Validation of commercial antimicrobial intervention technologies to control *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC) on pre-rigor, skin-on market hog carcasses and chilled pork wholesale cuts

by

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B.S., Pan-American School of Agriculture, 2017

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science
Animal Sciences & Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

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Abstract

Even though swine-associated foodborne disease outbreaks have been reported less frequently than in other meat products, the potential that swine represent a source of *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC) infections in human beings cannot be disregarded. The control of pathogens using antimicrobial technologies during processing is of increasing interest by pork processors. The first study compared eight carcass antimicrobial washes or sprays, and six chilled subprimal/trim sprays, applied using commercial equipment and parameters, to quantify their ability to reduce *Salmonella* contamination in raw pork. Hogs were harvested to provide skin-on carcasses, and eight sides (per replication) were inoculated with a *Salmonella* cocktail (ca. 5 log CFU/cm^2). Each side was treated in a commercial Chad cabinet using a spray [low-volume: 3% lactic acid (*l*LA), 400 ppm peracetic acid (*l*PAA), or acidified 400 ppm peracetic acid (*l*aPAA)] or wash [high volume: ambient water (*h*AW), 400 ppm PAA (*h*PAA), 400 or 600 ppm hypobromous acid (*h*DBDMH), or 71°C water (*h*HW)] treatment within a randomized complete block study design. Post-treatment *Salmonella* reductions were compared. Chilled subprimals and trim from each side were inoculated and treated with antimicrobial sprays [AW, 400 ppm PAA, 400 ppm aPAA, 400 and 600 ppm DBDMH, or 2% LA] in a subprimal spray cabinet or ribbon mixer (trim). Reductions were determined over 14 days of vacuum packaged subprimal storage and four days of trim storage, along with lactic acid bacterial populations, TBARS and color determinations on non-inoculated products. For the exterior skin-on carcass surface treatments, *h*PAA, *h*DBDMH$_{600}$, and *h*HW resulted in larger (P≤0.05) *Salmonella* reductions (2.8 to 3.1 log CFU/cm^2) compared to AW (1.5 log CFU/cm^2). *Salmonella* was reduced by 0.4-0.6 and 0.1-0.3 logs on subprimals and trim, respectively (P>0.05). *Salmonella* control was less evident at the chilled subprimal and trim stages of processing. None of the treatments had a negative impact on the subprimal quality factors evaluated. The second study compared the same carcass antimicrobial intervention technologies

to quantify their ability to reduce STEC contamination on market hog carcasses. Hogs were harvested to provide skin-on carcass sides, and eight sides (per three replications) were inoculated with a 7-strain STEC cocktail (ca. 5 log CFU/cm^2). Each side was randomly assigned to a final pre-chill wash treatment administered in a commercial Chad carcass cabinet within a randomized complete block study design. Post-treatment and post-chilling STEC reductions were compared. Post-treatment color changes were determined on lean, adipose, and skin carcass surfaces before and after chilling. The *h*HW, *h*PAA, and *h*DBDMH$_{600}$ deluge washes achieved the greatest external surface STEC reductions (3.8, 3.4, and 3.2 log CFU/cm^2, respectively), and were significantly ($P \leq 0.05$) more effective than the other intervention technologies, including the 1.7-log reduction achieved by the ambient water control. The carcass washes and sprays were less effective at reducing STEC populations attached to interior body cavity smooth lean tissue. None of the treatments negatively impacted instrumental carcass color. All pre-rigor carcass interventions provided beneficial *Salmonella* and STEC reductions, allowing processors flexibility in their operations.

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Acknowledgements

I want to thank my advisors, Dr. Sara Gragg and Dr. Randall Phebus, for allowing me to work on such a great project and pursue my degree at Kansas State University. I also would like to thank them for all their support in anything I could ever need during my time here at K-State. I also want to thank my committee member Dr. Valentina Trinetta for her guidance and assistance in the study.

I also want to thank the biosafety, the project management, and the engineering staff at the K-State Biosecurity Research Institute for all their support in the study. A special note of gratitude to Mr. John Wolf, K-State Meat Lab Manager, for his assistance in swine harvesting and carcass fabrication activities under strict biocontainment protocols. Additionally, the undergraduate and graduate student workers in the K-State Food Safety and Defense Laboratory, Daniel Vega, Francisco Najar-Villarreal, Josh Maher, Kellen Habib, Nick Sevart, Brock Brethour, Yashoda Subedi, Mackenzie Mayo, Olivia Harrison, April Molitor, Gabriela Magossi Boccato, Brittany Ostmeyer, Krysteena Redman, Erika Kringen, and Kellen Marcum for assisting in project preparation and sample analyses.

Finally, I would like to thank my family, my parents Jorge Pozuelo and Cecilia Bonilla, and my brother Jorge Pozuelo, who have always supported me in everything I do. I also want to thank my boyfriend and my friends who have supported me and help me in many ways.

Chapter 1 - Introduction and research questions

Salmonella spp. and Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens of concern for public health due to the severity of the illness they can cause and their ability to cause large outbreaks. Food-producing animals such as chickens, turkeys, pigs, and cattle are the most common reservoirs for many foodborne pathogens including *Salmonella* and STEC (Heredia and García, 2018). There are more than 2,500 serovars described for *Salmonella*, however, less than 100 account for most human infections (Centers for Disease Control and Prevention, 2020). *Salmonella* causes three major diseases in humans: non-invasive non-typhoidal salmonellosis, invasive non-typhoidal salmonellosis, and typhoid fever (Kurtz et al., 2017). Each year, according to the Centers for Disease Control and prevention (2020), *Salmonella* is estimated to cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States of America (U.S.). Hogs are among the main animal species that contribute to human salmonellosis transmission (Bonardi, 2017).

On the other hand, STEC are estimated to cause more than 265,000 illnesses each year with approximately 3,600 hospitalizations and 30 deaths in the U.S. (Centers for Disease Control and Prevention, 2016). Even though these numbers are smaller compared to *Salmonella*, STEC infections have a great impact on public health because they can cause severe clinical diseases, that can lead to acute renal failure and even death, such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Tseng et al., 2014). *E. coli* O157:H7 is the most widely recognized due to the severity of the foodborne illness it causes. However, non-O157 STEC serogroups have also been shown to cause foodborne illnesses, and both O157 and the "big six" (O26, O45, O103, O111, O121, and O145) STEC serogroups are consider by the Food Safety and Inspection Service (FSIS)

as adulterants in non-intact beef and ground beef products (Food Safety and Inspection Service, 2011). Cattle are regarded as the natural reservoir of STEC; however, other animals such as pigs, can also harbor this pathogen (Persad and Lejeune, 2014). In the U.S., the prevalence of *E. coli* O157:H7 in domestic swine is less than 2 %; however, swine have been shown to harbor and shed STEC for up to 2 months (Persad and Lejeune, 2014). Nevertheless, there is still potential for human infection regardless of the low prevalence of pathogenic STEC serogroups from domestic swine (Persad and Lejeune, 2014).

Cross-contamination can happen at various steps in pork carcass processing; however, the evisceration process has the greatest potential for microbial contamination (Dickson et al., 2019). It has been demonstrated that a final antimicrobial wash has the potential to reduce bacterial populations on animal carcasses (Dickson et al., 2019). Organic acids are commonly used as antimicrobial treatments for the decontamination of animal carcasses (Sohaib et al., 2016). The efficacy of organic acid spray washes is determined principally by water pressure, water temperature, chemicals concentration and exposure time (Sohaib et al., 2016). Some studies have investigated the effectiveness of different antimicrobial interventions on beef carcasses to control different foodborne pathogens. Those interventions included spray carcass washes consisting of hot water, lactic acid, citric acid, acetic acid, acidified sodium chlorite, peracetic acid, sodium hypochlorite, chlorine dioxide, and aqueous ozone (Kocharunchitt et al., 2020; Signorini et al., 2018; Yoder et al., 2012).

However, research on these interventions' effectiveness on pork carcasses and chilled pork is limited (Eastwood et al., 2020). Therefore, the main objectives reported in this thesis were (i) to validate the efficacy of selected USDA-approved antimicrobial interventions applied as high-volume washes (peracetic acid, hypobromous acid, ambient water, and 160°F hot water) or low-

volume sprays (acidified peracetic acid, lactic acid, and peracetic acid) to reduce *Salmonella* and STEC populations on pre-rigor skin-on pork carcass sides, (ii) to investigate the effectiveness of the selected antimicrobial sprays to reduce *Salmonella* populations on vacuum-packaged pork subprimals and fresh pork trimmings, and (iii) quantify treatment impacts on quality and shelf-life during storage of fresh pork wholesale cuts.

Research questions

1. Are there significant differences in mean reductions of *Salmonella* and STEC populations achieved by the application of interventions on pre-rigor pork carcasses compared to the control (ambient water)?
2. Which intervention is the most effective at reducing *Salmonella* and STEC on pre-rigor pork carcasses?
3. Are there significant differences in mean reductions of *Salmonella* populations achieved by the application of interventions on vacuum-packaged pork subprimal and fresh pork trimming compared to the control (ambient water)?
4. Are meat quality characteristics (microbial spoilage, color, and lipid oxidation) negatively affected by the application of interventions on pre-rigor pork carcasses, vacuum-packaged pork subprimals, and fresh pork trimmings?
5. Do *Salmonella* and STEC population levels differ from inoculation to the time when the carcass is removed from the cooler for fabrication for each intervention (how do microbial populations subjected to the pre-rigor final wash interventions change during treatment and chilling)?
6. Are there any significant differences in total mean reductions of *Salmonella* and STEC

populations on pre-rigor pork carcasses at different anatomical locations (top, middle, and bottom) of the carcass sides after antimicrobial applications, and after 18 hours of carcass chilling at 2 °C?

7. Are there any significant differences in total mean reductions of *Salmonella* and STEC populations on pre-rigor pork carcasses between the exterior skin-on surface (top, middle, and bottom) and the lean tissue of the internal cavity?

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Chapter 2 - Literature Review

2.1 *Salmonella* background information

Salmonella was originally discovered and isolated in 1855 by Theobald Smith from swine intestines infected with swine fever and it was named after Dr. Daniel Elmer Salmon (Eng et al., 2015). *Salmonella* is a Gram-negative, facultative anaerobe that is oxidase negative, catalase positive, and a non-spore forming rod member of the *Enterobacteriaceae* family (Ryan et al., 2017). The nomenclature for this pathogen is complex and it has evolved from the first model suggested by Kauffmann, where each serovar was considered a separate species, to the current nomenclature system used by the WHO, ASM, and CDC as approved in 2005 (Ryan et al., 2017).

It was not until 1973 that a development in *Salmonella* taxonomy occurred, when it was demonstrated by DNA-DNA hybridization that all serovar and subgenera were related at the species level (Brenner et al., 2000). In the current nomenclature system, the *Salmonella* genus is split into two species: *Salmonella enterica* and *Salmonella bongori* (Ryan et al., 2017). *Salmonella enterica* is subdivided into six subspecies which are referred to by a Roman numeral and name: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indic*a (Centers for Disease Control and Prevention, 2011).

The serotype or serovar can indicate both the antigen formula of the subspecies and the formal names assigned to isolates of *S. enterica* subsp. *enterica* (subspecies I); where most of the names are usually based upon the geographic region where the bacterium was first isolated (Ryan et al., 2017). Conversely, the remaining *S. enterica* subspecies and *S. bongori* serovar names are designated by the antigenic formula, which includes the subspecies designation (in Roman letters)

and the antigenic formulae as follows: O (somatic), Vi (if present), H (flagellar) antigens (phase 1), and H antigens (phase 2, if present) (Ryan et al., 2017).

Salmonella serovars show variations in their pathogenicity and can cause illnesses to a particular host based on their virulence genes (Campbell-Platt, 2011). *S.* Typhi and *S.* Paratyphi A, B, and C are considered human-adapted (primarily infect humans) serovars, which can cause diseases in humans after the ingestion of viable bacterial cells. These serovars can cause high mortality (10-15%) for untreated patients (Campbell-Platt, 2011). Some *Salmonella* serovars can be considered host-adapted, which means they usually cause disease in a limited number of related species (Uzzau et al., 2000). Examples of host-adapted *Salmonella* serovars are *S.* Gallinarum, *S.* Pollorum (poultry); *S.* Choleraesuis (pigs); *S.* Abortus-ovis (sheep) and *S.* Dublin (cattle) (Campbell-Platt, 2011). Other *Salmonella* serovars can be considered unadapted (no host preference), including most of the serovars causing diseases in humans, such as *S.* Typhimurium and *S.* Enteritidis (Campbell-Platt, 2011).

According to the Centers for Disease Control and Prevention (2020), there are more than 2,500 *Salmonella* serovars, however, less than 100 account for most human infections. This pathogen can be transmitted through fecal-oral routes after the consumption of contaminated food and water (Gut et al., 2018). To be fully pathogenic, *Salmonella* must have a complete lipopolysaccharide coat, the ability to invade cells and replicate intracellularly, and the ability to produce toxins (Giannella, 1996).

Moreover, the ability of different *Salmonella* serovars to survive in different host environments depends on several interconnected factors, such as commensal organisms present, the host environment and immune system, and the pathogen genetics (Foley et al., 2013). After ingestion, *Salmonella* colonizes the distal ileum and proximal colon, using its flagella to move and

target the enterocyte cells to adhere to them (Gut et al., 2018). Once attached, the bacteria are phagocytized into the cells (Gut et al., 2018). This process is caused by the expression of type III secretion systems (T3SS) (Gut et al., 2018).

After invasion, most *Salmonella* serovars produce an acute inflammatory response, including the release of pro-inflammatory cytokines (Giannella, 1996). Aside from the invasion of cells, the production of toxins associated with diarrhea and intestinal mucosal surface damage (enterotoxin and cytotoxin) has been identified across all *Salmonella* subspecies (Gut et al., 2018). Salmonellosis, the infection caused by *Salmonella*, has become a major public health concern around the world. According to the Centers for Disease Control and Prevention (2020), in the U.S. this pathogen is estimated to cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths annually.

Salmonella causes three main diseases in humans: non-invasive, non-typhoidal salmonellosis; invasive, non-typhoidal salmonellosis; and typhoid fever (Kurtz et al., 2017). Non-typhoidal salmonellosis, or NTS, refers to any illnesses caused by all serovars except for the serovars Typhi and Paratyphi A-C (Kurtz et al., 2017). Non-invasive, non-typhoidal salmonellosis is described by Kurtz et al. (2017) as an acute enterocolitis accompanied by inflammatory diarrhea with symptoms developing after 6-72 hours following consumption of >50,000 bacteria. Commonly, symptoms will resolve within 5-7 days without treatment; however, oral or intravenous rehydration may be necessary (Kurtz et al., 2017).

Non-typhoidal, invasive salmonellosis or iNTS causes bloodstream infections accompanied by clinical features comparable to other febrile illnesses (Kurtz et al., 2017). The disease symptoms include fever, hepatosplenomegaly, and respiratory symptoms, which if left untreated, can become fatal (Balasubramanian et al., 2019). The majority of the estimated 3.4 million annual infections

and 618,316 deaths related to iNTS occur in Africa, where host risk factors such as HIV infections, malnutrition, and malaria are higher than in other regions (Balasubramanian et al., 2019). Uche et al. (2017) reported that iNTS is mainly caused by *S.* Typhimurium and *S.* Enteritidis, with these serovars being responsible for 91% of iNTS bacteremia in Africa.

According to the World Health Organization (2019), *Salmonella* Typhi is the serovar responsible for causing typhoid fever, which is a systemic infection after the ingestion of contaminated food or water. The acute disease symptoms are often clinically non-distinguishable from febrile illnesses, and include prolonged fever, headache, nausea, loss of appetite, and constipation or diarrhea (World Health Organization, 2019). Typhoid fever causes 21 million cases each year worldwide and an estimated 200,000 deaths, with most cases occurring in developing countries or among travelers to these countries (Kurtz et al., 2017)

2.2 Importance of *Salmonella* research

Salmonella in swine

Food-producing animals such as chickens, turkeys, pigs, and cattle are the most common reservoirs for many foodborne pathogens, including *Salmonella* (Heredia and García, 2018). *Salmonella* transmission in swine occurs mostly via the fecal-oral route; however, the respiratory tract can also be a portal of entry (Rostagno and Callaway, 2012). The first *Salmonella enterica* serovar isolated from pigs was known as Choleraesuis and it was considered to be the cause of swine fever (Demirbilek, 2017).

