment were analyzed for inoculated *Salmonella* counts following treatment application (0 h analysis) or, in the case of untreated control samples, immediately following the inoculation procedure. The three remaining samples per treatment were placed in sterile plastic containers that were covered

with aluminum foil (without it touching the product) and analyzed after a 24 ± 1 h storage period at 4°C.

Microbiological analysis

At each sampling time (0 h and 24 h), untreated (control) and treated samples were analyzed for *Salmonella* counts. Individual pork jowl samples were placed into a Whirl-Pak filter bag (24-oz, Nasco, Modesto, CA) with 75 mL of Dey/Engley neutralizing broth (Difco, BD), and then mechanically pummeled for 2 min (Masticator, IUL Instruments, Barcelona, Spain). Samples were serially diluted (10-fold) in 0.1% buffered peptone water (Difco, BD) and appropriate dilutions were surface-plated, in duplicate, onto XLD agar. Colonies were counted after 24 h of incubation of plates at 35°C. Three uninoculated and untreated pork jowl samples were also analyzed on each of the inoculation and treatment application days, for levels of the natural microflora (on tryptic soy agar; Acumedia-Neogen), and for any naturally present *Salmonella* populations (on XLD agar) on the pork jowl samples. The detection limit of the microbiological analysis was 0.2 log CFU/cm^2 (1.5 CFU/cm^2).

Statistical analysis

The study was designed as an 8 (treatments) × 2 (sampling times) factorial for each inoculation level (low, high), blocked by trial day. It was repeated on two separate days for each inoculation level, and three samples were analyzed per treatment and sampling time (0 h and 24 h) in each trial (i.e., a total of six samples per treatment and sampling time). For each inoculation level, recovered *Salmonella* counts from treatments were analyzed within and across the two sampling times (0 h, 24 h). Bacterial populations were expressed as least squares means for log CFU/cm^2 under the assumption of a log-normal distribution of plate counts. Data were analyzed

using the CRAN-R package (Lenth, 2018) in R (version 3.5.1). All differences are reported using a significance level of $\alpha = 0.05$.

Results and Discussion

Bacterial counts of uninoculated and untreated pork jowls

Analysis of uninoculated and untreated pork jowl samples indicated absence (i.e., below the detection limit: <0.2 log CFU/cm^2) of any naturally occurring *Salmonella* populations. Therefore, colony counts recovered with the XLD agar from inoculated untreated and treated samples (Tables 2.1 and 2.2) were those of the inoculated pathogen. Aerobic plate counts of the uninoculated and untreated pork jowls used for the study ranged from 2.4 to 4.6 log CFU/cm^2, with a mean of 3.2 ± 0.5 log CFU/cm^2.

Pork jowls inoculated at a 6 to 7 log CFU/cm^2 inoculation level

Salmonella populations recovered from untreated and treated jowls that were inoculated at the 6 to 7 log CFU/cm^2 target inoculation level are shown in Table 2.1. The actual inoculation level achieved on jowls, as determined by microbial analysis of untreated samples, was 6.2 log CFU/cm^2. Pathogen counts recovered from untreated samples stored at 4°C for 24 h were not ($P \geq 0.05$) different than those obtained at the 0-h sampling time.

All seven spray treatments tested effectively ($P < 0.05$) reduced initial (0 h) inoculated (6.2 log CFU/cm^2) pathogen populations (Table 2.1). Overall, at the 0-h sampling time, surviving populations on treated samples ranged from 5.4 (water; 0.8 log CFU/cm^2 reduction) to 4.3 (PAA acidified with SSS; 1.9 log CFU/cm^2 reduction) log CFU/cm^2. No ($P \geq 0.05$) differences in efficacy were noted between the water treatment and formic acid treatment. Compared to the

untreated control, formic acid, SSS, and PAA lowered ($P < 0.05$) *Salmonella* populations by 1.2, 1.6, and 1.5 log CFU/cm^2, respectively. No ($P \geq 0.05$) differences in efficacy were obtained between SSS, PAA, and any of the acidified PAA treatments (i.e., PAA acidified with acetic acid, formic acid, or SSS). Spray treatment of jowls with PAA acidified with formic acid was found to be more ($P < 0.05$) effective than treating the jowls with formic acid on its own.

Overall, within each treatment, pathogen counts for samples analyzed after the refrigerated storage period were, in general, similar ($P \geq 0.05$) to counts of corresponding treatments at 0 h. The only exception was for the water treatment where counts of samples analyzed after 24 h were 0.5 log CFU/cm^2 lower ($P < 0.05$) than the corresponding 0-h samples.

Pork jowls inoculated at a 3 to 4 log CFU/cm^2 inoculation level

Inoculated *Salmonella* populations recovered from untreated and treated jowls that were inoculated at the lower target inoculation level of 3 to 4 log CFU/cm^2 are shown in Table 2.2. The actual inoculation level achieved was 3.5 log CFU/cm^2. Recovered counts from the untreated samples stored at 4°C for 24 h were not ($P \geq 0.05$) different than those obtained at the initial sampling time.

As seen for the 6 to 7 log CFU/cm^2 inoculation level, all seven spray treatments evaluated effectively ($P < 0.05$) reduced the initial (0 h) inoculated (3.5 log CFU/cm^2) *Salmonella* populations. Pathogen counts of spray-treated samples ranged from 2.8 (water; 0.7 log CFU/cm^2 reduction) to 1.8 (PAA acidified with SSS; 1.7 log CFU/cm^2 reduction) log CFU/cm^2 at the 0-h sampling time (Table 2.2). Counts of samples treated with formic acid were again not ($P \geq 0.05$) different from those of the water-treated samples. *Salmonella* counts of jowls treated with the individual chemical treatments were 1.0 (formic acid), 1.3 (SSS), and 1.4 (PAA) log CFU/cm^2

lower ($P < 0.05$) than counts of the untreated control. No ($P \geq 0.05$) differences in efficacy were noted between formic acid on its own and the formic acid-acidified PAA treatment. Similarly, no ($P \geq 0.05$) differences in antimicrobial efficacy were obtained between PAA on its own and any of the three acidified PAA treatments. On the other hand, spray treatment of jowls with PAA acidified with SSS was found to be more ($P < 0.05$) effective than treating the jowls with SSS on its own.