Pigs can become infected with *Salmonella* and show clinical symptoms or become asymptomatic carriers during the pre-harvest stage (Bonardi, 2017). In pigs, clinical salmonellosis can be separated into two forms: septicemia caused by host limited *S. enterica* serovars (e.g., *S.*

Choleraesuis) and enterocolitis caused by broad host limit serovars (e.g., *S.* Enteritidis) (Demirbilek, 2017). Moreover, according to Rostagno and Callaway (2012), intermittent shedding of *Salmonella* and asymptomatic intestinal carriage are characteristic of most subclinical infected swine herds around the world.

Swine infections with *Salmonella* are of concern for two reasons, the first of which is clinical disease in swine and the second is the broad range of serovars that can be a potential source of human illness (Dickson et al., 2019). It has been shown that the prevalence of *Salmonella* in mesenteric lymph nodes ranges from 7.4% to 26%, moreover, the prevalence in fecal contents ranges from 20% to 30% (Bonardi, 2017). According to Naberhaus et al. (2019), the five serovars most commonly isolated from swine in 2017 were *S.* 4,[5],12:i:-, *S.* Typhimurium, *S.* Derby, *S.* Choleraesuis, and *S.* Infantis.

Salmonella in pork

Pork is the most consumed meat worldwide through a wide variety of products (Baer et al., 2013). The United States is one of the top five annual pork exporters in the world since 2000 (Economic Research Service, 2019). According to the USDA Livestock, Dairy and Poultry Outlook by Hahn (2020), the total pork production expectations for the first half of 2020 were 28.6 billion pounds, which represents an increase of almost 4 percent compared with 2019. Since pork products are widely consumed in the United States and worldwide, ensuring pork supply safety is essential (Baer et al., 2013).

Cross-contamination can occur at various steps during the processing of pork carcasses (Miller et al., 2005). In EU countries, *Salmonella* levels from 0.3% to 17.4% have been detected on pig carcasses (Pala et al., 2019). According to Ferrari et al. (2019), the most prevalent *Salmonella* serovars in pork in Europe, Oceania, Asia, and North America are Typhimurium and

Derby. Moreover, in Africa, the most prevalent serovar is Hadar and in Latin America is Meleagridis (Ferrari et al., 2019).

Hogs are among the main animal species that contribute to human salmonellosis transmission (Bonardi, 2017). In Southern Europe, *Salmonella* source attribution studies estimate that 43.6% of all reported salmonellosis cases are caused by pigs (Bonardi, 2017). Moreover, according to the Centers for Disease Control and Prevention (2017) Surveillance for Foodborne Disease Outbreaks United States Annual Report (data from 2015), pork meat was the second most common source attributed to *Salmonella* outbreaks. Furthermore, pork was the fourth most common source attributed to *Salmonella* outbreak-associated deaths in 2017, with five outbreaks, 55 illnesses, 13 hospitalizations, and one death (Centers for Disease Control and Prevention, 2019).

Public health impact of Salmonella

NTS serovars are estimated to cause approximately 78 million cases of illness, 59,000 deaths, and 4 million Disability-Adjusted Life Year (DALYs) per year globally (Havelaar et al., 2015). According to Havelaar et al. (2015), in the European Region, NTS occupied the first position of DALYs and deaths, causing approximately 107,000 DALYs and 1,854 deaths per year. In addition, NTS represents the third most common cause of foodborne illnesses in Europe, causing around 1.7 million cases of illness annually (Havelaar et al., 2015).

Similarly, according to the Centers for Disease Control and Prevention (2020), in the U.S. *Salmonella* is estimated to cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths annually. Further, NTS were the leading cause of hospitalization and death, representing 35% and 28% from the total cases, respectively, in the U.S. (Scallan et al., 2011). Moreover, in the U.S, *Salmonella* is known to be the second most common cause of outbreaks, accounting for 133 outbreaks and 3,007 illnesses in 2017 (Centers for Disease Control and Prevention, 2019).

Salmonella outbreaks in pork

It has been estimated that the consumption of contaminated pork meat is responsible for 525,000 foodborne infections, 2,900 hospitalizations, and 82 deaths each year in the U.S. (Self et al., 2017). Pork products have also been associated with several outbreaks, according to Self et al. (2017), with 288 outbreaks attributed to pork that resulted in 6,372 illnesses, 443 hospitalizations, and four deaths in the period between 1998-2015. In addition, *Salmonella* was the pathogen most commonly confirmed in pork-related outbreaks (44%) and caused the most outbreak-associated illnesses (35% of illnesses in pork-associated outbreaks) (Scallan et al., 2011).

According to Campos et al. (2019), *Salmonella* Typhimurium is among the major serovars associated with human salmonellosis (ranked second). *Salmonella* Typhimurium is also one the serovars most frequently associated with the pork industry. In a 2015 outbreak, *Salmonella* Typhimurium caused 280 illnesses and one death in North Carolina. The outbreak was linked to pork barbecue and illnesses were reported in six states and 21 North Carolina counties (Self et al., 2017). In 2011, *S.* Typhimurium also caused an outbreak attributed to smoked pork tenderloin in Denmark, with 22 laboratory confirmed cases (Oktawia P Wójcik et al., 2012). The isolation of *S.* Typhimurium is more common from pigs than from pork, which is likely why a lower *Salmonella* prevalence is reported for pork (Bonardi, 2017).

Another serovar of concern is *Salmonella* 4,[5],12:i:-, which was first reported in the 1980s and is a variant of *Salmonella* Typhimurium that lacks the second-phase flagellar antigens (Marin et al., 2020). According to Self et al. (2017), *Salmonella* 4,[5],12:i:- has emerged as the most frequent etiology of pork-associated outbreaks since 1998 and is especially concerning because this pathogen is often multi-drug resistant. In 2013, *Salmonella* I 4,[5],12:i:- caused the largest outbreak associated with pork (333 illnesses), and was linked to cooked pork sausages from a

restaurant in Nevada (Self et al., 2017). This *Salmonella* serovar was again associated with an outbreak in 2015, where a total of 192 cases were reported from five states and approximately 30 individuals were hospitalized, becoming the largest *Salmonella* outbreak reported in Washington (Kawakami et al., 2016).

Salmonella Derby is also one of the most frequent causes of gastroenteritis in humans (Sévellec et al., 2020). According to Sévellec et al. (2020), pigs and poultry are the main reservoirs of this serovar worldwide, which has been found to contaminate 28.5% of European swine. In 2015, *S.* Derby accounted for 22.9% of isolates from the European pork sector (Sévellec et al., 2020) and one of the largest outbreaks caused by this serovar occurred in Germany between November 2013 to January 2014, when 145 people (mainly elderly) were affected with raw pork sausage as the source of infection (Simon et al., 2018). In 2011, *S.* Derby cause an outbreak in Spain associated with the consumption of dried pork sausage (three positive cases) (Arnedo-pena et al., 2015).

Economic impact of Salmonella

Besides the impact that *Salmonella* has on public health, its economic implications worldwide are also a significant concern. Foodborne illnesses are responsible for high societal costs such as reduced productivity, treatment, hospitalization, and death (Van der Gaag, 2004). According to Hoffman (2015), foodborne illnesses cause an estimated economic burden of $15.5 billion annually in the United States. The cost of *Salmonella* related foodborne illnesses was estimated to be $3.7 billion annually, where deaths of people who do not survive the infection account for 89% of the economic burden from this pathogen (Hoffman, 2015). Similarly, according to the European Food Safety Authority (2014), the overall economic burden of human salmonellosis in the European Union is estimated to be about € 3 billion anually.

2.3 Shiga toxin-producing *Escherichia coli* (STEC) background information

Bacterium coli commune was first characterized in 1885 by Theodor Escherich while conducting examinations of neonates' feces and meconium of infants who were breast-fed (Erjavec, 2019). The bacterium was renamed as *Escherichia coli* (*E. coli*) in 1919 by Castellani and Chalmers (Erjavec, 2019). *E. coli* is a Gram-negative, rod-shaped, facultative anaerobic bacterium that belongs to the family *Enterobacteriaceae* (Desmarchelier and Fegan, 2011). A well-known commensal, *E. coli* is among the first bacteria to colonize the gut after birth (Erjavec, 2019). However, some groups of *E. coli* can cause severe diarrheal diseases in humans.

In 1898, Kiyoshi Shiga described *Shigella dysenteriae* type 1 as the agent of epidemic bacterial dysentery (Kaper and O'Brien, 2014). Several studies following that discovery reported that Shigella's bacillus extracts paralyzed and killed rabbits, which led to the discovery of the Shiga toxin (Stx) (Kaper and O'Brien, 2014). Further, in 1977, Konowalchuk reported that verotoxins, which are cytotoxins that kill Vero cells, are produced by certain strains of diarrheagenic *E. coli* (Kaper and O'Brien, 2014). However, after discovering that the *E. coli* O157:H7 strain that caused an outbreak in the U.S. produced a Shiga-like toxin (Stx) that was the same as the Verotoxin (Vtx) described in 1977, the routes of research on Stxs and Vtxs fused in 1983 (Kaper and O'Brien, 2014). Thus, the production of Vtx or Stx are considered synonyms for the same group of toxins, either referring to their toxicity for Vero cells or to their similarities with the *Shigella dysenteriae* type 1 Shiga toxin (Piérard et al., 2012).

STEC serogroups produce two types of Stxs, Shiga toxin type 1 (Stx1) and Shiga toxin type 2 (Stx2) (Terajima et al., 2017). According to epidemiological evidence, Hemolytic Uremic Syndrome (HUS) is more frequently associated with strains of STEC O157:H7 producing Stx2 than strains producing Stx1 (Terajima et al., 2017). A new category of virulent

"enterohemorrhagic" *E. coli* (EHEC) was proposed after the discovery of the ability of STEC serogroups to induce attaching and effacing (A/E) lesions, along with the production of Stxs, and a characteristic large plasmid encoding EHEC hemolysin (Ehx) (Kaper and O'Brien, 2014).

The most recognized pathogenic groups are divided into enterotoxigenic E. coli (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive E. coli (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (U.S. Food and Drug Administration, 2019). However, EHEC is the group that is often implicated in major foodborne outbreaks worldwide (U.S. Food and Drug Administration, 2019). *E. coli* O157:H7 is the most widely recognized from the EHEC group due to the severity of the foodborne illness it causes (Amezquita-Lopez et al., 2018). However, non-O157 STEC serogroups have also been shown to cause foodborne illnesses (Food Safety and Inspection Services, 2011b). Both O157 and the "big six" (O26, O45, O103, O111, O121, and O145) STEC serogroups are considered by the Food Safety and Inspection Service (FSIS) as adulterants in raw non-intact beef and ground beef products (Food Safety and Inspection Services, 2011b).

STEC can also be classified by immunological serotyping, which is based on combinations of 174 somatic surface antigens (O-antigens) and 53 flagellum (H-antigens) (Amezquita-Lopez et al., 2018). More than 400 different STEC serogroups have been isolated and have been further classified into five seropathotypes (A through E), based on their association with human diseases, outbreaks, and HUS (Scheutz, 2014). Seropathotype A is considered the most virulent, followed by B, similar to Seropathotype A, but diseases and outbreaks occur at a lower frequency (Scheutz, 2014). Seropathotype C serogroups are associated with outbreaks but not often implicated with HUS (Amezquita-Lopez et al., 2018). Serogroups belonging to seropathotype D are involved in

sporadic cases of diarrhea, and seropathotype E is composed of serogroups that have not been implicated in human diseases (Amezquita-Lopez et al., 2018; Scheutz, 2014).

Human exposure to *E. coli* O157 is usually associated with the consumption of undercooked ground beef or another feces-contaminated foodstuff (Ho et al., 2013), such as leafy green produce and non-pasteurized juices. The infectious dose of *E. coli* O157:H7 is very low, as it is estimated to be less than 100 organisms (<10–100 cells) (Kuruwita et al., 2020). Some individuals can be asymptomatic, and others can develop severe cases of infection characterized by abdominal cramps and bloody diarrhea (hemorrhagic colitis) within three days after the ingestion of contaminated food (Ho et al., 2013). Children (<5 years old), the elderly, and immune-compromised persons typically develop the most severe cases of infection (Ho et al., 2013).

In severe cases of *E. coli* O157:H7 infection, individuals can present hemolytic uremic syndrome (HUS), especially in young children, where the illness is the leading cause of pediatric acute renal failure (Petruzziello-Pellegrini and Marsden, 2012). This complication develops in approximately 10% of cases and with a 25% mortality rate (Petruzziello-Pellegrini and Marsden, 2012). EHEC-associated HUS typically presents as thrombocytopenia, hemolytic anemia, and acute renal failure, which develops within 2-12 days after the first appearance of diarrhea (Karpman et al., 2017). Moreover, besides the life-threatening illness that HUS can cause, it can also become a lifelong chronic disease (Ameer et al., 202).

2.4 Importance of STEC research

STEC in swine

Swine can be colonized with various STEC serogroups, including O157, and as in humans, can be highly pathogenic to pigs (Persad and Lejeune, 2014). Different from ruminants, pigs

possess Stx-positive vascular receptors and can develop edema disease after intestinal colonization with a STEC strain (Persad and Lejeune, 2014). The edema disease is more frequently associated with the STEC serogroups O138, O139, O141, and O147 (Tseng et al., 2014). This disease usually affects healthy animals after weaning and is characterized by swollen eyelids, a typical snoring sound, neurologic signs, and subcutaneous and submucosal edema in several tissues (Arancia et al., 2019). According to Arancia et al. (2019), the occurrence of this disease in swine depends on several variables and has a lethality rate from 50% to 90% with frequent relapses.

In the U.S., there are approximately five million feral swine, which have also been associated with human STEC diseases (Persad and Lejeune, 2014). In 2007, feral swine were first identified as a reservoir for STEC O157:H7 in California, where they were isolated from 14.9% of swine specimens tested (Persad and Lejeune, 2014). These isolates were identical to an isolate obtained from an outbreak associated with spinach (Persad and Lejeune, 2014). Identifying STEC from feral swine indicates that swine play a role in the epidemiology of STEC infections; they can contaminate vegetables and act as vectors for STEC between livestock (Persad and Lejeune, 2014).

STEC in pork

According to Bardasi et al. (2017), the lower intestinal tract of ruminants is considered the primary natural reservoir for STEC. Therefore, most human infections occur primarily by ingestion of contaminated food of bovine origin (Bardasi et al., 2017). However, some outbreaks have been associated with the consumption of other food products, including pork, contaminated with STEC (Bardasi et al., 2017). According to Persad and Lejeune (2014), it is still unknown whether the contamination of pork products occurs during swine processing or via cross-contamination from other food products contaminated with the pathogen. In the U.S., the prevalence of STEC O157:H7 in domestic swine is less than 2%; however, swine have been shown

to harbor and shed STEC for up to 2 months (Persad and Lejeune, 2014). Nevertheless, there is still potential for human infection regardless of the low prevalence rate of pathogenic STEC serogroups from domestic swine (Persad and Lejeune, 2014).

However, there is limited information about STEC occurrence in other domestic animals besides cattle (Ercoli et al., 2015). Some epidemiologic studies have been performed in different countries to determine the prevalence of STEC in the swine population, the presence of STEC in pork products, and the incidence of STEC outbreaks associated with pork; however, the data between these studies are not directly comparable due to differences in study designs, sampling methods, and STEC detection/isolation protocols (Ercoli et al., 2015). Pork is the most consumed meat worldwide through a wide variety of products; therefore, ensuring pork supply safety is essential (Baer et al., 2013).

Public health impact of STEC

STEC represents a substantial challenge for public health because of the severity of the illness and its ability to cause large outbreaks (Bruyand et al., 2018). According to a World Health Organization (2019) report, STEC causes more than 1 million illnesses, 128 deaths, and nearly 13,000 Disability-Adjusted Life Years (DALYs) per year globally. Furthermore, in the U.S., STEC serogroups are estimated to cause more than 265,000 illnesses, 3,600 hospitalizations and 30 deaths annually (Centers for Disease Control and Prevention, 2016a).

According to Bruyand et al. (2018), STEC infections are the leading cause of HUS in children. It is estimated that the proportion of patients with STEC infections who develop HUS is 5 to 15%; however, this depends on host factors like age, STEC serogroup, and the Shiga toxin (Stx) profile (Bruyand et al., 2018). Most STEC outbreaks and cases of HUS in the U.S. have been

attributed to *E. coli* O157:H7, being responsible for about 36% of all STEC infections annually (Centers for Disease Control and Prevention, 2016a).

STEC outbreaks in pork

According to Ho et al. (2013), STEC outbreaks can be attributed to their low infectious dose (<100 organisms) and high transmissibility. However, pork products have been confirmed as the source involved in outbreaks of STEC infections only occasionally (Tseng et al., 2014). One example is a 2011 outbreak in Ontario, Canada, where 29 people developed gastrointestinal illness, six cases developed bloody diarrhea, and seven were hospitalized after eating leftover pork from a pig roast (Lise A Trotz-Williams et al., 2012). The STEC serogroup O157:H7 was isolated from 11 out of 29 stool samples from symptomatic patients and from leftover pork (Lise A Trotz-Williams et al., 2012).

E. coli O157:H7 was again linked to contaminated pork products in 2014 in Alberta, Canada (Honish et al., 2017). This was the third largest *E. coli* O157:H7 and the second largest foodborne outbreak ever reported in Canada (Honish et al., 2017). The outbreak was associated with 119 infections, and 19% of the patients were hospitalized, with six patients developing HUS; no deaths were reported (Honish et al., 2017). While there are a few reports implicating pork as the source of STEC outbreaks, considering such products as potential STEC transmission sources is warranted (Ercoli et al., 2015).

2.5 Pork processing

Areas of contamination

Cross-contamination of pork carcasses occurs mainly through redistribution of bacteria at various slaughter stages, which can be attributed to the pathogens that the pigs harbor on their skin

or in their oral/nasal cavity, feces, or lymph nodes (Rodríguez and Suárez, 2014). It has been demonstrated that the level of contamination of swine carcasses at the exsanguination stage is high in many studies (Arguello et al., 2013). For example, a study conducted by Van Hoek et al. (2012) found that the prevalence of *Salmonella* contaminated carcasses was 96.6% after exsanguination. According to Wheatley et al. (2014), this might result from ineffective cleaning and lack of sanitization procedures during transportation.

Scalding, dehairing, singeing, and polishing are processing steps typically applied to reduce visual and microbial contamination (Arguello et al., 2013). However, during scalding of slaughtered pigs, the water can move into the lungs and contaminate the oral cavity, which can then contaminate the carcass during removal of the lungs at evisceration (Rodríguez and Suárez, 2014). Further, during the dehairing process, the carcass can become contaminated when the rotating dehairing flails accumulate feces and other contaminated organic matter (Arguello et al., 2013). According to Wheatley et al. (2014), even though scalding and singeing are often considered as Critical Control Points (CCPs) within Hazard Analysis and Critical Control Points (HACCP) systems, the bacterial population decrease following singeing is alternated by a significant bacterial increase in the subsequent processing steps.

Among all the slaughter stages, the evisceration process has the greatest potential for microbial contamination (Dickson et al., 2019). Many studies have reported an increase in microbial populations after evisceration, including pathogens such as *Salmonella* and STEC (O'Connor et al., 2012; Tseng et al., 2014; Warriner et al., 2002). According to Wheatley et al. (2014), the direct or indirect spillage of fecal material from rupture of the gut represents the primary contamination risk during evisceration, next to the removal of the pharynx, tonsils, and tongue. A final carcass wash combined with the application of antimicrobial treatments is one

strategy that has demonstrated potential for reducing microbial populations on animal carcasses (Dickson et al., 2019).

Wash interventions

After the 2002 USDA directive requesting beef slaughter plants to reevaluate their HACCP plans, the beef industry began testing and using several interventions to reduce the likelihood of *E. coli* O157:H7 being on the carcass (Buege and Ingham, 2003). These interventions consisted of trimming, steam vacuuming, and carcass washing (hot water rinses, organic acid rinses, and steam pasteurization) (Buege and Ingham, 2003). Several studies have shown the effectiveness of organic acids at reducing the presence of pathogens on beef carcass surfaces prior to chilling (Kalchayanand et al., 2011; Signorini et al., 2018; Williams et al., 2010).

According to Dickson and Acuff (2017), the reduction in microbial populations on meat surfaces, depending on the treatment applied, will be because of physical removal of the microorganisms, a killing effect of the antimicrobial solution, or both factors. However, bacterial attachment onto meat surfaces and surface fat characteristics can influence the carcass wash interventions' effectiveness (Dickson and Acuff, 2017). Even though antimicrobial interventions are now commonly used on beef carcasses and trimmings, research on the effectiveness of these interventions to control pathogens on pork carcasses and chilled pork is limited (Eastwood et al., 2020). Therefore, this literature review will also highlight intervention effectiveness as it relates to the beef industry. However, some studies have already begun to demonstrate the effectiveness of some antimicrobial solutions to control *Salmonella*, STEC, and other pathogens on pork carcasses (Cleyton, 2002; Eggenberger-Solorzano et al., 2002; Orange et al., 2018) and pork cuts (Choi et al., 2009; Eastwood et al., 2020), which will also be highlighted.

Hot water

Hot water is the most common antimicrobial intervention used for microbial control in beef processing (Williams et al., 2010). According to Wheeler et al. (2014), the mode of action of heat treatments is primarily the denaturation of enzymes vital for bacterial life and by causing DNA strand breakage, as well as RNA degradation. Many studies have shown the potential for hot water washes to reduce bacterial populations on beef carcasses. A study conducted by Kalchayanand et al. (2012) obtained the largest STEC reductions (3.2 to 4.2 log) on beef carcasses with hot water (85 °C) compared to other antimicrobial interventions. Hot water is usually applied at temperatures between 65.6 °C (150 °F) and 82.2 °C (180 °F) on beef carcasses (Buege and Ingham, 2003). Hot water sprays have also resulted in significant bacterial reductions on hog carcasses. A study conducted by Eggenberger-Solorzano et al. (2002) demonstrated reductions of approximately 2 log CFU/cm^2 of *Enterobacteriacea* populations after a hot water rinse (82.2 °C) of pork carcasses. The same study suggested that hot water followed by an organic acid wash could significantly improve bacterial reductions on pork carcasses (Eggenberger-Solorzano et al., 2002).

Lactic acid

Some of the more widely studied antimicrobials are organic acids, including lactic acid (Wheeler et al., 2014). Organic acids are commonly used for decontamination of meat surfaces because of their availability, cost effectiveness approach, and efficiency without any negative impacts on human health (Sohaib et al., 2016). Lactic acid has a unique combination of low acid dissociation constant (pK_a) and low hydrophobicity (miscible with water), which allows this acid to easily diffuse across the bacterial cell wall (Boomsma et al., 2015). Once inside the cell at neutral pH, lactic acid dissociates into ions and decreases the internal pH; thus, to survive, the bacteria spend energy pumping out the charge ions which will eventually kill the bacteria (Boomsma et al.,

2015). The acid stress caused by lactic acid can also reversibly and irreversibly damage cellular macromolecules, which may inflict sublethal and lethal injury to the microbial cell (Wheeler et al., 2014).

The use of lactic acid (up to 5%) as an antimicrobial for meat animal carcasses is approved by the Food Safety and Inspection Service (FSIS) (Food Safety and Inspection Services, 2020). Several studies have shown the effectiveness of a variety of concentrations up to 5% of lactic acid at controlling different pathogens on livestock carcasses and meat cuts. A study by Reynolds (2003) reported reductions of 66 percent in the prevalence of *Salmonella* on market hogs to be used for sausage making following a 2% lactic acid spray. In another study that evaluated the effect of warm (55°C) 2.5 and 5.0% lactic acid on skin-on pork carcass, STEC was reduced by 0.9 and 1.2 CFU/cm^2, respectively. Organic acid applied as a carcass rinse at temperatures between 50 to 55 °C have been shown to be generally most effective on beef surfaces (Koutsoumanis and Skandamis, 2013).

Peracetic acid and acidified peracetic acid

Peracetic acid (PAA) is an oxidizing biocide that maintains its efficacy in the presence of organic soil and can inactivate both Gram-negative and Gram-positive bacteria, fungi, and yeast depending on the chemical concentration (Centers for Disease Control and Prevention, 2016b). Oxidative biocides, such as PAA, are suggested to have multiple targets within a cell, including denaturation of protein and enzymes, disruption of cell wall permeability, and oxidation of sulfhydryl and sulfur bonds in proteins, and other metabolites, which eventually leads to cell death (Freeman and Auer, 2012). Many processing plants use PAA for carcass decontamination (Han et al., 2020). The use of PAA on beef and pork carcasses is approved by the FSIS at concentrations not higher than 400 ppm (Food Safety and Inspection Services, 2020).

On non-chilled pork, *Salmonella* Typhimurium reductions of 1.3 log CFU/cm^2 and 0.8 log CFU/cm^2 were observed after a treatment with 400 ppm PAA on skinless pork and skin-on pork, respectively (Eastwood et al., 2020). Information about the efficiency of PAA solutions that are pH adjusted with organic or inorganic acids (acidified PAA) at reducing pathogens on beef is limited (Britton et al., 2020), and to our knowledge, there are no published studies describing its efficacy on pork. Britton et al. (2020) sprayed beef samples with PAA and acidified PAA and observed STEC surrogate population reductions ($P \leq 0.05$) of 1.7 to 1.9 log CFU/cm^2, respectively.

Hypobromous acid (HOBr)

In aqueous solution, 1,3-Dibromo-5,5-dimethylhydantoin (DBDMH) hydrolyzes to hypobromous acid (active antimicrobial agent) and dimethylhydantoin (Ulfig and Leichert, 2020). Bromine compounds, including hypobromous acid (HOBr), are very effective oxidizing biocides that cause oxidative damage to proteins, DNA, and lipids, which ultimately leads to cell death (Ulfig and Leichert, 2020). Hypobromous acid has been used for a long time in water processing and coolers (Wheeler et al., 2014). Hypobromous acid is allowed at concentrations up to 900 ppm for the beef industry; however, it is usually used at 300 ppm for carcass decontamination (Wheeler et al., 2014). Kalchayanand et al. (2009) reported *E. coli* O157:H7 reductions of 1.6 to 2.1 log CFU/cm^2, and *Salmonella* reductions of 0.7 to 2.3 log CFU/cm^2 on fresh beef and beef hearts after hypobromous acid spray treatments. The literature is limited regarding efficacy of HOBr on hog carcasses and fresh pork products. A study by Orange et al. (2018) evaluated multiple interventions to control *Salmonella* on fresh pork skin that was warmed and held at the pre-rigor temperature of 37 ^{o}C. The study reported that HOBr (300 ppm) achieved significant *Salmonella* reductions of 1.6 and 2 log CFU/cm^2 five minutes and 24 hours post-treatment, respectively (Orange et al., 2018).

2.6 USDA FSIS pathogen control actions

Current pork microbiological baseline data by the USDA-FSIS

The current microbiological baseline data collection program on market hogs was conducted by the FSIS from August 2010 to August 2011 and included 3,920 sponge samples from market hog carcasses (1,960 at Pre-Evisceration and 1,960 at Post-Chill) at 152 establishments under federal inspection (Food Safety and Inspection Services, 2011a). The samples collected were analyzed for positive percent rate and levels of *Enterobacteriaceae*, *Salmonella*, generic *Escherichia coli*, Aerobic Plate Count (APC), and total coliforms. The FSIS found a 69.6% *Salmonella* positive rate at pre-evisceration, whereas at post-chill it was reduced to 2.7% ($P \leq 0.05$) (Food Safety and Inspection Services, 2011a). Moreover, the most frequent *Salmonella* serovar at pre-evisceration and post-chill was Derby with 26.7% and 17%, respectively (Food Safety and Inspection Services, 2011a). Overall, the prevalence of *Salmonella* in market hogs is estimated to be about 1.66% (Food Safety and Inspection Services, 2011a).

Healthy People 2030 goals for Salmonella and STEC

The U.S. Department of Health and Human Services (HHS) Healthy People initiative helps to improve the health and well-being of communities, organizations, and individuals focusing on addressing public health challenges (Office of Disease Prevention and Health Promotion, 2020b). This initiative includes objectives related to the reduction of foodborne illness in the United States (Office of Disease Prevention and Health Promotion, 2020b). The results of Healthy People 2010 final review show that *Salmonella* infection increased by 10.3% (from 13.6 to 15.0 per 100,000 population), moving away from the 6.8 per 100,000 population target (National Center for Health Statistics, 2012). Similarly, the 2010 target to reduce *Salmonella* Enteritidis outbreaks was not

meet (24 outbreaks): however, the outbreaks were reduced by 44.9% (from 49 to 27) (National Center for Health Statistics, 2012). Conversely, the rate of O157 STEC infections decreased 52.4% (from 2.1 to 1.0 per 100,000 population), meeting the target of 1.0 per 100,000 population; however, the outbreaks cause by this pathogen increased from 10 to 32, moving away from the 5 outbreaks target (National Center for Health Statistics, 2012).

From the Healthy People 2020 available results it can be observed that the 11.4 cases per 100,000 population target for *Salmonella* species infections has not been met yet; however, a little change was observed from 2006–2008 to 2017 (15.0 and 14.5 cases per 100,000 population, respectively) (National Center for Health Statistics, 2016; Office of Disease Prevention and Health Promotion, 2020a). Similarly, the target of 0.6 cases per 100,000 population of. *E. coli* O157:H7 infections has not been achieved; however, a little change was also observed from 2006–2008 to 2017 (1.2 and 0.9 cases per 100,000 population, respectively) (National Center for Health Statistics, 2016; Office of Disease Prevention and Health Promotion, 2020a).

The results from the Healthy People objectives from the past years show the need for continued improvement of the food safety system and highlights the critical role that research on the control of foodborne pathogens has to accomplish this goal. Healthy People 2030 was launched on August 18, 2020 and is the fifth iteration of the Healthy People initiative (Office of Disease Prevention and Health Promotion, 2020b). The goals to reduce foodborne illness includes the reduction of the incidence of laboratory-diagnosed, domestically acquired *Salmonella* infections, with a target of 11.1 per 100,000 population over a baseline of 14.8 per 100,000 population (Office of Disease Prevention and Health Promotion, 2020b).

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Chapter 3 - Validation of Commercial Antimicrobial Intervention Technologies to Control *Salmonella* on Skin-on Market Hog Carcasses and Chilled Pork Wholesale Cuts

*To be submitted in the International Journal of Food Microbiology

Abstract

Pork is a substantial contributor to salmonellosis, and *Salmonella* control using antimicrobial technologies during processing is of increasing interest by processors. This study compared eight carcass antimicrobial washes or sprays, and six chilled subprimal/trim sprays, applied using commercial equipment and parameters, to quantify their ability to reduce *Salmonella* contamination in raw pork. Market weight hogs were harvested to provide skin-on carcasses, and eight sides (per replication) were inoculated with a *Salmonella* cocktail (ca. 5 log CFU/cm^2). Each side was treated in a commercial Chad cabinet using a spray [low-volume: 3% lactic acid (*l*LA), 400 ppm peracetic acid (*l*PAA), or acidified 400 ppm peracetic acid (*l*aPAA)] or wash [high volume: ambient water (*h*AW), 400 ppm PAA (*h*PAA), 400 or 600 ppm bromous acid (*h*DBDMH), or 71 °C water (*h*HW)] treatment within a randomized complete block study design. Post-treatment carcass *Salmonella* population reductions were compared before and after air chilling. Chilled subprimals and trim fabricated from each side were reinoculated and treated with antimicrobial sprays [AW, 400 ppm PAA, 400 ppm aPAA, 400 and 600 ppm DBDMH, or 2% LA] in a subprimal spray cabinet or ribbon mixer (trim). *Salmonella* reductions were determined over 14 days of vacuum packaged subprimal storage and four days of trim storage, along with lactic acid bacterial populations, TBARS and color determinations on non-inoculated products. For the

exterior skin-on carcass surfaces, treatments *h*PAA, *h*DBDMH$_{600}$, and *h*HW resulted in larger ($P \leq 0.05$) *Salmonella* reductions (2.8 to 3.1 log CFU/cm^2) compared to AW (1.5 log CFU/cm^2). Other treatments provided 2.0-2.4 log cycle reductions. *Salmonella* populations were reduced by 0.4-0.6 and 0.1-0.3 log cycles on subprimals and trim, respectively ($P>0.05$). None of the treatments had a negative impact on the subprimal quality factors evaluated. All pre-rigor carcass interventions provided beneficial *Salmonella* reductions, allowing processors flexibility in their operations. *Salmonella* control was less evident at the chilled subprimal and trim stages of processing.