Salmonella counts of jowls treated with PAA on its own, or PAA acidified with acetic acid or SSS and stored at 4°C for 24 h were similar ($P \geq 0.05$) to the counts obtained at the 0-h sampling time for each treatment. For jowls treated with SSS, formic acid, or PAA acidified with formic acid, pathogen counts obtained after 24 h of refrigerated storage were 0.6 log CFU/cm^2 lower ($P < 0.05$) than the corresponding 0-h counts. Statistical comparison of *Salmonella* counts obtained at the 24 h sampling time indicated no ($P \geq 0.05$) differences between any of the chemical spray treatments.

As previously mentioned, use of PAA for reducing bacterial pathogen contamination on beef and poultry has been extensively investigated (Ellebracht et al., 2005; King et al., 2005; Nagel et al., 2013; Scott et al., 2015; Mohan and Pohlman, 2016; Kim et al., 2017; Britton et al., 2018; Ramirez-Hernandez et al., 2018). To the best of our knowledge, this is the first report addressing antimicrobial effects of PAA against *Salmonella* contamination on pork. The antimicrobial effect of PAA depends on concentration, contact time, target bacteria, and tissue type (Zoellner et al., 2018). From the results of the current study, PAA applied at 400 ppm as a spray treatment (approximately 1.0 gpm flow rate, 18 to 19 psi, 10 s contact time) onto pork jowls, reduced ($P < 0.05$) high (6.2 log CFU/cm^2) and low (3.5 log CFU/cm^2) *Salmonella* contamination levels by 1.5 and 1.4 log CFU/cm^2, respectively. Under conditions of the study,

acidification of the PAA with 1.5% acetic acid or formic acid, or with pH 1.2 SSS, did not ($P \geq 0.05$) affect the decontamination efficacy of PAA.

Pohlman et al. (2019) evaluated the efficacy of 200 ppm PAA compared with PAA (200 ppm) followed (after a 3 min drip time) by different 3% organic acid solutions (i.e., malic, pyruvic, and octanoic acid) against *Salmonella* inoculated on beef trimmings. The treatments were applied using a conventional spray application (0.1 mL/g), and after treatment, trimmings were ground twice, and samples were analyzed for *Salmonella* (Pohlman et al., 2019). In general, reductions ranged from 0.92 (PAA followed by 3% malic) to 1.76 (PAA) log CFU/g (Pohlman et al., 2019). In another study (Yeh et al., 2018), *Salmonella*-inoculated (3.5 log CFU/g) beef trim was treated by pipetting 5 mL of 400 ppm PAA as an individual treatment or a combination of PAA (400 ppm) and 5% lactic acid. After antimicrobial treatments were applied, samples were stored at 5°C for 1.5 h and were then ground twice (Yeh et al., 2018). Treatment with PAA alone, or in combination with lactic acid did not significantly decrease *Salmonella* counts when compared to an untreated control treatment (Yeh et al., 2018). A potential reason for the ineffectiveness of PAA in this study could be the application method used to treat the beef trim.

Antimicrobial effects of SSS have been evaluated mostly on beef and poultry products (Geornaras et al., 2012a; Geornaras et al., 2012b; Schmidt et al., 2014; Scott et al., 2015; Acuff, 2017; Kim et al., 2017; Scott-Bullard et al., 2017; Yang et al., 2017; Muriana et al., 2019). Yang et al. (2017) inoculated prerigor beef carcass surface tissue with a mixture of six *Salmonella* serotype strains and treated them with SSS (pH 1.1, 21°C) using a spray cabinet (5 s, 15 lb/in^2, 33 mL/s flow rate). The SSS treatment reduced initial *Salmonella* levels (6.3 log CFU/cm^2) by 2.0 log CFU/cm^2 (Yang et al., 2017). Schmidt et al. (2014) evaluated SSS (1.0%) and PAA (220 ppm), applied as immersion treatments (1, 2.5, or 5 min), for their antimicrobial effects against

Salmonella populations inoculated on adipose and lean tissue surfaces of beef cheek meat. After SSS treatment, reductions of counts ranged from 1.1 to 1.5 log CFU/cm^2 (adipose surfaces) and 1 to 1.3 log CFU/cm^2 (lean surfaces) with no ($P \geq 0.05$) differences between the immersion times (Schmidt et al., 2014). Similarly, following PAA treatment, reductions of counts ranged from 0.8 to 1.1 log CFU/cm^2 (adipose surfaces) and 0.6 to 1.0 log CFU/cm^2 (lean surfaces) (Schmidt et al., 2014). No ($P \geq 0.05$) differences in antimicrobial efficacy were observed between the SSS and PAA treatments, regardless of the immersion time or tissue type (Schmidt et al., 2014). One study (McCullough, 2016) reports on the decontamination efficacy of SSS on pork. In this study, pH 1.5 or 1.0 solutions of SSS were applied, using a spray cabinet (11 s, 20 psi, and 0.14 gpm flow rate), onto pork shoulder portions inoculated with the same mixture of *Salmonella enterica* serotype strains used in the current study. Treatments reduced inoculated pathogen counts (6.46 log CFU/g) by 0.76 (pH 1.5 SSS) and 0.86 (pH 1.0 SSS) log CFU/g (McCullough, 2016).