3.1 Introduction

Food-producing animals such as pigs are major reservoirs for foodborne pathogens, particularly *Campylobacter* species, non-typhoidal serovars of *Salmonella enterica,* Shiga toxin-producing strains of *Escherichia coli,* and *Listeria monocytogenes* (Heredia and García, 2018). Pork is the most widely consumed protein source worldwide; therefore, ensuring the safety of these products is critical (Baer et al., 2013). Microbiological cross-contamination can occur at various steps in pork carcass processing, such as scalding, dehairing and polishing, singeing, carcass washing, evisceration, carcass rinsing post evisceration, and chilling (Miller et al., 2005). The evisceration process is one step that has the greatest potential for microbial contamination (Dickson et al., 2019). A study by Schmidt et al. (2012) performed in two large U.S. commercial pork processing plants revealed that the overall prevalence of *Salmonella* on pork carcasses at pre-scald, pre-evisceration, and post-chilling was 91.2%, 19.1%, and 3.7%, respectively. Similarly, in European Union countries, pork carcass contamination rates by *Salmonella* ranged from 0.3 to 17.4% (Pala et al., 2019).

Salmonella's economic implications worldwide are significant; according to Hoffman (2015), the cost of *Salmonella* is estimated to be $3.7 billion annually, where deaths of people who do not survive the infection account for 89% of the economic burden from this pathogen. Moreover, *Salmonella* also has a significant impact on public health. According to the Centers for Disease Control and Prevention (2020), *Salmonella* causes an estimated 1.35 million infections, 26,500 hospitalizations, and 420 deaths annually in the U.S. Hogs are among the main animal species that contribute to human salmonellosis transmission (Bonardi, 2017). Self et al. (2017) estimated that consumption of pork meat is responsible for 525,000 foodborne infections, 2,900 hospitalizations, and 82 deaths each year in the U.S.

Thus, the implementation of an effective intervention on pork carcasses is needed to improve the microbiological safety of pork products. It has been demonstrated that a final wash combined with the application of an antimicrobial treatment can potentially reduce bacteria populations on animal carcasses (Dickson et al., 2019). Additionally, a study by Miller et al. (2005) showed that rinsing carcasses at various temperatures with and without sanitizer is profitable from a social economic perspective. Several studies have explored the effectiveness of various antimicrobial interventions on beef carcasses to control different foodborne pathogens. Those interventions included spray carcass washes with hot water, acetic acid, citric acid, lactic acid, peroxyacetic acid, acidified sodium chlorite, chlorine dioxide, sodium hypochlorite, and aqueous ozone (Kocharunchitt et al., 2020; Signorini et al., 2018; Yoder et al., 2012).

There is limited published information regarding the effectiveness of intervention treatments on pork carcasses during slaughter under commercial conditions (Loretz et al., 2011). However, application of food grade antimicrobials such as acidic electrolyzed oxidizing water (Fabrizio and Cutter, 2004), lactic acid (Van Ba et al., 2019), and combinations of hot water and acetic acid

(Eggenberger-Solorzano et al., 2002) have demonstrated effective control of pathogen levels on pork carcasses. Some studies have investigated the efficacy of organic acids to control *Salmonella* on pork wholesale cuts. Choi et al. (2009) treated inoculated pork loins with 3% acetic acid and 3% lactic acid, which reduced *Salmonella* Typhimurium by 0.75 and 0.82 log CFU/g, respectively. In a similar manner, Kang et al. (2003) treated aerobically packaged pork loins with spray applications of lactic acid, citric acid, and acetic acid, achieving *Salmonella* Typhimurium reductions between 0.4-1.5 CFU/cm^2.

The objectives of this study were to (i) validate the efficacy of selected antimicrobial interventions: high-volume washes (peracetic acid, hypobromous acid, and hot water) or low-volume sprays (acidified peracetic acid, lactic acid, and peracetic acid) to reduce *Salmonella* populations on pre-rigor skin-on pork carcass sides, (ii) investigate the effectiveness of the selected antimicrobial sprays to reduce *Salmonella* risks associated with vacuum-packaged pork subprimals and fresh pork trimmings, and (iii) quantify treatment impacts on raw pork quality and shelf-life during wholesale storage.

3.2 Materials and methods

3.2.1 Experimental design

All inoculated carcass and fabricated pork studies were conducted within the biosafety level-3 meat animal harvest floor and chilled meat processing room contained within the Biosecurity Research Institute biocontainment facility at Kansas State University by trained personnel according to University Research Compliance Office approvals (BRI; Manhattan, KS). The experiment was designed as a randomized complete block with repeated measurements. Three replications served as the blocking factor. For the carcass level study, four market weight hogs (ca. 108.9 kg ea.) were harvested and processed per replication following a standard USDA-approved

protocol, including scalding/dehairing and singeing, and the eight carcass sides were randomly assigned to the eight wash/spray treatments. A *Salmonella* cocktail inoculum was uniformly applied to all pre-rigor carcass surfaces. *Salmonella* populations were determined on each carcass side at four anatomical locations (top, middle, bottom and inside body cavity) at three time points (pre-treatment, post-treatment and post-chilling).

For the subprimal level study, six spray treatments were compared. Each treated chilled carcass side was separately fabricated (with processing area, equipment and personnel receiving a wash-down and sanitizing between sides). From each side, the pork butt and loin were removed and trimmed to industry specifications. The subprimal spray treatment was always matched to the previous carcass-level wash/spray treatment. The loin remained uninoculated and was treated with the assigned chemical spray treatment and then lactic acid bacterial (LAB) populations, thiobarbituric acid test (TBARS) values, and Hunter L*, a*, and b* color values were determined on days 0 (pre- and post-treatment), 7, 14, 21, and 28 of vacuum-packaged storage at 2 °C to characterize shelf-life and quality impacts. To provide five separate sampling day sub-units for vacuum-packaged storage for each treatment, the post-treatment loin was divided aseptically into five equal portions that were separately vacuum-packaged and stored.

The butt from each treated side was inoculated with a five strain *Salmonella* cocktail inoculum and treated with the assigned antimicrobial spray. *Salmonella* population levels were determined on days 0 (pre- and post-treatment), 7 and 14 of vacuum-packaged storage at 2 °C. To provide three separate sampling day sub-units for vacuum-packaged storage for each spray treatment, the post-treatment butt was divided aseptically into three equal portions that were separately vacuum-packaged and stored. The remainder of each carcass side was processed into pork trim (approximately 13.6 kg), with 6.8 kg assigned to a *Salmonella*-inoculated intervention

treatment application and 4.5 kg to a non-inoculated intervention treatment application (aerobically stored shelf-life and quality evaluation). After spray treatment of each trim batch while tumbling in a ribbon-blender, *Salmonella,* or total aerobic bacterial populations (APC counts) were assessed on days 0 (pre- and post-treatment), 2 and 4 of aerobic storage at 2 °C. Additionally, color measurements and TBARS values were collected at each aerobic storage time point of the non-inoculated samples.

3.2.2 Bacterial strains

Salmonella strains used in the study represented the following serovars: Weltevreden, Typhimurium, Worthington, Anatum, and monophasic 1,4,[5],12: i:-. All strains were isolated from swine and include some of the most prevalent serovars find on hogs and pork-associated outbreaks (Naberhaus et al., 2019). The strains used in the study were obtained from Dr. Brian Lubbers (Kansas State University Veterinary Diagnostic Laboratory). Cultures were stored at -80 °C on cryoprotect beads (Key Scientific, Stamford, TX). To activate pure cultures for the study, a single bead was transferred to 10 mL of trypticase soy broth (TSB; Bacto™, Sparks, MD) and incubated 24 h at 37 °C to create parent strains, which were stored at 4 °C until needed. All pure cultures were confirmed to be *Salmonella* by PCR analysis (Applied Biosystems 7500 FAST Real-Time PCR System, Rapidfinder™ software; Thermo Fisher Scientific, Waltham, MA).

3.2.3 Inoculum preparation

A 5-strain inoculum cocktail was prepared for carcasses by transferring one 10-µl loopful from each stored parent strain broth culture into separate 10-mL tubes of TSB and incubating at 37 °C for 24 h. A second 10-µl transfer of each strain was then made from each 10-mL tube into two separate 40-mL tubes of TSB followed by incubation at 37 °C for 24 h. The five individual *Salmonella* strains (80 mL of each for a total of 400 mL) were combined and mixed with 3,600

mL of Phosphate Buffered Saline (PBS; VWR™ International, LLC, Radnor, PA) to form the carcass cocktail inoculum containing ca. 7 log CFU/mL.

Subprimal inoculum was prepared as described above, by performing a second transfer from each 10-mL tube into another 10-mL tube of TSB, followed by incubation at 37 °C for 24 h. The five tubes containing individual *Salmonella* strains were combined (50 mL total) and mixed with 950 mL of PBS to form the subprimal cocktail inoculum containing ca. 7 log CFU/mL.

A second transfer was performed to prepare pork trim inoculum, made from each 10-mL tube into a 20-mL tube of TSB followed by incubation at 37 °C for 24 h. The five tubes containing individual *Salmonella* strains were combined (100 mL total) and mixed with 900 mL PBS to form the trim cocktail inoculum containing ca. 7 log CFU/mL.

Aliquots of the individual pure culture solutions and the inoculum cocktails were analyzed prior to use to verify an approximately equal ratio of strains in the cocktail and the final *Salmonella* concentration in the inoculation solution. Briefly, individual strains and the cocktails were diluted in 0.1% peptone water (PW; Bacto™ Peptone, Becton, Dickinson and Company, Sparks, MD) and spread plated on Xylose Lysine Deoxycholate agar (XLD; Thermo Scientific™ Remel, Waltham, MA) that had been overlaid 24 h earlier with 14 mL of trypticase soy agar (TSA; Difco™, Sparks, MD) (XLD+TSA) to improve recovery of sublethally injured *Salmonella* cells (Kang and Fung, 2000). The XLD+TSA plates were incubated at 37 °C for 24 h and black colonies were counted.

3.2.4 Inoculation of carcass sides, subprimals, and trim

After evisceration and splitting, each pre-rigor (hot) carcass side was inoculated top to bottom, and both external skin surfaces and internal body cavity surfaces, with 500 mL of the inoculum cocktail to achieve ca. 5 log CFU/cm^2. A 4-inch high-density polypropylene foam paint miniroller specified for all paint finishes (Master, Bestt Liebco®, Cleveland, OH) was used to apply

a heavy coverage of the inoculum solution to all surfaces. Inoculated carcass sides were allowed to hang for ca. 30 min on the harvest floor rail to facilitate bacterial attachment prior to applying the final pre-rigor carcass wash or spray experimental treatments. Carcass inoculations were staggered in time (ca. 30 min between inoculations) to ensure similar pre-treatment attachment times across all treatments and to allow intervention treatment changes at the Chad cabinet.

Each chilled butt was individually placed on a sanitized tray that was placed inside a large biohazard bag. The butt was misted evenly using a previously primed and calibrated 8-oz spray bottle (iGo, The Bottle Crew, Bloomfield, MI), applying 15 mL of the cocktail inoculum to the top (fat cap) and 15 mL to the bottom (lean trimmed) surfaces to achieve ca. 5 log CFU/cm^2. Inoculated pork butts were held on the trays for 30 min at a processing room temperature of 10 °C to allow bacterial attachment, again using a time-staggered approach, prior to applying the specified antimicrobial spray treatments.

For pork trim inoculation, 6.8 kg of trim per treatment (approximately equal proportions of lean and fat and cut to an approximate size of 10 x 7 x 4 cm) were placed into a sanitized plastic meat lug, which was placed inside a large biohazard bag. Each sample was mist inoculated evenly with the aforementioned spray bottle, applying a total of 68 mL of inoculum cocktail to achieve a target of ca. 6 log CFU/g. Midway through the trim misting, the lug inside the biohazard bag was shaken to mix the meat portions, with the remaining inoculum subsequently sprayed onto the trim surfaces. A final shaking of the lug was done to uniformly disperse inoculum across all trim pieces. The attachment protocol described for butts was followed for trim. After utilization of each mixed cocktail inoculation solution (which required approximately 4 h of processing activities), an aliquot of the inoculation solution was collected and enumerated as previously described to verify similar starting and ending *Salmonella* concentrations.

3.2.5 Antimicrobial treatment solutions

Table 3.1 provides details of the antimicrobial intervention treatments chosen for evaluation in this study. Peracetic acid solutions (PAA) were prepared according to manufacturer's recommendation to achieve the target concentration. Peracetic acid concentrate (Microtox Plus™, Zee Company, Chattanooga, TN) was mixed with ambient tap water and target concentration was verified using a Peroxyacid/Tsunami Test Kit 311 (Ecolab, Saint Paul, MN). For the acidified peracetic acid solution (aPAA), the peracetic acid concentrate was mixed with ambient tap water to achieve the target concentration and verified by titration as described above, and a pH of 1.2 (carcass) and 1.4 (subprimal) was achieved by slowly blending in the acidifier Titon™ (Zoetis Inc., Florham Park, NJ), which is a proprietary blend of sulfuric acid and sodium sulfate. 88% L-lactic acid concentrate (Corbion, Purac®, Lenexa, KS) was mixed with 55 °C tap water to achieve 3% (v/v) for carcass application and 2% (v/v) for subprimal and trim applications (LA). Target lactic acid concentrations were verified by titrating 5 mL of solution with 0.25 N NaOH (Sigma-Aldrich, San Luis, MO) using phenolphthalein (Sigma-Aldrich) as an indicator. Hypobromous acid solutions (PorciBrom®; Passport Food Safety Solutions, West Des Moines, IA) were prepared using a feeder pump with 1,3-dibromo-5,5-dimethytlhydantoin (DBDMH) pellets, achieving 400 or 600 ± 30 ppm for application. Target concentrations were verified using a HI 96711 titration kit (HANNA instruments, Woonsocket, RI). For the hot water (HW) and ambient water (AW) wash treatments, long-lead thermocouples were mounted at a spray nozzle within the Chad carcass wash cabinet to verify water temperature (Table 3.1).

Table 3.1. Antimicrobial intervention technologies applied to skin-on market hog carcass sides as a pre-chill final wash (*h*) or spray (*l*), and as a surface spray drench for subprimals, mimicking commercially applied parameters.

Intervention	**Concentration**	**Temperature**	**Application**	**Products**
Water (Control) (*h*AW)	N/A	Ambient (ca. 16 °C)	High-Volume	Carcass Subprimals/trim
Peracetic acid (*h*PAA)	400 ± 30 ppm[1]	Ambient (ca. 16 °C)	High-Volume	Carcass Subprimals/trim
Hypobromous acid (*h*DBDMH$_{400}$)	400 ± 30 ppm [2]	Ambient (ca. 16 °C)	High-Volume	Carcass Subprimals/trim
Hypobromous acid (*h*DBDMH$_{600}$)	600 ± 30 ppm [2]	Ambient (ca. 16 °C)	High-Volume	Carcass Subprimals/trim
Hot water (*h*HW)	N/A	71.1 °C	High-Volume	Carcass
Acidified peracetic acid (*l*aPAA)	400 ± 30 ppm 1.2 pH[3] for carcass 1.4 pH[3] for subprimal	Ambient (ca. 16 °C)	Low-Volume	Carcass Subprimals/trim
Lactic acid[4] (*l*LA$_{3\%}$) *(*LA$_{2\%}$*)*	3 ± 0.1% for carcasses 2 ± 0.2% for subprimals	55 °C	Low-Volume	Carcass Subprimals/trim
Peracetic acid (*l*PAA)	400 ± 30 ppm	Ambient (ca. 16 °C)	Low-Volume	Carcass

[1] Microtox Plus™ (Zee Company; Chattanooga, TN)
[2] PorciBrom® (Passport Food Safety Solutions; West Des Moines, IA)
[3] Microtox Plus™ acidified with Titon™ (Zoetis Inc; Florham Park, NJ)
[4] 88% L-lactic acid (Corbion, Purac®; Lenexa, KS)

3.2.6 Antimicrobial application

For carcass-level intervention applications, a three-stage commercial grade Chad wash cabinet (Chad Co. Inc., Olathe, KS) was used. Treatments were categorized as either a high-volume deluge wash (ambient water, 400 ppm peracetic acid, 400 and 600 ppm DBDMH, and hot water) or a low-volume spray (3% lactic acid, 400 ppm peracetic acid, and acidified 400 ppm acidified peracetic acid) (Table 3.1). Table 3.2 provides operational details of the Chad carcass cabinet and Zoetis subprimal spray cabinet. High-volume treatments were applied in the Chad cabinet's second stage that incorporates a vertical oscillating spray bar on each side of the cabinet. The low-volume carcass treatments were applied in the third cabinet stage, where a fixed vertical spray bar on each side of the cabinet delivers a light spray. Stage one of the carcass cabinet, which is a high-volume ambient horizontally oscillating ambient water wash, was not utilized in these studies. To apply the antimicrobial intervention sprays, carcass sides were manually positioned (centered within the spray zone) in the cabinet at each stage (two or three) being used and remained in that positing during the defined time period for each antimicrobial intervention.

Chilled subprimal (non-inoculated loins and *Salmonella*-inoculated butts) antimicrobial spray applications were applied and were matched to the corresponding carcass level treatment (for six of the eight carcass-level treatments). High-volume HW and low-volume 400 ppm peracetic acid carcass treatments were excluded as subprimal matched treatments. For subprimals, LA treatment concentration was reduced to 2% and aPAA pH was raised to 1.4 at the suggestion of the respective technology providers based on commercial observations of raw product quality. All interventions were applied with a commercial style subprimal spray cabinet engineered by Zoetis, Inc. (Parsippany, NJ). As a rubber band-style transfer belt moved each subprimal through

the cabinet, the nozzles sprayed the subprimal with the designated antimicrobial from the top, bottom and sides of the cabinet (Table 3.2).

For the pork trim antimicrobial application, a 4-L hand-pump garden sprayer (Chapin International, Inc., Batavia, NY) was utilized. Antimicrobial solutions were sprayed onto inoculated trim batches (6.8 kg receiving 120 mL) and non-inoculated trim batches (4.5 kg receiving 80 mL) while trim was tumbled in a ribbon mixer. This was done to mimic industry tumbler systems used to apply chemical interventions (personal communication with various technology and meat processing company representatives).

The high-volume *h*PAA solution was prepared for treatment application through the Chad cabinet using municipal water and chemical concentrate and held in a 492-L external tank connected to the Chad carcass cabinet via a high-volume pump unit. The *h*DBDMH$_{400}$ and *h*DBDMH$_{600}$ solutions were generated by a feeder system (provided by Passport Solutions) to fill the external tank. The low-volume carcass spray treatments were prepared in a 113-L external tank connected to the Chad cabinet via a small pump unit. Both the 492-L and the 113-L external tanks were supplied by Birko/Chad Company (Chad Co. Inc., Olathe, KS). The subprimal cabinet used two sequenced Dosatron© models D14MZ2 and D14MZ10 (Dosatron International, LLC; Clearwater, FL) pumps to achieve the target concentration of each antimicrobial intervention. The DBDMH feeder pump was connected directly to the subprimal cabinet via a hose to deliver target concentrations.

Table 3.2. Spray cabinet specifications for the antimicrobial intervention technologies applied to skin-on market hog carcass sides and subprimals.

Treatment delivery	psi	Flow rate L/min	Residence time (s)	Volume delivered*	Number of nozzles	Nozzle model
High-volume carcass applications	50	302.83	12	60.57 L	36	VeeJet 50/20
Low-volume carcass applications	30	9.24	16	2.50 L	20	110015
Subprimal applications	15	0.38	11	4.16 L	24**	550011/8k 1.0 PVC

* Total volume delivered per carcass side.

** 8 nozzles positioned above, 8 below, and 4 on each side.

3.2.7 Sample collection and microbial analysis

Three carcass-level sampling points were evaluated using a surface tissue excision protocol to determine treatment-induced changes in the *Salmonella* population: post-inoculation (after a 30-min attachment period), post-antimicrobial spray application (after a 10-min on-rail drip time before chilling), and post-chilling (after 18 h of air-chilling at 2 °C). Tissue excision samples were obtained from three exterior anatomical locations [representing a pork skin surface at the top (rump), middle (midline) and bottom (shoulder) of each carcass side] and one interior body cavity location (lean tissue surface at the diaphragm region) per carcass side at each sampling point. A sterilized 5.2-cm diameter stainless steel coring device (delineating a 21.2 cm^2 tissue surface area) was used to make two distinct surface tissue scores (2-3 mm deep) at the targeted external anatomical location. Both surface tissue cores were extracted using a sterile forceps and scalpel

and combined into a single collection bag to represent a total of 42.4 cm^2 per external sample collected. The coring device, forceps and scalpel were sterilized between each collected sample. For the internal body cavity carcass sample, a smaller 3.5-cm diameter coring device (delineating a 9.6 cm^2 tissue surface area) was used and two cores were combined for a total of 19.2 cm^2 of tissue surface per sample. The sample was introduced in a 200-mL sterile homogenizer blender filter bags (Whirl-Pak®, Madison, WI) containing 50 mL (large cores) or 35 mL (small cores) of chilled Dey-Engley neutralizing broth (D/E broth; Difco™, Sparks, MD). All samples were stored at 4 °C for <3 h and transported to the laboratory. For analysis, sample bags were stomached for 1 min in an AES Blue Line Smasher™ (bioMerieux, Marcy-I'Etoile, France), serially diluted in PW and plated in duplicate on XLD+TSA. All plates were incubated at 37 °C for 24 h and black colonies were counted. The detection limits for exterior carcass and interior carcass cavity samples were 0.1 and 0.3 log CFU/cm^2, respectively.

For the subprimal-level sampling, surface tissue excisions were collected over four time points for *Salmonella*-inoculated butt samples: pre-treatment after a 30-min bacterial inoculation attachment period, after the antimicrobial wash/spray application with 10-min drip time, and after vacuum packaged storage of 7 and 14 days at 2 °C. The 3.5-cm diameter coring device was used to score two 9.6 cm^2 areas on the top side (fat side) and two 9.6 cm^2 areas on the bottom side (lean) of each butt, and a forceps and scalpel were used to extract the four tissue cores, providing a total excised sample area of 38.4 cm^2, that was placed into each sample bag containing 50 mL chilled D/E broth. The same procedure for stomaching, plating and incubation previously described for *Salmonella* enumeration was followed.

Loin subunits from each treatment were excision sampled, as described for butts, over six time points: before antimicrobial application (T_0), post-antimicrobial application with a 10-min

drip (day 0), and after vacuum packaged chilled storage of 7, 14, 21, and 28 days at 2 °C. All surface sample cores were placed in bags with 50 mL of chilled D/E broth, stomached, and serially diluted in DeMan, Rogosa and Sharpe broth (MRS; Sigma-Aldrich, San Luis, MO). Dilutions were plated in duplicate on Petrifilm™ Aerobic Count plates (3M™ Corporation, St. Paul, MN; APC), which were anaerobically incubated at 35 °C for 48 h to estimate lactic acid bacteria (LAB) population levels. The detection limit for plating of loin samples was 0.8 log CFU/cm^2.

For analysis of both non-inoculated (shelf-life) and *Salmonella*-inoculated pork trim, a surface excision protocol was utilized that was generally similar to N-60 style excision sampling (USDA FSIS, 2015). The 6.8 kg of treated inoculated trim, or 4.5 kg of treated non-inoculated trim, were laid out in a single layer on a sanitized metal pan and sterilized forceps and a butcher's knife were used to slice off approximately 2.5 x 5 cm surfaces (2-3 mm deep) of 20-30 trim pieces, representing both lean and fat tissues in approximately equal ratio. Slices were weighed into filter stomacher bags containing 200 mL chilled D/E broth until reaching 100 ± 10 g meat per treatment bag. Trim samples from each spray treated batch were collected over four time points: before treatment after a 30-min inoculum attachment period, post-antimicrobial spray application after a 10-min drip time, and after 2 and 4 d of aerobic storage at 2 °C. Sample bags were stomached for 1 min and sample homogenates were serially diluted in PW. Dilutions were plated in duplicate on Petrifilm™ Aerobic Count plates incubated at 35 °C for 24 h to determine total aerobic bacteria levels, or onto XLD+TSB plates to enumerate the viable *Salmonella* population. The detection limit for non-inoculated trim samples was 2.0 log CFU/g.

3.2.8 Color evaluation

Color measurements were collected from each non-inoculated loin subunit over the six time points (day 0 pre- and post-treatment, and days 7, 14, 21 and 28 of storage). A calibrated

MSEZ 4500L spectrophotometer (Hunter Associated Laboratories Inc., Reston, VA) was used to obtain CIE Lab Color L*, a*, and b* values at each sampling point. D65 illuminant was used with a 10° observer. Color readings were obtained from the left and right sides of each loin subunit, representing cut lean surfaces, which were averaged to obtain a single L*, a*, and b* value for statistical analysis. The readings were taken approximately 3 min after opening vacuum bags at each sampling time.

3.2.9 TBARS analysis

Thiobarbituric acid tests (TBARS) were performed as described by Ahn et al. (1998), with minor modifications, to estimate lipid oxidation that could have resulted from the antimicrobial spray applications to subprimals and trim during chilled storage. The N-60 style surface excision protocol was again used to collect lean tissue (ca. 50 g) from five pieces of trim into treatment-labeled, small Whirl Pak bags at each sampling time. For subprimals (non-inoculated loins), ca. 10 g of lean meat was thinly sliced from the cross-sectional cut meat surface of both ends of the loin subunit using a scalpel. Trim and loin samples were individually stored at -80 °C to stop oxidation reactions until analysis was performed. Each frozen sample was ground into a powder using a Waring 7011G 2-speed blender (Waring Commercial, Torrington, CT). The meat powder (1 g) was then combined with 5 mL of double-distilled water and homogenized with a CAT X120 hand-held homogenizer (CAT Scientific, Inc., Paso Robles, CA) for 15 sec at 10,000 rpm. Sample homogenates were centrifuged at 2,000 x g for 5 min and the supernatant was filtered through Whatman #1 filter paper (Tisch Scientific, North Bend, OH). Filtered supernatants (0.4 mL aliquot) were transferred to microcentrifuge tubes where 0.8 mL of TBA/TCA stock solution (20 Mm TBA and 15% TCA) were added. The tubes containing these samples were incubated at 70 °C in a water bath for 30 min and cooled on ice for 5 min. After cooling, samples were centrifuged

a second time at 10,000 x g for 5 min. The supernatant was placed into a cuvette and the absorbance was measured at 532 nm against a blank containing MQ water in a GENESYS 10S UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The final value was displayed as mg malondialdehyde (MDA)/kg of muscle tissue.

3.2.10 Statistical analysis

Data were analyzed under the linear mixed model. When applicable, interactions between model fixed effects were examined using type III tests at the 0.05 level. Least squares (LS) means and standard errors for fixed effects were reported (*Salmonella* log CFU/cm^2 or g reductions). Pair-wise comparisons between two levels of a fixed effect were performed based on the two-sided test for non-zero difference in means. For the subprimal study, disregarding the significance of treatment-by-time interaction, the effect of treatment was examined at each time point; the effect of time was examined for each treatment. When treatment-by-time interaction was not significant, the main effect of treatment was reported. Tukey's multiplicity adjustment was applied. SAS statistical analysis was executed via Statistical Analysis Software (SAS version 9.4; Cary, NC) PROC MIXED with option DDFM=KR.

3.3 Results

3.3.1 Carcass study

For the exterior skin-on carcass surfaces, the main effect of treatment was significant (P=0.04). As Figure 3.1 depicts, *h*PAA, *h*DBDMH$_{600}$, and *h*HW deluge washes achieved the largest *Salmonella* reductions (2.8 to 3.1 log CFU/cm^2), which were significantly ($P \leq 0.05$) greater than the 1.5-log cycle reduction achieved by the *h*AW control treatment. While the remaining intervention technologies, both low- and high-volume, also produced larger *Salmonella* reductions

(2.0 to 2.4 log CFU/cm^2) than the *h*AW control, the reductions were statistically similar (P>0.05). Although the time x treatment interaction was not significant (P=0.185), it is interesting to note that *Salmonella* populations declined by an additional 0.8 and 1.4 log CFU/cm^2 on exterior skin areas after 18 h of carcass chilling for *l*aPAA (P=0.042) and *l*LA (P=0.002) treatments, respectively. The *Salmonella* reductions achieved on exterior skin-on surfaces for final carcass wash/spray plus 18-h chilling ranged from 2.7-3.2 log CFU/cm^2 for all chemical wash/spray treatments, except for *l*PAA (2.2 log cycle total reduction). Even though the time x treatment interaction was not significant (P=0.185) for the exterior skin-on carcass surfaces; *l*aPAA and *l*LA reduced *Salmonella* populations by an additional 0.8 and 1.4 log CFU/cm^2, respectively, after 18 h of chilling.

For the inside cavity of the carcass sides (Figure 3.2), the main effect of treatment was significant (P=0.006). Applying *h*PAA produced a 1.7 log CFU/cm^2 *Salmonella* reduction, which was significantly more effective than all other intervention technologies ($P \leq 0.05$) except 3% *l*LA and *h*DBDMH$_{600}$ that resulted in similar (P>0.05) 1.2 and 1.1 log CFU/cm^2 reductions, respectively. Inside carcass cavity *Salmonella* reductions achieved by the remaining intervention technologies ranged from 0.5 to 1.0 log CFU/cm^2, whereas the *h*AW control wash reduced the *Salmonella* population by only 0.1 log CFU/cm^2. The time x treatment interaction for inside carcass cavity was not significant (P=0.096); however, it is noteworthy to mention that *l*LA reduced *Salmonella* populations by an additional 0.7 log CFU/cm^2 (P=0.021) after 18 h of chilling resulting in a total reduction of 1.5 log CFU/cm^2 from carcass inoculation to post-chill. This is the same total reduction achieved by the combined final carcass wash plus chilling using *h*PAA.

3.3.2 Pork subprimal and trim study

The mixed-strain *Salmonella* population inoculated onto chilled pork subprimals (butts) was not reduced by the AW control spray (0.0 log CFU/cm^2) on day 0; however, the other five chemical intervention sprays evaluated (aPAA$_{400}$, PAA$_{400}$, LA$_{2\%}$, DBDMH$_{400}$ and DBDMH$_{600}$) reduced the population by 0.4-0.6 log CFU/cm^2. *Salmonella* population reductions on the chilled subprimals ranged from 0.8-1.2 log CFU/cm^2 after 14 days of vacuum packaged storage, compared to 0.4 log CFU/cm^2 achieved by the AW control over the same storage period. While *Salmonella* reductions are being reported for the intervention technologies, the main effect of treatment (P=0.13) and the treatment x time interaction (P=0.85) were not significant for *Salmonella* reductions on the chilled subprimals. Although the time x treatment interaction was not significant, it is noteworthy that some intervention technologies produced significant reductions on days 0 and 14 in comparison to the AW control. More specifically, DBDMH$_{600}$ significantly reduced *Salmonella* by 0.6 (P=0.03) and 1.1 log CFU/cm^2 (P=0.034) on days 0 and 14, respectively. On day 14, aPAA, PAA$_{400}$, and 2% LA significantly reduced *Salmonella* by 1.2 (P=0.01), 1.1 (P=0.02), and 1.1 (P=0.03) log CFU/cm^2, respectively.

LAB populations on the loins remained at $\leq$2.5 log CFU/cm^2 during the 28-d storage period. The main effect of treatment (P=0.50) and the treatment x time interaction (P=0.28) were not significant. Loin LAB counts after 28 d of vacuum packaged storage at 2 °C across treatments remained low (1.0-2.1 log CFU/cm^2). Moreover, trim APC counts after 4 d of storage at 2 °C were $\leq$3.7 log CFU/g.

Salmonella reductions on inoculated trim ranged from 0-0.2 log and 0.2-0.5 log CFU/g after 0 and 4 d of aerobic 2 °C storage, respectively. In comparison, the AW control reduced *Salmonella* on trim by 0 and 0.2 log CFU/g on days 0 and 4, respectively. The main effect of

treatment (P=0.71) and the treatment x time interaction (P=0.67) were not significant for *Salmonella* reductions. Similarly, APC populations on non-inoculated aerobically stored (2 °C) trim were ≤3.7 log CFU/g during 4 d of storage and were not impacted by treatment (P=0.42), nor was the treatment x time interaction significant (P=0.97).

3.3.3 Lipid oxidation analysis

TBARS was measured on refrigerated (2 °C) pork trim and loins after 4 days of aerobic and 28 days of vacuum packaged storage, respectively. TBARS values ranged from 0.3-0.4 and 0.4-1.0 mg MDA/kg during storage for the non-inoculated loins and trim, respectively. It is important to note that TBARS values for loins and trim were not impacted by treatment (P=0.91 and P=0.27, respectively). The treatment x time interaction was also not significant for loin (P=0.76) or trim (P=0.90) TBARS values.