In summary, results of this current study showed that all evaluated acid spray treatments effectively reduced both the high (6 to 7 log CFU/cm^2) and low (3 to 4 log CFU/cm^2) *Salmonella* contamination levels on pork jowls. Overall, similar immediate (0 h) reductions of the pathogen were obtained, regardless of the inoculum level. Pathogen reductions for the chemical treatments ranged from 1.2 to 1.9 log CFU/cm^2 (6.2 log CFU/cm^2 inoculation level) and 1.0 to 1.7 log CFU/cm^2 (3.5 inoculation level). Regardless of contamination level, no ($P \geq 0.05$) differences in efficacy were obtained between PAA on its own and any of the acidified PAA treatments evaluated.

Table 2.1: Mean (n = 6) *Salmonella* counts (log CFU/cm^2 ± standard deviation) for inoculated (six serotype strain mixture; 6 to 7 log CFU/cm^2) pork jowls that were left untreated (control) or were spray-treated (10 s, 18 to 19 psi) with various treatment solutions. Samples were analyzed for inoculated *Salmonella* populations after treatment (0 h) as well as after a 24 h storage period at 4°C.

Treatment	**Mean *Salmonella* counts (log CFU/cm^2 ± SD)**	
	0 h	**24 h**
Untreated (control)	$6.2 \pm 0.1^{a\text{-}z}$	$6.0 \pm 0.1^{a\text{-}z}$
Water	$5.4 \pm 0.1^{b\text{-}z}$	$4.9 \pm 0.2^{b\text{-}y}$
Formic acid (1.5%)	$5.0 \pm 0.3^{bc\text{-}z}$	$4.7 \pm 0.3^{bc\text{-}z}$
SSS (pH 1.2)	$4.6 \pm 0.2^{cd\text{-}z}$	$4.8 \pm 0.2^{bc\text{-}z}$
PAA (400 ppm)	$4.7 \pm 0.4^{cd\text{-}z}$	$4.5 \pm 0.3^{bcd\text{-}z}$
PAA (400 ppm) acidified with acetic acid (1.5%)	$4.5 \pm 0.3^{cd\text{-}z}$	$4.5 \pm 0.2^{bcd\text{-}z}$
PAA (400 ppm) acidified with formic acid (1.5%)	$4.5 \pm 0.4^{d\text{-}z}$	$4.3 \pm 0.3^{cd\text{-}z}$
PAA (400 ppm) acidified with SSS (pH 1.2)	$4.3 \pm 0.2^{d\text{-}z}$	$4.1 \pm 0.1^{d\text{-}z}$

SSS: sulfuric acid and sodium sulfate blend; PAA: peroxyacetic acid

$^{a\text{-}d}$ Least squares means in the same column without a common superscript letter are different ($P < 0.05$).

$^{y\text{-}z}$ Least squares means in the same row without a common superscript letter are different ($P < 0.05$).

Table 2.2: Mean (n = 6) *Salmonella* counts (log CFU/cm^2 ± standard deviation) for inoculated (six serotype strain mixture; 3 to 4 log CFU/cm^2) pork jowls that were left untreated (control) or were spray-treated (10 s, 18 to 19 psi) with various treatment solutions. Samples were analyzed for inoculated *Salmonella* populations after treatment (0 h) as well as after a 24 h storage period at 4°C.

Treatment	Mean *Salmonella* counts (log CFU/cm^2 ± SD)	
	0 h	24 h
Untreated (control)	3.5 ± 0.0^{a-z}	3.3 ± 0.1^{a-z}
Water	2.8 ± 0.1^{b-z}	2.5 ± 0.4^{b-y}
Formic acid (1.5%)	2.5 ± 0.2^{bc-z}	1.9 ± 0.3^{c-y}
SSS (pH 1.2)	2.2 ± 0.2^{cd-z}	1.6 ± 0.2^{c-y}
PAA (400 ppm)	2.1 ± 0.2^{cde-z}	1.9 ± 0.2^{c-z}
PAA (400 ppm) acidified with acetic acid (1.5%)	1.9 ± 0.1^{de-z}	1.7 ± 0.3^{c-z}
PAA (400 ppm) acidified with formic acid (1.5%)	2.1 ± 0.2^{cde-z}	1.5 ± 0.5^{c-y}
PAA (400 ppm) acidified with SSS (pH 1.2)	1.8 ± 0.3^{e-z}	1.5 ± 0.2^{c-z}

SSS: sulfuric acid and sodium sulfate blend; PAA: peroxyacetic acid

$^{a-e}$ Least squares means in the same column without a common superscript letter are different ($P < 0.05$).

$^{y-z}$ Least squares means in the same row without a common superscript letter are different ($P < 0.05$).

CHAPTER 3: ANTIMICROBIAL EFFECTS OF INDIVIDUAL AND COMBINED CHEMICAL TREATMENTS AGAINST *CAMPYLOBACTER JEJUNI* INOCULATED ON POULTRY WINGS

Summary

A study was conducted to (i) evaluate decontamination efficacy of five chemical treatments when applied to chicken wings inoculated with *Campylobacter jejuni* and (ii) determine antimicrobial efficacy of the treatments as a result of applying the test solutions by immersion or spraying. Skin-on chicken wings were surface-inoculated with a six-strain mixture of *C. jejuni* of poultry origin. The target inoculation level was 3 to 4 log CFU/mL of wing rinsate. Following inoculation, samples remained untreated (control) or were treated by immersion (500 mL solution per wing; 5 s) or spray application (10 to 12 psi; 4 s) with water, a proprietary sulfuric acid and sodium sulfate blend (SSS; pH 1.2), formic acid (1.5%), peroxyacetic acid (PAA; 550 ppm), PAA (550 ppm) acidified with SSS (pH 1.2), or PAA (550 ppm) acidified with formic acid (1.5%). Samples were analyzed for *C. jejuni* counts immediately after treatment application (0 h) and following 24 h of refrigerated (4°C) storage. All five acid treatments evaluated in this study were effective ($P < 0.05$) at reducing the initial inoculated (3.9 log CFU/mL) *C. jejuni* populations on chicken wings, regardless of the antimicrobial treatment application method. Pathogen counts for samples spray-treated with one of the chemical solutions and analyzed immediately (0 h) after treatment ranged from 3.4 (SSS; 0.5 log CFU/mL reduction) to 2.7 (PAA acidified with formic acid; 1.2 log CFU/mL reduction) log CFU/mL. When the chemical treatments were applied by immersion, *C. jejuni* counts of 2.2 (SSS; 1.7 log CFU/mL reduction) to 1.7 (PAA, and PAA acidified with SSS; 2.2 log CFU/mL reduction) log