3.3.4 Product color analysis

For L*, a* and b*, the main effect of treatment was not significant (P>0.05). While the time x treatment interaction was not significant for L*, a* or b* (P>0.05), it is noteworthy that L* was significantly affected by $DBDMH_{600}$ and aPAA spray treatments of loins. More specifically, $DBDMH_{600}$ lightened the loin on day 14 as seen by an L* of 63.0 in comparison to the 59.0 value reported for AW (P=0.01). Similarly, aPAA significantly (P=0.02) lightened the loin on day 28 (L*=62.8) in comparison to the AW control (L*=58.0).

3.4 Discussion

3.4.1 Carcass study

The *h*AW deluge wash served as a control to quantify physical removal effects on *Salmonella* cells attached to carcass surfaces using the high-volume Chad cabinet, as opposed to the bactericidal component of the overall reduction achieved due to chemical or hot temperature. Conversely, the low-volume treatments would result in a very low level, if any, of physical detachment of attached *Salmonella* cells from carcass surfaces, thus, virtually all of the observed reductions can be attributed to the bactericidal effects of the chemical spray treatments. With the exception of *l*PAA and *h*AW, the $\leq$0.5 log cycle difference among treatments applied to the exterior, skin-on surface suggests that all treatments were similar in *Salmonella* control on exterior surfaces by the end of the carcass chilling stage of processing.

The reductions on the interior carcass surface were lower in magnitude than those observed for the exterior, skin-on surface. The skin represents a different attachment surface than internal lean (smooth) tissue, which may contribute to differences in intervention efficacy. However, it is also important to consider the impact that structural differences may have and, more specifically, the limited spray contact angle or possible shielding that may reduce antimicrobial contact to the inoculated interior cavity surfaces in the diaphragm area. As shown in Figure 3.2, no discernable trend was observed for greater treatment efficacy when comparing high-volume washes versus low-volume sprays for the inside body cavity, suggesting that most of the *Salmonella* population reduction was attributed to the chemical being applied. Higher average populations of *Salmonella* Typhimurium were also reported by Cleyton (2002) on the jowl area of pork carcasses compared to the ham and belly. This suggests that the jowl was the most difficult area for each treatment to

access due to the shape of this region of the swine carcass interfering with an equal distribution of antimicrobial during spray application.

In meat processing, the cumulative effect of multiple antimicrobial interventions in series is commonly relied upon to achieve increasing levels of risk reduction relating to bacterial pathogens from the carcass to the final fabricated raw products. As depicted in the treatment effects (Figures 3.1 and 3.2) for final carcass washes, selection of a single treatment application to simultaneously achieve optimal *Salmonella* reductions on exterior and interior surfaces of skin-on market hog carcasses may not be straight forward. This study revealed *h*PAA (400 ppm) and *h*DBDMH (600 ppm) to be within the top three applications for both exterior and inside cavity to reduce attached *Salmonella* populations, suggesting that they would suffice as a single technology carcass wash. However, the *h*HW wash, although similar ($P>0.05$) to *h*PAA and *h*DBDMH$_{600}$ on exterior surfaces, was inferior ($P\leq0.05$) for reducing the *Salmonella* population level on the inside carcass cavity. Thus, the application of a secondary chemical carcass spray to inside body cavities of carcass sides would likely improve *Salmonella* control efforts at the final wash stage of processing. Although the use of mixed technologies in sequence was not evaluated in the current study, considering the greater residual reductions observed during 18 h of carcass chilling for 3% *l*LA and *l*aPAA sprays for external and inside body cavities, these two secondary treatments may be warranted.

Reynolds (2003) reported reductions of 50 and 66% in the prevalence of *Salmonella* on market hogs to be used for sausage making following 180-200 ppm peroxyacetic acid and 2% lactic acid sprays, respectively, when applied using a low-pressure/low-volume garden sprayer. However, prior to application of these interventions, all carcasses were washed with 50 ppm chlorinated water. It is difficult to compare this study to the current due to it being a prevalence

versus magnitude of *Salmonella* population reduction, differences in sampling technique (swabbing versus excision), and no indication of the microbiological plating protocol being reported. Epling et al. (1993) investigated the effect of a 2% lactic acid spray electrostatically applied (150 mL per side) on 75 commercially slaughtered pork carcasses and reported a reduction in *Salmonella* prevalence at all anatomical locations after treatment of pre-rigor carcasses and after 24 h of air chilling compared to non-treated control carcasses, with 2% lactic acid having a notable reduction after chilling carcasses at the shoulder and ham areas. A similar observation occurred in the current study as 3% LA resulted in the greatest *Salmonella* reduction during air chilling (an additional 1.4 log cycle reduction over the immediate post-treatment pre-rigor final carcass spray reduction). In an inoculated study, Cleyton (2002) demonstrated *Salmonella* Typhimurium reductions of 3.71 log CFU/cm^2, 2.28 log CFU/cm^2, and 2.27 log CFU/cm^2 after treating pork carcasses with 53 ºC (127 ºF) hot water, 50 ppm chlorine, and 2% lactic acid sprays, respectively. In comparison, the 71.1 ºC (160 ºF) hot water used in the present study achieved 2.2 log CFU/cm^2, which is 1.5 logs less than the 53 ºC (127 ºF) hot water used by Cleyton (2002). Similarly, the present study used a more concentrated 3% lactic acid than the 2% lactic acid used by Cleyton (2002); however, both studies report similar reductions of ca. 2.2 to 2.3 log CFU/cm^2. Cleyton (2002) also reported an additional reduction of 3.23 log CFU/cm^2 after 24 h of chilling for the 2% lactic acid treatment. This additional post-treatment *Salmonella* population reduction during chilling is consistent with, although substantially greater than, the additional reductions reported herein after 18 h of air chilling for the external and internal carcass surfaces subjected to the low volume 3% lactic acid intervention. Furthermore, Eggenberger-Solorzano et al. (2002) investigated the effect of a combination of hot water and 2% acetic acid interventions on hog

carcasses during processing. They reported that hot water washing followed by organic acid rinsing significantly reduced the microbial populations on skinned and scalded pork carcasses.

3.4.2 Pork subprimal and trim study

The chilled subprimal data reported herein suggest that all intervention technologies were similar ($P>0.05$) in efficacy as treating the chilled butts with AW. The reductions observed for subprimals were similar to those previously reported in the literature. Choi et al. (2009) demonstrated *Salmonella* Typhimurium reductions on inoculated boneless pork loins of 0.75 and 0.82 log CFU/g following treatment with 3% acetic acid and 3% lactic acid, respectively. Similar *Salmonella* Typhimurium reductions were reported by Fabrizio and Cutter (2004) on inoculated pork bellies stored under vacuum-packaged conditions for 7 days at 4 °C, where a 2% lactic acid treatment was the most effective with a 1.76 log CFU/cm^2 reduction. *Salmonella* Typhimurium reductions between 0.4-1.5 log CFU/cm^2 have also been reported after 2% lactic acid, 2% citric acid, and 2% acetic acid spray applications on aerobic-packed pork loins after 24 h at 4 °C (Kang et al., 2003). However, the *Salmonella* reductions (0.9-1.2 log CFU/cm^2) observed in the current study after the 14 days of storage (2 °C) are still relevant because, even though recontamination can happen throughout the fabrication process, the pathogen levels at this stage are expected to be low (<1 log CFU/cm^2).

The five intervention technologies applied to chilled pork trim were not effective at reducing *Salmonella* or APC. These data suggest that applying these specific intervention technologies to pork trim at the volume, concentration and dwell time used in the current study, using a hand-held garden sprayer and ribbon mixer, is no more effective at reducing *Salmonella* or APC populations than applying the AW control. Published research describing the efficacy of antimicrobial interventions on pork trim is still limited. Castelo et al. (2001) observed that ground

pork prepared from pork trim treated with a combination of water and 2% lactic acid (15 °C, 75 s) reduced APC, LAB, and coliform populations by 2.17, 1.16, and 1.87 log CFU/g, respectively. Much like APC on trim, the lack of a treatment (P=0.50) effect suggests that these intervention technologies are no more effective than the AW control at reducing LAB populations on chilled subprimals during vacuum-packaged storage. According to Kirsch et al. (2014), the low bacterial population reductions observed on chilled (<5 °C) meat surfaces could be the result of bacteria attaching more strongly to cold meat surfaces, making the antimicrobial interventions less effective. However, other considerations such as chemical-meat surface interaction and bacterial physiological state while chilled likely impact the antimicrobial effect of the spray interventions.

3.4.3 Lipid oxidation analysis

Thresholds to detect rancidity have been published, and raw ground beef TBARS values above 1.0 are typically associated with a detectable, oxidized odor and off-flavor when cooked (American Meat Science Association, 2012). According to Xiong et al. (2020), off-flavors in pork are unlikely to be detected by consumers at values below 0.5 mg MDA/kg. In the present study, trim samples were stored aerobically, which may explain why TBARS values above the 0.5 mg MDA/kg threshold were detected. This suggests that lipid oxidation in trim may be detectable by consumers; however, the lack of a treatment effect for trim (P=0.27) indicates that applying the intervention technologies to trim did not increase lipid oxidation beyond TBARS values observed for the AW control.

3.4.4 Product color analysis

Despite significant differences in L* during shelf life, overall, the color data indicate that application of these intervention technologies did not significantly alter the L*, a* or b* parameters of pork loin color and, therefore, do not have a negative impact on the product. Grajales-Lagunes

et al. (2012) studied the effect of lactic acid on meat quality properties and taste of the pork *Serratus ventralis* muscle, and also concluded that the treatments had no significant effect on product color. Another experiment performed on veal calf carcasses suggested that concentrations of up to 1.25% lactic acid did not produce unacceptable discoloration, and concentrations up to 2.00% were not significantly different from the control in terms of cooked flavor (Woolthuis and Smulders, 1985).

3.5 Conclusions

The intervention technologies applied to pre-chill carcasses as a final wash were generally less effective on the inside cavity lean tissue than on the exterior skin-on surface. This may be the result of anatomical differences that impact the ability of spray interventions to sufficiently reach the inside cavity, or due to differences in the buffering capacity of lean versus pork skin. Future research should investigate this hypothesis, which may include the application of a secondary carcass wash intervention to more effectively cover the inside carcass surface.

Further evaluations for the technologies investigated in the current study for chilled pork trim should include using a larger volume of antimicrobial solution in relation to the trim weight being treated. Additionally, enhanced efficacy might be achieved by utilizing spraying and mixing equipment to provide greater and more uniform coverage of individual trim pieces.

3.6 Acknowledgements

The authors acknowledge the undergraduate and graduate student workers in the K-State Food Safety and Defense Laboratory for assisting in project preparation and sample analyses. Special notes of gratitude are extended to Dr. Qing Kang, Director of the K-State Statistical Consulting Laboratory, for her extensive assistance with statistical analysis and data interpretation, and to Mr. John Wolf, K-State Meat Lab Manager, for his assistance in swine harvesting and carcass

fabrication activities under strict biocontainment protocols. The authors also extend our appreciation for the technical guidance and supplies provided by the antimicrobial technology and equipment companies mentioned in this study. Finally, the assistance of biosafety, project management and engineering staff at the K-State Biosecurity Research Institute was greatly appreciated. This work was supported in part by the USDA National Institute of Food and Agriculture, Hatch project 1014344 and Hatch Multistate project 1018371. This is contribution no. 21-049-J from the Kansas Agricultural Experiment Station. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by Kansas State University.

Funding

This work was financially supported by the Foundation for Meat and Poultry Research and Education. In-kind support of engineering time, new system components, and shipping/installation to upgrade the Chad cabinet system was provided by Chad Company (Lenexa, KS). Additional in-kind funding for intervention technology support equipment, supplies and expert consulting time was received from Arm and Hammer (Passport Solutions), Zee, Birko, Corbion, and Zoetis companies.

3.7 Figures

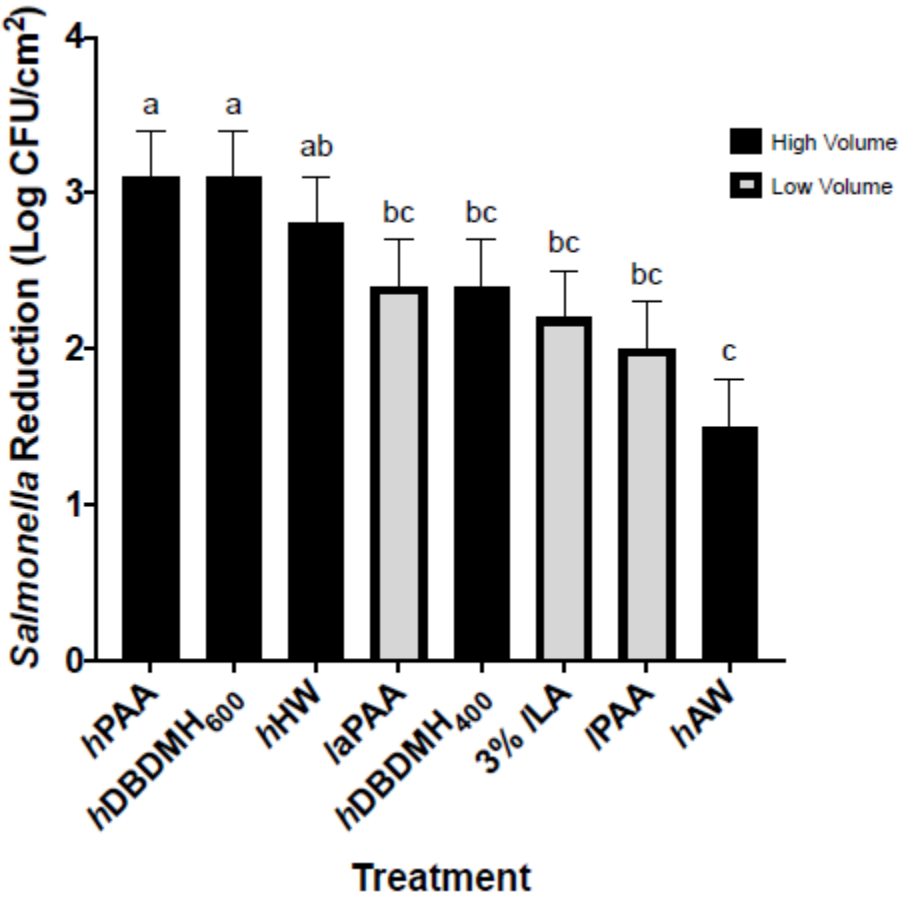

Figure 3.1. *Salmonella* reductions achieved by antimicrobial intervention technologies on the exterior surface of skin-on pork carcasses. Carcass core samples were excised and enumerated on xylose lysine desoxycholate agar (XLD) with a tryptic soy agar (TSA) overlay to allow for enumeration of injured cells. The main effect of treatment was significant (P=0.044).

[a-c] Treatments that do not share a superscript differ (P≤0.05).

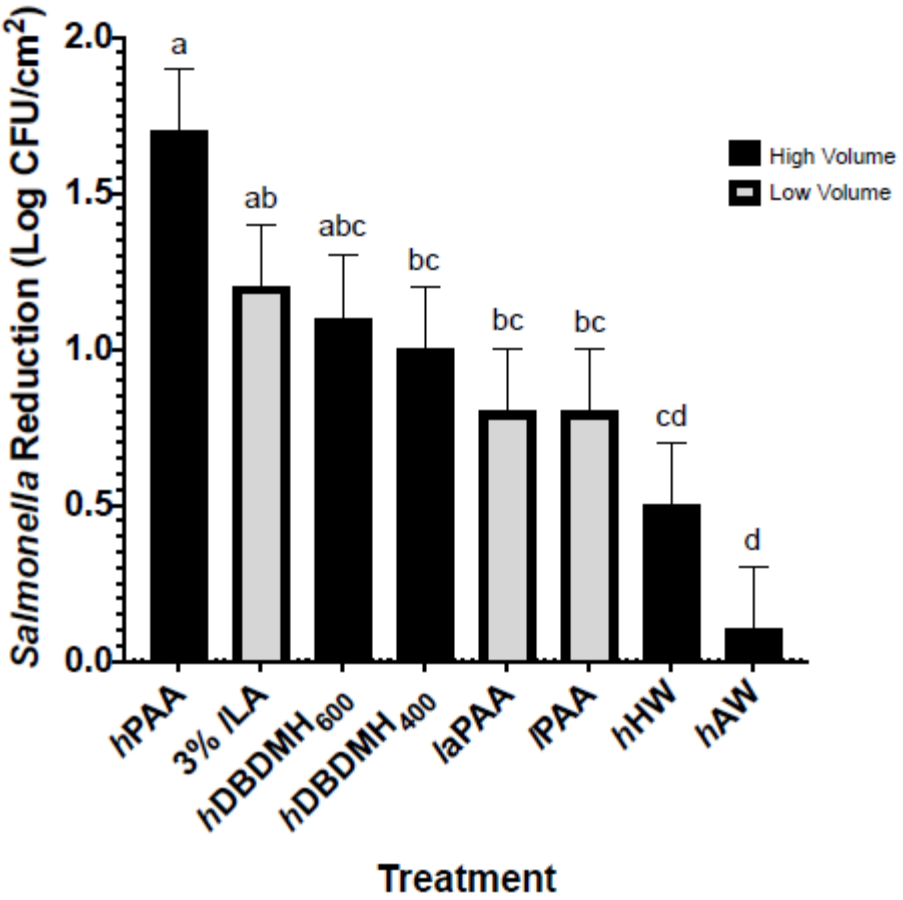

Figure 3.2. *Salmonella* reductions achieved by antimicrobial intervention technologies on the inside body cavity (lean tissue surface) of pork carcasses. Carcass core samples were excised and enumerated on xylose lysine desoxycholate agar (XLD) with a tryptic soy agar (TSA) overlay to allow for enumeration of injured cells. The main effect of treatment was significant (P=0.006).