CFU/mL were obtained for wings analyzed at the 0-h sampling time. The PAA and acidified PAA treatments (PAA acidified with SSS or formic acid) were equally ($P \geq 0.05$) effective at reducing initial *C. jejuni* populations, regardless of treatment application method. However, following the refrigerated storage period, samples that had been treated with SSS- or formic acid-acidified PAA had lower ($P < 0.05$) pathogen counts than those that had been treated with the non-acidified PAA treatment. Additionally, *C. jejuni* counts of wings that had been immersion-treated with SSS, formic acid, PAA acidified with SSS, and PAA acidified with formic acid and stored for 24 h were lower ($P < 0.05$) than those recovered from the corresponding 0-h samples. Findings of this study should be useful to the poultry industry as they consider new interventions against *Campylobacter* contamination on chicken parts.

Introduction

The Centers for Disease Control and Prevention (CDC) estimates that *Campylobacter* spp. are responsible for 1.5 million food-related illnesses each year in the United States (Scallan et al., 2011; CDC, 2020b). The Foodborne Diseases Active Surveillance Network (FoodNet) of CDC's Emerging Infections Program reported that *Campylobacter* was the leading bacterial cause of foodborne illness in the United States in 2018 (Tack, 2019). Out of 25,606 total cases of foodborne illness that were laboratory-diagnosed in that year, 9,723 were due to infection with *Campylobacter* (Tack, 2019). Furthermore, *Campylobacter jejuni* is responsible for at least 80% of campylobacteriosis enteric infections (Moore et al., 2005; Dodd et al., 2017).

Campylobacter infections are primarily associated with consumption of unintentionally undercooked contaminated poultry products (Acheson and Allos, 2001; Arritt et al., 2002). Moreover, *Campylobacter* in poultry is the number one pathogen-food combination in terms of

annual illness burden, with a total of 608,231 infections and an estimated cost of more than $1 billion (Morris et al., 2011). In an attempt to further control pathogen contamination in poultry products, the U.S. Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) implemented a testing program for the prevalence of *Salmonella* and *Campylobacter* in not-ready-to-eat poultry products (USDA-FSIS, 2011). This verification program is based on a preventive approach with a scientific risk assessment, and findings are reported quarterly (USDA-FSIS, 2011). Since more than 85% of poultry meat in the United States is sold as parts, the FSIS included in this testing program sampling sites for both pathogens in the cut-up room to test poultry parts (USDA-FSIS, 2016a; Ramirez-Hernandez et al., 2018). The performance standard set the maximum acceptable *Campylobacter*-positive samples at 7.7% for chicken parts (USDA-FSIS, 2019a). Thus, the poultry industry is reevaluating current antimicrobial interventions used for pathogen control and is looking for new ones to apply to meet the stricter regulations (Chen et al., 2014; Ramirez-Hernandez et al., 2018).

There are numerous published studies (Laury et al., 2009; Scott et al., 2015; Sukumaran et al., 2015; Ramirez-Hernandez et al., 2018) on the antimicrobial effects of various chemical treatments against inoculated *Salmonella* populations on whole chicken carcasses and parts. However, research studies on the effect of such treatments against *Campylobacter*, and in particular, on chicken parts, are limited. Additionally, studies of chemical treatments that combine two or more modes of action against pathogens are limited, regardless of food-matrix application. Therefore, objectives of this study were to (i) evaluate antimicrobial effects of a proprietary sulfuric acid and sodium sulfate blend (SSS), formic acid, peroxyacetic acid (PAA), and PAA acidified with SSS or formic acid, when applied to chicken wings inoculated with *C. jejuni* and (ii) determine antimicrobial efficacy of the treatments as a result of applying the test

solutions by immersion or spraying. Additionally, antimicrobial effects against inoculated populations were evaluated immediately after treatment application (0 h) and after 24 h of storage at 4°C.

Materials and Methods

Bacterial strains and inoculum preparation

The inoculum consisted of a mixture of six *C. jejuni* strains of poultry origin (Table 3.1). Working cultures of the strains were maintained at 4°C on plates of Campy Cefex Agar, Modified (mCCA; Hardy Diagnostics, Santa Maria, CA) that were held within anaerobic containers (AnaeroPack Rectangular Jar; Mitsubishi Gas Chemical America, New York, NY) with a microaerophilic environment (mixture of approximately 6 to 12% O_2 and 5 to 8% CO_2) generating gas pack (AnaeroPack-MicroAero sachet, Mitsubishi Gas Chemical America).

The six strains were separately activated by transferring colonies from the mCCA plate into 10 mL of Bolton broth (Hardy Diagnostics). Inoculated Bolton broth was incubated at 42°C for 48 h under microaerophilic conditions (Oxoid CampyGen sachet, Thermo Scientific, Basingstoke, UK) and then subcultured once by transferring a 0.1 mL aliquot of the activated broth culture into 10 mL of fresh Bolton broth. After incubation (42°C, 48 h, microaerophilic environment), cultures of the six *C. jejuni* strains were combined, and cells were harvested by centrifugation (6,000 × g, 15 min, 25°C; Sorvall Legend X1R centrifuge, Thermo Scientific, Waltham, MA). Following centrifugation, supernatant was decanted, and the cell pellet was washed with 10 mL of phosphate-buffered saline (PBS; pH 7.4; Sigma-Aldrich, St. Louis, MO). The cell suspension in PBS was centrifuged again (6,000 × g, 15 min, 25°C), and the supernatant again decanted. This washing step was repeated once more for a total of two cell pellet washes with PBS. The final washed cell pellet

comprised of all six strains was resuspended in 60 mL of PBS. This cell suspension (7 to 8 log CFU/mL concentration) was then diluted 10-fold in PBS, and the diluted inoculum was used to inoculate the chicken wings.