[a-d] Treatments that do not share a superscript differ (P≤0.05).

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Chapter 4 - Validation of Commercial Antimicrobial Intervention Technologies to Control Shiga Toxin-producing *Escherichia coli* (STEC) on Skin-on Market Hog Carcasses

*To be submitted in the International Journal of Food Microbiology

Abstract

Although swine are less associated with STEC foodborne disease outbreaks, the potential for swine to serve as a source of STEC infections in human beings cannot be disregarded. This study compared eight USDA-approved antimicrobial intervention technologies to quantify their ability to reduce STEC contamination on market hog carcasses. Hogs were harvested to provide skin-on carcass sides, and eight sides (per three replications) were inoculated with a 7-strain STEC cocktail (ca. 5 log CFU/cm^2 across all external and body cavity surfaces). Each side was randomly assigned to a final pre-chill wash treatment administered in a commercial Chad carcass cabinet using a low-volume spray [3% lactic acid (*l*LA; 130°F), 400 ppm peracetic acid (*l*PAA), or acidified 400 ppm peracetic acid (*l*aPAA)] or a high-volume wash [ambient water (*h*AW), 400 ppm PAA (*h*PAA), 400 or 600 ppm hypobromous acid (*h*DBDMH), or 71 °C water (*h*HW)] treatment within a randomized complete block study design. Post-treatment (after a 10-min hanging drip) and post-chilling (18 h at 2 °C) STEC reductions were compared for external skin-on surfaces and internal body cavity lean surface tissue. Post-treatment color changes were determined for lean, adipose, and skin carcass surfaces before and after chilling. The *h*HW, *h*PAA, and *h*DBDMH$_{600}$ deluge washes achieved the greatest external surface STEC reductions (3.8, 3.4, and 3.2 log CFU/cm^2, respectively), and were significantly ($P \leq 0.05$) more effective than the other

intervention technologies, including the 1.7-log reduction achieved by the ambient water control. The carcass interventions were less effective at reducing STEC populations attached to interior body cavity smooth lean tissue, with post-chill populations reduced by 0.9-2.2 log cycles, while the *h*AW control wash achieved a 0.6-log reduction. None of the treatments negatively impacted instrumental carcass color. All market hog carcass interventions reduced STEC populations, thus equipping pork processors with information to support decision-making when selecting an intervention technology.

4.1 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are a substantial challenge for public health because of the potential severity of illness and association with large outbreaks. The severe clinical diseases that STEC infections can cause include hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), which can lead to acute renal failure (Tseng et al., 2014). Around 2010, estimates indicate that STEC caused more than 1 million illnesses, 128 deaths, and loss of almost 13,000 Disability-Adjusted Life Years (DALYs) (World Health Organization, 2019). In the United States, this pathogen is estimated to cause more than 265,000 illnesses each year, with more than 3,600 hospitalizations and 30 deaths (Centers for Disease Control and Prevention, 2016). STEC are commonly isolated from the gastrointestinal tract (hindgut) of a wide variety of animal species (Persad and Lejeune, 2014).

Cattle are regarded as the primary natural reservoir of STEC; however, other ruminant species can also serve as reservoirs (Persad and Lejeune, 2014). Birds, swine, dogs, and horses are considered spillover hosts, meaning they are susceptible to colonization and may transmit disease; however, once they are no longer exposed to STEC, they do not maintain this colonization (Persad and Lejeune, 2014). Remfry et al. (2021) evaluated 598 fecal samples from finisher pigs collected

from 10 pig flows and analyzed them for the presence of *stx*$_1$ and *stx*$_2$ genes, followed by cultural isolation of STEC cultures where they found 178 isolates representing 23 STEC serogroups. These authors concluded that healthy finisher pigs shed several serogroups associated with pig-related illness, but the prevalence of STEC of public health significance was low. Nevertheless, there is still potential for human infection regardless of the low prevalence of pathogenic STEC serogroups from swine (Persad and Lejeune, 2014).

Antimicrobial interventions are commonly used on beef carcasses and trimmings; however, research on the effectiveness of these interventions at controlling pathogens on market hog carcasses and chilled pork is limited (Eastwood et al., 2020). To date, the published pork intervention research focuses on efficacy against *Salmonella* more so than STEC, which is in response to the greater degree of recognition *Salmonella* has received from the pork industry in recent years. Research has documented that organic acid-based carcass washes decrease *Salmonella* populations (Baer et al., 2013); however, these reductions are not significantly better than those achieved by washing carcasses with hot water alone (Baer et al., 2013; Eggenberger-Solorzano et al., 2002).

Because STEC have been associated with swine, and little research has documented the efficacy of interventions against this pathogen in pork processing, additional research is necessary to validate a variety of antimicrobial intervention technologies on a commercial scale for effectiveness against STEC. The objectives of this study were (i) to validate the efficacy of selected antimicrobial interventions, both high-volume washes (peracetic acid, hypobromous acid, and hot water) and low-volume sprays (acidified peracetic acid, lactic acid, and peracetic acid), at reducing STEC populations on pre-rigor, skin-on pork carcass sides, and (ii) to evaluate the impact of each antimicrobial intervention on carcass color quality.

4.2 Materials and methods

4.2.1 Experimental design

Pathogen-inoculated studies were conducted using a high-level biocontainment meat animal harvest and processing facility (biosafety level-3) located at the Kansas State University Biosecurity Research Institute (BRI; Manhattan, KS). Carcass samples collected at the BRI were transported and analyzed at the K-State Food Safety & Defense Laboratory (Manhattan, KS). This study implemented a randomized complete block design with repeated measurements. The three replications serve as the blocking factor. For each replication, four market weight hogs (ca. 108.9 kg ea.) were harvested and processed according to a standard USDA-approved protocol, including hot water scalding/dehairing and singing, and all harvest procedures were approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC). The eight carcass sides obtained were randomly assigned to the eight final carcass wash/spray treatments. After evisceration and splitting, a STEC cocktail was uniformly applied to pork carcass surfaces and allowed to attach prior to treatment. The STEC population was enumerated at three anatomical locations on the exterior skin-on carcass surface [top (rump), middle (split line), and bottom (shoulder)] and from one location on the interior body cavity (lean tissue at diaphragm region) at three processing time points [pre-treatment (T_0), post-treatment (T_1), and post-chill (T_2)]. Carcass color measurements (L*, a* and, b*) were collected from three tissue types (adipose, lean and, skin) at each sampling time.

4.2.2 Bacterial strains

The STEC strains used in the study included *E. coli* O157:H7 (ATCC 35150) and non-O157 strains O145: NM (83-75), O121:H19 (CDC 97-3068), O111:H- (JBI-95), O103:H2 (CDC

90-3128), O45:H2 (CDC 90-3285), and O26:H11 (H30). All strains were isolated from human samples and obtained from Dr. John Luchansky (USDA, ARS, Eastern Regional Research Center, Wyndmoor, PA). The STEC serogroups used in this study are often implicated in major foodborne outbreaks worldwide and are known to cause the most severe clinical diseases to humans (U.S. Food and Drug Administration, 2019; Amezquita-Lopez et al., 2018). Cultures were stored at -80 °C on cryoprotect beads (Key Scientific, Stamford, TX). To activate pure cultures, a single bead was transferred to 10 mL of trypticase soy broth (TSB; Bacto™, Sparks, MD) and incubated for 24 h at 37 °C to create parent strains, which were stored at 4 °C until needed. All pure cultures were confirmed to be STEC using the Genetic Detection System (Assurance GDS®, MilliporeSigma, Burlington, MA). Pure cultures were also streaked on MacConkey agar (Thermo Scientific™ Remel, Waltham, MA) that had been overlaid 24 h earlier with 14 mL of trypticase soy agar (TSA; Difco™, Sparks, MD) (MacConkey + TSA) to document appearance and ensure proper colony counting during the inoculation study. Pure cultures grew pink and, in the case of heavy growth, yellow to colorless growth was also observed.

4.2.3 Inoculum preparation

To prepare the working inoculum, one 10-µl loopful from each parent strain broth culture was transferred separately into a 10-mL tube of TSB and incubated at 37 °C for 24 h. After incubation, another 10-µl loopful was transferred from each 10-mL tube into two separate 40-mL tubes of TSB followed by incubation at 37 °C for 24 h. The seven individual STEC strains were combined, and 400 mL of the master inoculum were then mixed with 3,600 mL of phosphate-buffered saline (PBS; VWR™ International, LLC, Radnor, PA) to prepare the 7-serogroup cocktail inoculum containing ca. 7 log CFU/mL. This cocktail inoculum was prepared in bulk for each study replication and subdivided into eight equal portions to be applied to the eight sides. An

aliquot of the first and eighth portions, corresponding to the first and eighth side processed (an approximate 4-h difference in time), was analyzed to confirm that STEC population levels in the inoculation solutions did not change from start to finish of the treatment applications. Aliquots of the individual pure culture solutions were also analyzed prior to use to verify an approximately equal ratio of strains in the cocktail solution. Briefly, individual strains and the cocktails were diluted in 0.1% peptone water (PW; Bacto™ Peptone, Becton, Dickinson and Company, Sparks, MD) and spread plated on MacConkey + TSA to improve recovery of sublethally injured STEC cells (Kang and Fung, 2000). The MacConkey + TSA plates were incubated at 37 °C for 24 h and colonies (pink and yellow to colorless) were counted.

4.2.4 Carcass inoculation

After evisceration and splitting, each pre-rigor (hot) carcass side was inoculated top to bottom, both external skin surfaces and internal body cavity surfaces, with the inoculum cocktail to achieve ca. 5 log CFU/cm^2. Briefly, a 4-inch high-density polypropylene foam paint miniroller specified for all paint finishes (Master, Bestt Liebco®, Cleveland, OH) was used to apply heavy, uniform coverage of the inoculum to all carcass surfaces. Inoculated carcass sides were allowed to hang for ca. 30 min on the harvest floor rail to allow bacterial attachment prior to applying the final pre-rigor carcass wash/spray experimental treatments. Carcass side inoculations were staggered in time (ca. 30 min between inoculations) to ensure similar pre-treatment attachment times across all treatments while the research team switched from one chemical spray treatment to the next (mixing new chemical solutions, adjusting concentration/temperature/pH as needed, and flushing/priming Chad cabinet lines).

4.2.5 Antimicrobial treatment solutions evaluated

Peracetic acid solutions (PAA) were prepared according to manufacturer's recommendation to achieve the target concentration (Table 4.1). Peracetic acid concentrate was mixed with ambient tap water and target concentration was verified using a Peroxyacid/Tsunami Test Kit 311 (Ecolab, Saint Paul, MN). For the acidified peracetic acid solution, the peracetic acid concentrate was mixed with ambient tap water to achieve the target concentration (Table 4.1) verified by titration as described above, and a pH of 1.2 was achieved by slowly blending in the acidifier Titon™, which is a proprietary blend of sulfuric acid and sodium sulfate marketed by Zoetis, Inc. (Florham Park, NJ). 88% L-lactic acid concentrate was mixed with 55 °C tap water to achieve 3% (v/v) (Table 4.1). Target concentrations were verified by titrating 5 ml of solution with 0.25 N NaOH (Sigma-Aldrich, San Luis, MO) using phenolphthalein (Sigma-Aldrich) as an indicator. Hypobromous acid solutions were prepared using a feeder pump with 1,3-Dibromo-5,5-Dimethylhydantoin (DBDMH) pellets, achieving 400 or 600 ± 20 ppm for application (Table 4.1). Target concentrations were verified using a HI 96711 titration kit (HANNA instruments, Woonsocket, RI). For the *h*HW and *h*Aw wash treatments, long-lead thermocouples were mounted at a spray nozzle within the Chad cabinet to verify water temperature (Table 4.1). Target wash/spray pressures and nozzle outputs were confirmed throughout each study replication by checking the pump controller panel screen and by measuring flow rates (average volume of water obtained per nozzle in 30 seconds), respectively.

Table 4.1. Antimicrobial intervention technologies applied to skin-on market hog carcass sides as a pre-chill final wash (*h*) or spray (*l*), mimicking commercially applied parameters.

High-Volume Wash (*h*)			**Low-Volume Spray (*l*)**		
Treatment	**Concentration**	**Abbreviation**	**Treatment**	**Concentration**	**Abbreviation**
Ambient Water	Water (Control)	*h*AW	Acidified Peracetic Acid[3]	400 ppm ± 20 ppm at pH of 1.2	*l*aPAA
Peracetic Acid[1]	400 ± 20 ppm	*h*PAA	Lactic Acid[4]	3% ± 0.1 at 130°F (54.4°C)	*l*LA
Hypobromous Acid[2]	400 ± 20 ppm	*h*DBDMH$_{400}$	Peracetic Acid[1]	400 ± 20 ppm	*l*PAA
Hypobromous Acid[2]	600 ± 20 ppm	*h*DBDMH$_{600}$			
160°F (71°C) Water	Water	*h*HW			

[1] Microtox Plus™ (Zee Company; Chattanooga, TN)

[2] PorciBrom® (Passport Food Safety Solutions; West Des Moines, IA)

[3] Microtox Plus™ acidified with Titon™ (Zoetis Inc; Florham Park, NJ)

[4] 88% L-lactic acid (Corbion, Purac®; Lenexa, KS)

4.2.6 Antimicrobial application

A three-stage commercial grade Chad wash cabinet (Chad Co. Inc., Olathe, KS) was used to apply all carcass interventions. Treatments were categorized as either a high-volume (*h*) deluge wash or a low-volume (*l*) spray, as described in Table 4.1. Table 4.2 provides the spray cabinet's operational specifications during operation, which were achieved by working with Birko/Chad Company engineers to approximate typical industry operations. High-volume treatments were applied in the Chad cabinet's second stage,

which included a vertical oscillating spray bar on each side of the cabinet. The low-volume treatments were applied in the third cabinet stage, which was comprised of fixed vertical spray bars on each side of the cabinet. Stage one of the carcass cabinet, which is a high-volume ambient horizontally oscillating ambient water wash, was not utilized in these studies. To apply the antimicrobial intervention sprays, carcass sides were manually positioned (centered within the spray zone) in the cabinet at each stage (two or three) being used and remained in that positing during the defined time period for each antimicrobial intervention.

The *h*PAA intervention was held in a 492-L external tank connected to the cabinet through a high-volume pump unit. A feeder system provided by Passport Food Safety Solutions (West Des Moines, IA) generated the *h*DBDMH$_{400}$ and *h*DBDMH$_{600}$ solutions that were used to fill the external tank. All low-volume carcass spray treatments were prepared in a 113-L external tank connected to the cabinet through a small pump unit. Both the 492-L and the 113-L external tanks were supplied by Birko/Chad Company (Chad Co. Inc., Olathe, KS).

Table 4.2. Spray cabinet specifications for the antimicrobial intervention technologies applied to market hog carcass sides.

Treatment Delivery	**psi**	**Flow rate (gal/min)**	**Residence time (sec)**	**Volume (gallons)***	**Number of nozzles**	**Nozzle model**
High-volume	50	80	12	16	36	VeeJet 50/20
Low-volume	30	2.44	16	0.66	20	110015

*Total volume delivered per carcass side.