Inoculation of chicken wings

Skin-on whole chicken wings were purchased from a wholesale food distributor. Wings were stored at 2°C and were used for the study within six days of receipt. Two trials (repetitions) of the study, conducted on two separate days, were performed. On the first day of each trial, chicken wings were randomly assigned to one of seven antimicrobial treatments to be applied by immersion or spraying. Before inoculation, the wing tip of each wing was aseptically removed using an ethanol-sterilized knife. For each antimicrobial treatment and application method, six samples were placed on trays lined with alcohol-sterilized aluminum foil and were inoculated under a biological safety cabinet. A 0.1-mL (100 µL) aliquot of the diluted *C. jejuni* inoculum was deposited, with a micropipette, on one side of each wing and then spread over the entire surface with a sterile disposable spreader. After a 10 min bacterial cell attachment period, samples were turned over, using sterile forceps, and inoculated on the second side using the same procedure. The second inoculated side was also left undisturbed for 10 min to allow for inoculum attachment. The target inoculation level was 3 to 4 log CFU/mL of wing rinsate.

Antimicrobial treatment of chicken wings

Inoculated chicken wings were left untreated, to serve as controls, or they were treated by immersion or spray application with water (room temperature), SSS (pH 1.2; Amplon, Zoetis, Florham Park, NJ), formic acid (1.5%; BASF Corporation, Florham Park, NJ), PAA (550 ppm; Kroff, Pittsburgh, PA), PAA (550 ppm) acidified with SSS (pH 1.2), or PAA (550 ppm) acidified

with formic acid (1.5%). Antimicrobial treatment solutions were prepared according to the manufacturers' instructions, and the pH of solutions was measured (Orion Star A200 Series pH meter and pH electrode, Thermo Scientific, Schaumburg, IL). Average pH values of the SSS, formic acid, and PAA solutions were 1.2, 2.9, and 3.2, respectively. For the SSS-, and formic acid-acidified PAA solutions, average pH values were 1.2 and 2.8, respectively. The PAA concentration was verified using a hydrogen peroxide and peracetic acid test kit (LaMotte Company, Chestertown, MD).

For immersion application of the test solutions, inoculated wings were individually immersed for 5 s in 500 mL of the solution in a Whirl-Pak bag (55-oz; Nasco, Modesto, CA). Fresh, unused solution was used to immersion-treat each sample. Spray application of the water and acid treatments was performed using a custom-built spray cabinet (Birko/Chad Equipment, Olathe, KS) fitted with two 0.1-gpm (gallons per minute) floodjet spray nozzles (Spraying Systems Co., Glendale Heights, IL) positioned above the product belt. The inoculated wings were placed on a cutting board on top of the product belt of the cabinet and were sprayed with the test solution at a pressure of 10 to12 psi and a product contact time of 4 s.

Immersion- and spray-treated wings were placed on sterile wire racks for 5 min to allow excess solution to drip off samples before microbial analysis or refrigerated storage. For each of the two trials of the study, three of the six samples per treatment were analyzed for inoculated *C. jejuni* counts following treatment application (0 h analysis), and the three remaining samples were placed in individual 24-oz Whirl-Pak bags (Nasco) and analyzed after a 24 ± 1 h storage period at 4°C.

Microbiological analysis

At each sampling time (0 h and 24 h), untreated (control) and treated samples were analyzed for *C. jejuni* counts. For microbial analysis of 0 h samples, untreated and treated wings were placed in a Whirl-Pak bag (24-oz) containing 150 mL of neutralizing buffered peptone water (nBPW; Acumedia-Neogen, Lansing, MI; USDA-FSIS, 2016b). For the 24 h samples, which were already in Whirl-Pak bags, 150 mL of nBPW was aseptically poured into each bag. Sample bags containing individual wings were vertically shaken by hand with a strong downward force 60 times to recover cells from the wing surface. Rinsates were serially diluted in 0.1% buffered peptone water (Difco, Becton Dickinson and Company, Sparks, MD) and appropriate dilutions were surface-plated, in duplicate, onto pre-warmed (42°C) mCCA plates. Plates were placed into anaerobic containers (AnaeroPack Rectangular Jar) with an appropriate number of microaerophilic environment generating gas packs (AnaeroPack-MicroAero), per manufacturer instructions, and were incubated at 42°C for 48 ± 1 h. Three uninoculated and untreated chicken wings also were analyzed on each of the inoculation and treatment application days, for levels of the natural microflora (on Tryptic Soy Agar; Acumedia-Neogen), and for any naturally present *Campylobacter* populations (on mCCA) on the chicken wings used in the study. The detection limit of the microbiological analysis was 1 CFU/mL.

Statistical analysis

The study was designed as a 7 (antimicrobial treatments) × 2 (sampling times) factorial for each solution application method (immersion, spraying), blocked by trial day. It was repeated on two separate days, and three samples were analyzed per treatment and sampling time (0 h and 24 h) in each trial (i.e., a total of six samples per treatment and sampling time). For each solution

application method, recovered *C. jejuni* counts were analyzed within and across the two sampling times (0 h, 24 h). Bacterial populations were expressed as least squares means for log CFU/mL of sample rinsate solution under the assumption of a log-normal distribution of plate counts. Data were analyzed using the CRAN-R package (Lenth, 2018) in R (version 3.5.1). All differences are reported using a significance level of $\alpha = 0.05$.

Results and Discussion

Uninoculated and untreated chicken wings

Naturally-occurring *Campylobacter* populations were not detected (i.e., below the detection limit of 1 CFU/mL) in five of the six uninoculated and untreated chicken wings analyzed. The remaining sample had a *Campylobacter* count of 1 CFU/mL, which was much lower than the *C. jejuni* counts recovered from any of the inoculated samples (Tables 3.2 and 3.3). As such, bacterial counts recovered with the mCCA culture medium from inoculated control (untreated) and immersion- or spray-treated samples (Tables 3.2 and 3.3) were those of the inoculated pathogen. Aerobic plate counts of the uninoculated and untreated wings used for the study ranged from 2.6 to 4.3 log CFU/mL, with a mean of 3.6 ± 0.7 log CFU/mL.

Inoculated untreated chicken wings

Immersion and spray application methods of the test solutions were evaluated on the same experiment day; therefore, the same set of untreated controls were used for both application methods (Tables 3.2 and 3.3). The inoculation level of *C. jejuni* on the wings following the inoculation procedure, as determined by microbial analysis of inoculated untreated samples, was 3.9 log CFU/ml (Tables 3.2 and 3.3). *C. jejuni* counts for untreated wings stored aerobically at

4°C for 24 h were 0.2 log CFU/mL lower than those obtained at the 0-h sampling time (Tables 3.2 and 3.3).

Inoculated chicken wings treated by immersion application of antimicrobial treatments

Inoculated *C. jejuni* populations recovered from untreated and immersion-treated wings immediately after treatment (0 h) and after 24 h of refrigerated (4°C) storage are shown in Table 3.2. Compared to the untreated control, all six immersion treatments effectively ($P < 0.05$) reduced initial (0 h) inoculated (3.9 log CFU/mL) *C. jejuni* populations. Surviving populations after treatment ranged from 3.4 (water; 0.5 log CFU/mL reduction) to 1.7 (PAA, and PAA acidified with SSS; 2.2 log CFU/mL reduction) log CFU/mL. Moreover, counts recovered from wings that had been treated with any of the five tested acid solutions were 1.2 (SSS) to 1.7 (PAA, PAA acidified with SSS) log CFU/mL lower ($P < 0.05$) than the counts of samples that had been treated with water. No ($P \geq 0.05$) differences in efficacy against *C. jejuni* were observed between the SSS, formic acid, and formic acid-acidified PAA treatments. Additionally, formic acid, PAA, and the two acidified PAA treatments were equally ($P \geq 0.05$) effective against *C. jejuni*, reducing initial inoculated populations by 1.8 (formic acid) to 2.2 (PAA, and PAA acidified with SSS) log CFU/mL.

Within each immersion treatment, pathogen counts of samples analyzed after the refrigerated storage period were similar (water, PAA; $P \geq 0.05$) or lower (SSS, formic acid, PAA acidified with SSS, PAA acidified with formic acid; $P < 0.05$) than the counts of corresponding 0-h samples (Table 3.2). More specifically, at the 24 h sampling time, pathogen counts of wings that had been treated with SSS, formic acid, PAA acidified with SSS or PAA acidified with formic acid were 0.6, 0.9, 0.8, and >1.2 log CFU/mL lower ($P < 0.05$), respectively, than those

obtained for corresponding treatments at the 0-h sampling time. Additionally, *C. jejuni* counts for wings that had been treated with SSS-acidified PAA or formic acid-acidified PAA and stored for 24 h were lower (by 0.5 and >0.8 log CFU/mL, respectively; $P < 0.05$) than the counts of PAA-treated samples stored for 24 h.

Peroxyacetic acid is an extensively used antimicrobial intervention treatment in the poultry industry, and several research studies (Bauermeister et al., 2008; Nagel et al., 2013; Chen et al., 2014; Purnell et al., 2014; Kim et al., 2017; Ramirez-Hernandez et al., 2018; Kumar et al., 2020) have reported on its antimicrobial effects against bacterial pathogen contamination on poultry products. To the best of our knowledge, however, this is the first report on the antimicrobial effects of an acidified PAA product against *C. jejuni* on poultry. With regards to previously conducted studies with PAA, Nagel et al. (2013) evaluated it as a post-chill immersion treatment of whole, chilled poultry carcasses. In this study (Nagel et al., 2013), carcasses inoculated (approximately 5 log CFU/mL inoculation level) on the breast portion with *C. jejuni* or *Salmonella* Typhimurium were immersed for 20 s (1,453 L; 4 rpm) in a post-chill dip tank containing 400 ppm or 1,000 ppm PAA. The investigators reported *C. jejuni* reductions of 1.93 and 2.03 log CFU/mL for carcasses subjected to the 400 ppm or 1,000 ppm PAA treatment, respectively, and similar reductions were obtained for the *Salmonella* Typhimurium inoculum (2.02 and 2.14 log CFU/mL, respectively) (Nagel et al., 2013). Based on their findings, the authors suggested that the optimal concentration of PAA in a post-chill immersion tank for reduction of *Campylobacter* and *Salmonella* contamination is less than 1,000 ppm and around 400 ppm (Nagel et al., 2013). In another study (Kim et al., 2017), PAA (750 ppm) and SSS (pH 1.4) were evaluated as post-chilling immersion (15 s) treatments for reduction of naturally-occurring *Campylobacter* populations on whole chicken carcasses. Carcass rinsate samples

collected before the immersion treatments served as a control (Kim et al., 2017). Counts of *Campylobacter* recovered from PAA- and SSS-treated carcasses were 2.2 and 1.5 log CFU/chicken rinsate lower, respectively, than counts obtained for the control samples (Kim et al., 2017).

Antimicrobial efficacy of PAA (700 ppm) and SSS (pH 1.1) against *Salmonella* inoculated on chilled chicken wings was investigated by Scott et al. (2015). Untreated and immersion-treated (20 s) samples were analyzed for *Salmonella* counts immediately after treatment and after 24 h of aerobic storage at 4°C (Scott et al., 2015). Immediately after treatment, pathogen counts of samples treated with PAA or SSS were 1.5 and 1.2 log CFU/mL of rinse solution lower, respectively, than counts of untreated samples (Scott et al., 2015). Counts of SSS-treated wings after 24 h of refrigerated storage were 0.6 CFU/mL lower ($P < 0.05$) than those obtained immediately after treatment (0 h). In contrast, *Salmonella* counts recovered from PAA-treated samples after storage were not ($P \geq 0.05$) different from those of corresponding 0-h samples (Scott et al., 2015).

Published studies on use of formic acid as a decontamination treatment for poultry are limited. Riedel et al. (2009) evaluated the antimicrobial efficacy of 2% formic acid, applied as an immersion treatment, against *C. jejuni* inoculated on chicken skin. Inoculated populations were reduced by 1.6 log CFU/mL following a 1-min exposure time to the treatment. After 24 h of storage at 5°C, counts of treated samples were >2.6 log CFU/mL (*C. jejuni* was not detected in chicken skin rinses) lower ($P < 0.05$) than counts obtained immediately after treatment application (0-h sampling time) (Riedel et al., 2009).

Inoculated chicken wings treated by spray application of antimicrobial treatments

In the current study, inoculated *C. jejuni* counts for untreated and spray-treated wings immediately after treatment (0 h) and after storage at 4°C for 24 h are shown in Table 3.3. All six spray treatments tested effectively ($P < 0.05$) lowered initial (0 h) inoculated (3.9 log CFU/mL) pathogen populations. Overall, at the 0-h sampling time, surviving counts on treated samples ranged from 3.6 (water; 0.3 log CFU/mL reduction) to 2.7 (PAA acidified with formic acid; 1.2 log CFU/mL reduction) log CFU/mL. No ($P \geq 0.05$) differences in efficacy against the inoculated pathogen were noted between the water treatment and SSS treatment. Furthermore, formic acid and PAA were equally ($P \geq 0.05$) effective, reducing ($P < 0.05$) inoculated populations by 0.7 and 0.9 log CFU/mL, respectively. Spray treatment of wings with PAA acidified with formic acid or PAA acidified with SSS were found to be more ($P < 0.05$) effective than treating the wings with formic acid or SSS alone. No ($P \geq 0.05$) differences in antimicrobial efficacy were obtained between PAA on its own and the two acidified PAA treatments (i.e., PAA acidified with SSS or formic acid).

Following storage at 4°C, *C. jejuni* counts for wings that had been spray-treated with water, SSS, or PAA were similar ($P \geq 0.05$) to the counts obtained for corresponding 0-h samples (Table 3.3). For wings treated with formic acid, SSS-acidified PAA, or formic acid-acidified PAA, pathogen counts obtained after 24 h of refrigerated storage were 0.2, 0.4, and 0.6 log CFU/mL lower ($P < 0.05$), respectively, than the counts obtained immediately after treatment (0 h). Furthermore, counts recovered from wings that were spray-treated with SSS-acidified PAA or formic acid-acidified PAA and stored for 24 h were lower (by 0.4 and 0.7 log CFU/mL, respectively; $P < 0.05$) than those recovered from stored samples that had been spray-treated with the non-acidified PAA treatment.

Studies reporting on the use of spray application of chemical treatments for decontamination of chilled poultry carcasses or parts are limited. Ramirez-Hernandez et al. (2018) inoculated skin-on chicken thighs with *Salmonella* using an immersion inoculation procedure. Samples were then spray-treated with PAA (200 ppm or 400 ppm; at 21°C) for 15 s in a commercially equivalent spray cabinet (20 psi, 2.7 gpm flow rate). Treatments were applied to both sides of the chicken thighs with nozzles located above and below the conveyor belt (Ramirez-Hernandez et al., 2018). The authors reported that, regardless of concentration level, *Salmonella* counts of PAA-treated samples were not ($P \geq 0.05$) different from counts obtained for a water control treatment applied in the same manner (Ramirez-Hernandez et al., 2018). A potential reason for the ineffectiveness of PAA in this study could be the immersion inoculation procedure used to inoculate the chicken thighs.

Overall, in the current study, all five acid treatments evaluated were effective ($P < 0.05$) at reducing inoculated *C. jejuni* populations on chicken wings, regardless of the antimicrobial treatment application method. Spray application of the acid treatments resulted in immediate (0-h sampling time) pathogen reductions ranging from 0.5 to 1.2 log CFU/mL, whereas their application by immersion resulted in reductions ranging from 1.7 to 2.2 log CFU/mL. Smith et al. (2015) also used immersion and spraying for evaluating the antimicrobial effect of 200 ppm PAA against *C. jejuni* inoculated on chicken carcasses. Inoculated carcasses were either immersed for 60 s in 6.05 L of solution, or, they were sprayed with 460 mL of solution (62 s) using a 2-gallon garden sprayer (Smith et al., 2015). *C. jejuni* populations were reduced from 5.6 to 4.2 log CFU/mL (1.4 log CFU/mL reduction) with the immersion application method and from 5.4 to 4.8 (0.6 log CFU/mL reduction) with the spray application procedure (Smith et al., 2015). In a similar study (Kumar et al., 2020), the efficacy of PAA (500 and 1,000 ppm)

immersion and spray treatments were evaluated against inoculated *Campylobacter coli* on chicken breasts. Skinless chicken breast fillets were inoculated (4.94 log CFU/mL) and then either immersed for 4 s in 3.5 L of PAA, or, they were sprayed for 5 s (15 mL/s) on each side of the sample (Kumar et al., 2020). Populations of *C. coli* were reduced by 0.85 (500 ppm) and 0.89 (1,000 ppm) log CFU/mL when the treatments were applied by immersion, and 0.78 (500 ppm) and 1.43 (1,000 ppm) log CFU/mL with the spray application method (Kumar et al., 2020). Therefore, depending on the concentration of PAA tested, some differences were noted between the two application methods.

In this study, PAA and acidified PAA treatments (PAA acidified with SSS or formic acid) were equally ($P \geq 0.05$) effective at reducing initial *C. jejuni* populations, regardless of treatment application method. However, after refrigerated storage (4°C, 24 h) of samples, pathogen counts for wings that had been treated with SSS- or formic acid-acidified PAA were lower ($P < 0.05$) than those of wings treated with the non-acidified PAA treatment. When treating samples with acidified PAA, regardless of the acidifier (i.e., SSS or formic acid), two mechanisms of action are used. PAA is an oxidizing agent that disrupts bacterial cell walls and essential enzyme functions (Imlay, 2003; Kitis, 2004). Formic acid and SSS cause cytoplasmic acidification, which results in accumulation of protons that leads to the cell using its energy to try to re-establish the intracellular pH (Stratford et al., 2009; FDA, 2012b; Mani-López et al., 2012). Therefore, use of this treatment, comprised of different modes of action, likely caused sublethal injury to cells, from which they were unable to recover under subsequent refrigerated, aerobic storage conditions.

Table 3.1: *Campylobacter jejuni* strains used in the study.

Strain ID	Origen	Source
FSIS21822450	Chicken leg drumsticks	USDA-FSIS[a]
FSIS21822588	Chicken leg drumsticks	USDA-FSIS
FSIS11815850	Ground chicken	USDA-FSIS
CVM N55886	Chicken wings	FDA-CVM[b]
CVM N56299	Chicken wings	FDA-CVM
CVM N16C024	Chicken breast	FDA-CVM

[a] Dr. Glenn Tillman, U.S. Department of Agriculture's Food Safety and Inspection Service, Office of Public Science, Eastern Laboratory, Microbiology Characterization Branch, Athens, GA.

[b] Dr. Shaohua Zhao, U.S. Food and Drug Administration, Center for Veterinary Medicine, Laurel, MD.

Table 3.2: Mean (n = 6) *Campylobacter jejuni* counts (log CFU/mL ± standard deviation [SD]) for inoculated (six-strain mixture; 3 to 4 log CFU/mL) chicken wings that were left untreated (control) or were immersion-treated (5 s, 500 mL of treatment solution per sample) with various treatment solutions. Samples were analyzed for inoculated *C. jejuni* populations after treatment (0 h) as well as after a 24 h storage period at 4°C.

Treatment	**Mean *C. jejuni* counts (log CFU/mL ± SD)**	
	0 h	**24 h**
Untreated (control)	$3.9 \pm 0.1^{a\text{-}z}$	$3.7 \pm 0.3^{a\text{-}z}$
Water	$3.4 \pm 0.1^{b\text{-}z}$	$3.2 \pm 0.2^{b\text{-}z}$
SSS (pH 1.2)	$2.2 \pm 0.1^{c\text{-}z}$	$1.6 \pm 0.2^{c\text{-}y}$
Formic acid (1.5%)	$2.1 \pm 0.2^{cd\text{-}z}$	$1.2 \pm 0.1^{cd\text{-}y}$
PAA (550 ppm)	$1.7 \pm 0.3^{d\text{-}z}$	$1.4 \pm 0.4^{c\text{-}z}$
PAA (550 ppm) acidified with SSS (pH 1.2)	$1.7 \pm 0.3^{d\text{-}z}$	$0.9 \pm 0.2^{de\text{-}y}$
PAA (550 ppm) acidified with formic acid (1.5%)	$1.8 \pm 0.2^{cd\text{-}z}$	$< 0.6 \pm 0.5^{f\text{-}y\ *}$

SSS: sulfuric acid and sodium sulfate blend; PAA: peroxyacetic acid

$^{a\text{-}f}$ Least squares means in the same column without a common superscript letter are different ($P < 0.05$).

$^{y\text{-}z}$ Least squares means in the same row without a common superscript letter are different ($P < 0.05$).

* One of the six samples analyzed had a *C. jejuni* count that was below the microbial analysis detection limit of 1 CFU/mL; therefore, the mean is reported as < (less than) the mean.

Table 3.3: Mean (n = 6) *Campylobacter jejuni* counts (log CFU/mL ± standard deviation [SD]) for inoculated (six-strain mixture; 3 to 4 log CFU/mL) chicken wings that were left untreated (control) or were spray-treated (4 s, 10 to 12 psi) with various treatment solutions. Samples were analyzed for inoculated *C. jejuni* populations after treatment (0 h) as well as after a 24 h storage period at 4°C.

Treatment	Mean *C. jejuni* counts (log CFU/mL ± SD)	
	0 h	**24 h**
Untreated (control)	$3.9 \pm 0.1^{a\text{-}z}$	$3.7 \pm 0.3^{a\text{-}y}$
Water	$3.6 \pm 0.1^{b\text{-}z}$	$3.5 \pm 0.2^{ab\text{-}z}$
SSS (pH 1.2)	$3.4 \pm 0.2^{bc\text{-}z}$	$3.3 \pm 0.2^{bc\text{-}z}$
Formic acid (1.5%)	$3.2 \pm 0.2^{cd\text{-}z}$	$3.0 \pm 0.2^{cd\text{-}y}$
PAA (550 ppm)	$3.0 \pm 0.2^{de\text{-}z}$	$2.8 \pm 0.2^{d\text{-}z}$
PAA (550 ppm) acidified with SSS (pH 1.2)	$2.8 \pm 0.1^{e\text{-}z}$	$2.4 \pm 0.5^{e\text{-}y}$
PAA (550 ppm) acidified with formic acid (1.5%)	$2.7 \pm 0.1^{e\text{-}z}$	$2.1 \pm 0.4^{e\text{-}y}$

SSS: sulfuric acid and sodium sulfate blend; PAA: peroxyacetic acid.

$^{a\text{-}e}$ Least squares means in the same column without a common superscript letter are different ($P < 0.05$).

$^{y\text{-}z}$ Least squares means in the same row without a common superscript letter are different ($P < 0.05$).

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