4.2.7 Microbial sampling

A surface tissue excision protocol was used at three sampling points: pre-treatment (T_0; after a 30-min attachment period), post-treatment (T_1; after a 10-min on-rail drip time), and post-chilling (T_2; after 18 h of air-chilling at 2 °C). At each sampling point, tissue excision samples were collected from three exterior locations (pork skin surface at the top, middle, and bottom) and one lean tissue surface from the interior body cavity per carcass side. A stainless steel coring device with a 5.2-cm diameter (21.2 cm^2) was used to score each target sampling area at a depth of 2-3 mm, and each core was removed using a sterile scalpel. Two cores were collected per exterior sampling location and combined for a total of 42.4 cm^2 per sample. For the internal body cavity, a coring device with a 3.5-cm diameter (9.6 cm^2) was used. The two cores, combined for a total of 19.2 cm^2 per sample from each anatomical location, were placed into a 200 mL sterile homogenizer blender filter bags (Whirl-Pak®, Madison, WI) containing 50 mL (for 21.2 cm^2 cores) or 35 mL (for 19.2 cm^2 cores) of chilled Dey-Engley neutralizing broth (D/E; Difco™, Sparks, MD). All sample bags were held at 4 °C for up to 3 h until they were transported to the Kansas State University Food Safety & Defense Laboratory (Manhattan, KS) for analysis. To enumerate STEC populations, sample bags were stomached for 1 min in an AES Blue Line Smasher™ (bioMerieux, Marcy-I'Etoile, France), serially diluted in 0.1% peptone water, and plated in duplicate on MacConkey agar + TSA. All agar plates were incubated at 37 °C for 24 h, and all colonies (pink and colorless) were counted. Representative colony types from plates were periodically confirmed by STEC latex agglutination (Pro-Lab Diagnostics, Round Rock, TX).

4.2.8 Color evaluation

Instrumental color was evaluated using CIE Lab Color L*, a*, and b* values from three anatomical locations: (1) ham, (2) sirloin, and (3) shoulder, corresponding to different tissue types,

including skin, lean, and adipose, respectively. These readings were assessed at each of the three time points (T_0, T_1, and T_2) on each carcass side. Duplicate readings were taken at each sampling location to obtain an averaged CIE value for statistical analysis. Color values were obtained using a hand-held calibrated MSEZ 4500L spectrophotometer, with D65 illuminant and a 10° observer (Hunter Associated Laboratories Inc., Reston, VA).

4.2.9 Statistical analysis

All experimental procedures were replicated three times on different days. For each replication, newly propagated STEC cultures were prepared, new working solutions of each chemical intervention were mixed and confirmed, and the spray parameters of the Chad cabinet were verified. Log reductions were calculated by comparing T_1 and T_2 STEC populations to T_0 populations. Data from three replications were analyzed under the linear mixed model. The model fixed effects were examined using type III tests at the 0.05 level. Least squares (LS) means and standard errors for fixed effects were reported. Pairwise comparisons between 2 levels of a fixed effect were performed based on the 2-sided test for non-zero difference in means. SAS Statistical analysis was executed via Statistical Analysis Software (SAS version 9.4; Cary, NC) PROC MIXED with option DDFM=KR.

4.3 Results

4.3.1 Carcass study

On the exterior, skin-on carcass surface, the main effects of treatment ($P<0.001$), anatomical location ($P=0.035$), and time ($P=0.002$) were significant. The treatment x time ($P=0.028$) interaction was the only significant interaction observed. Therefore, the impact of each treatment on STEC populations will be discussed according to sampling point (T_1 and T_2). Because

the main effect of anatomical sampling location was significant, and the anatomical location was not associated with any interactions, data from all sampling points and treatments have been combined into a single LS mean for the top, middle, and bottom of the exterior carcass surface (Fig. 4.1).

For the exterior, skin-on carcass surface, the intervention technologies *h*PAA, $hDBDMH_{600}$, and *h*HW achieved the largest STEC reductions (3.3, 2.8, and 2.8 log CFU/cm^2, respectively) post-treatment (T_1). These reductions were significantly ($P \leq 0.05$) greater than the 1.1-log cycle reduction achieved by the *h*AW control treatment (Table 4.3) at T_1. The *h*AW deluge wash served as a control to quantify physical detachment and removal of STEC cells from the carcass surfaces using a high-volume ambient water wash. Treatments *h*$DBDMH_{400}$ and *l*aPAA (2.4 and 2.3 log CFU/cm^2 reductions, respectively) were also significantly ($P \leq 0.05$) more effective against STEC than the *h*AW control at T_1. The remaining intervention technologies *l*PAA and *l*LA (1.6 and 0.7 log CFU/cm^2 reductions, respectively), were not significantly different from the control ($P > 0.05$) at T_1. After 18 h of carcass chilling (T_2), the *h*HW, *h*PAA, and *h*$DBDMH_{600}$ deluge washes achieved the greatest STEC reductions (3.8, 3.4, and 3.2 log CFU/cm^2, respectively), and these reductions were greater ($P \leq 0.05$) than the remaining intervention technologies, including the 1.7-log reduction achieved by the *h*AW control (Table 4.3). The *h*$DBDMH_{400}$ treatment also achieved a greater ($P \leq 0.05$) STEC reduction (2.4 log CFU/cm^2) compared to the *h*AW control at T_2. The remaining intervention technologies produced reductions (1.6-2.0 log CFU/cm^2) that were not significantly different ($P > 0.05$) from the control. As Figure 4.1 illustrates, STEC reductions were significantly greater on the bottom region of the carcass exterior surface (2.6 log CFU/cm^2) in comparison to the 2.1-log reduction achieved on the middle

(P=0.009). STEC reductions observed at the bottom of the carcass were greater than the marginally significant (P=0.056) 2.2-log reduction achieved at the top area of the carcass.

As summarized in Table 4.3, STEC populations declined (P≤0.05) by an additional 1.0 and 0.9 log CFU/cm^2 on the exterior skin surface during the 18-h carcass chilling period for sides treated with *h*HW (P=0.001) and *l*LA (P=0.004), respectively (Table 4.3). Small but insignificant (P>0.05) additional STEC reductions of 0.6 and 0.4 log CFU/cm^2 during chilling were noted for the *h*AW control and *h*DBDMH$_{600}$ treated carcasses. The remaining treatments demonstrated no additional STEC population changes during chilling compared to pre-chill population levels.

Table 4.3. STEC reductions on the exterior, skin-on surface of market hog carcass sides following antimicrobial spray intervention applications and after 18 h of chilling at 2 °C. Carcass core samples were excised and enumerated on MacConkey agar with a Tryptic Soy Agar (TSA) overlay to better enumerate sublethally injured cells.

	LS means ± SE STEC reductions (log CFU/cm^2)	
Treatments	**Post-treatment**	**Post-chilling**
*h*PAA	3.3 ± 0.3^{Aa}	3.4 ± 0.3^{Aa}
*h*DBDMH$_{600}$	2.8 ± 0.3^{Aab}	3.2 ± 0.3^{Aa}
*h*HW	2.8 ± 0.3^{Aab}	3.8 ± 0.3^{Ba}
*h*DBDMH$_{400}$	2.4 ± 0.3^{Ab}	2.4 ± 0.3^{Ab}
*l*aPAA	2.3 ± 0.3^{Abc}	2.0 ± 0.3^{Abc}
*l*PAA	1.6 ± 0.3^{Acd}	1.6 ± 0.3^{Ac}
*h*AW (control)	1.1 ± 0.3^{Ade}	1.7 ± 0.3^{Ac}
*l*LA	0.7 ± 0.3^{Ae}	1.6 ± 0.3^{Bc}

[abc] Values with different superscripts in the same column indicate significant differences between treatments at a sampling point.
[ABC] Values with different superscripts in the same row indicate significant differences between sampling points for a treatment.
Interaction between treatment and time was significant (P=0.028).

The main effects of treatment (P=0.131) and time (P=0.716) were not significant for the inside body cavity. However, the treatment x time interaction (P=0.030) was significant. Therefore, the impact of each treatment on STEC populations will be discussed according to sampling point. The intervention technologies were generally less effective on the inside cavity lean tissue in the diaphragm region than on the exterior, skin-on surface. The *h*PAA treatment achieved the largest STEC reduction (2.1 log CFU/cm^2) at T_1, which was significantly ($P \leq 0.05$) greater than *h*HW, *l*LA, and *h*DBDMH400 (0.8, 0.6, and 0.2 log CFU/cm^2, respectively; Table 4.4). However, *h*PAA was not significantly different ($P > 0.05$) from the remaining treatments, including the *h*AW control (1.0 log CFU/cm^2 reduction). After 18 h of carcass chilling (T_2), only the *h*PAA achieved a STEC reduction (2.2 log CFU/cm^2) that was statistically

significant (P≤0.05) in comparison to the control (0.6 log CFU/cm^2). The remaining intervention technologies produced reductions between -0.2 and 1.2 log CFU/cm^2.

On the inside body cavity of the carcass (Table 4.4), only the *l*LA carcass spray demonstrated a STEC population reduction after 18 h of chilling (T_2) compared to its pre-chill level (T_1). For this application, a significant (P=0.019) STEC population decline of 0.5-log cycle was observed. Conversely, small but significant (P≤0.05) post-chill STEC population increases of 0.4 log cycles were observed for carcasses receiving the *h*DBDMH400 and *h*AW (control) treatments. All other carcass intervention treatments demonstrated statistically insignificant (P>0.05) STEC population changes during chilling.

Table 4.4. STEC reductions on the internal body cavity (lean tissue surface at the diaphragm area) of market hog carcass sides following antimicrobial spray intervention applications and after 18 h of chilling at 2 °C. Carcass core samples were excised and enumerated on MacConkey agar with a Tryptic Soy Agar (TSA) overlay to better enumerate sublethally injured cells.

	LS means ± SE STEC reductions (log CFU/cm^2)	
Treatments	**Post-treatment**	**Post-chilling**
*h*PAA	2.1 ± 0.4^{Aa}	2.2 ± 0.4^{Aa}
*h*DBDMH$_{600}$	1.4 ± 0.4^{Aab}	1.2 ± 0.4^{Aab}
*l*PAA	1.0 ± 0.4^{Aab}	1.1 ± 0.4^{Aab}
*h*HW	0.8 ± 0.4^{Ab}	1.1 ± 0.4^{Aab}
*l*LA	0.6 ± 0.4^{Ab}	1.1 ± 0.4^{Bab}
*l*aPAA	1.0 ± 0.4^{Aab}	0.9 ± 0.4^{Aabc}
*h*AW (control)	1.0 ± 0.4^{Aab}	0.6 ± 0.4^{Bbc}
*h*DBDMH$_{400}$	0.2 ± 0.4^{Ab}	-0.2 ± 0.4^{Bc}

[abc] Values with different superscripts in the same column indicate significant differences between treatments at a sampling point.

[ABC] Values with different superscripts in the same row indicate significant differences between sampling points for a treatment.

Interaction between treatment and time was significant (P=0.030).

4.3.2 Color evaluation

The L*, a*, and b* color measurements collected from lean, skin, and adipose tissues were not significantly impacted by treatment ($P>0.05$), which indicates that these intervention technologies did not significantly alter the color on any of the carcass tissues studied; thus, treatments did not have a negative impact on the carcass appearance. The time x treatment interaction was also not significant ($P>0.05$) for L*, a*, or b* collected from lean, skin, and adipose tissues. The main effect of time was significant for L* ($P=0.003$), a* ($P<0.001$), and b* ($P<0.001$) for the skin tissue; a* ($P<0.001$) and b* ($P=0.010$) for lean tissue; and b* for adipose tissue, suggesting that carcass color changes occur over time regardless of intervention treatments applied. Because the objective of this research relative to quality impacts was to determine if the intervention technologies (treatment) impacted carcass color, the fact that color changed across time is less relevant, and these data will, therefore, not be discussed in detail hereafter.

4.4 Discussion

4.4.1 Carcass study

The objective of this research was to validate the effectiveness of selected antimicrobial intervention technologies at reducing STEC on skin-on market hog carcasses and their impact on carcass color when applied as a high-volume wash (peracetic acid, hypobromous acid, and hot water) or low-volume spray (acidified peracetic acid, lactic acid, and peracetic acid). The *h*HW, *h*PAA, and *h*DBDMH$_{600}$ deluge washes achieved the greatest post-chilling (T_2) STEC reductions (3.8, 3.4, and 3.2 log CFU/cm^2, respectively) on the exterior skin-on carcass surface, and were significantly ($P\leq0.05$) more effective than the other intervention technologies, including the 1.7-log reduction achieved by the *h*AW control wash. The intervention technologies were less

effective at reducing STEC populations attached to the lean tissue of the interior carcass cavity, with post-chilling (T_2) STEC populations reduced by 0.9-2.2 log cycles, while comparatively, the *h*AW control wash achieved a 0.6 log reduction. Moreover, the application of these intervention technologies did not have a negative impact on the carcass color.

Antimicrobial interventions are commonly utilized on beef carcasses and trimmings; however, research on the effectiveness of these interventions at controlling pathogens on market hog carcasses and chilled pork is limited (Eastwood et al., 2020). To date, the pork intervention research published explores efficacy against *Salmonella* more so than STEC, which is in response to the greater degree of recognition *Salmonella* has received from the pork industry in recent years. Some studies have evaluated the effectiveness of various antimicrobial interventions on pork carcass cuts to control foodborne pathogens, including STEC (Eastwood et al., 2020); however, the current study is one of the first evaluating antimicrobial intervention technologies for control of STEC on pork at a carcass level, with application parameters that represent commercial carcass applications.

Application of a high-volume wash of hot water (*h*HW; 71 ^{o}C) was one of the most effective treatments on the exterior skin-on surface, achieving post-treatment (T_1) and post-chilling (T_2) STEC reductions of 2.8 and 3.8 log CFU/cm^2, respectively. It is possible that STEC cells are injured by exposure to hot water and, therefore, not able to withstand the stress caused by 18 h of air-chilling. Eggenberger-Solorzano et al. (2002) also evaluated the use of hot water rinses on pork skin and reported that water temperatures of 55, 65, and 80°C reduced *Enterobacteriaceae* populations on inoculated, scalded carcasses by 1 to 1.5 log CFU/cm^2. Differences in efficacy may have been the result of wash application differences (*e.g.*, pressure) as well as target

microorganism; however, data presented from both studies suggests that applying hot water (55-80 °C) to pork carcasses reduces microbial contamination on the skin surface by > 1 log CFU/cm^2.

Two concentrations of hypobromous acid (400 and 600 ppm) were investigated in this study, and the 600 ppm (*h*DBDMH$_{600}$) wash was one of the most effective washes for reducing STEC on both the exterior skin-surface and the lean tissue of the internal body cavity. In general, the 400 ppm concentration (*h*DBDMH$_{400}$) was more effective on the external skin-on surface than the inside cavity. Hypobromous acid (300 ppm) was also investigated by Orange et al. (2018) as an intervention to control *Salmonella* on fresh pork skin that was warmed and held at the pre-rigor temperature of 37 °C, and a significant reduction of 1.6 log CFU/cm^2 five minutes post-treatment was reported (Orange et al., 2018). In comparison, a 2.8 log CFU/cm^2 and 2.4 log CFU/cm^2 reduction in STEC was achieved in the present study using 600 ppm and 400 ppm of hypobromous acid, respectively, ten minutes post-treatment (T_1) on the exterior skin-on surface. Both studies demonstrate that *Salmonella* and STEC can be significantly reduced on a pre-rigor pork skin surface following application of hypobromous acid. The discrepancies observed between these studies are likely due to differences in hypobromous acid concentrations (400 and 600 ppm versus 300 ppm), treatment application pressure [50 psi in the current study versus 37.4 psi used by Orange et al. (2018)], and target pathogen (STEC versus *Salmonella*).

Eastwood et al. (2020) applied warm (ca. 55 °C) 2.5 and 5.0% lactic acid to non-chilled, skin-on pork, and reported STEC reductions of 0.9 and 1.2 log CFU/cm^2, respectively, at 30-min post-treatment. At 10-min post-treatment (T_1) in the present study, STEC was reduced by 0.7 log CFU/cm^2 on the exterior skin-on surface of carcasses sprayed with warm (ca. 54.4 °C) 3% lactic acid (*l*LA), which is similar to the 0.9 log CFU/cm^2 STEC reduction reported by Eastwood et al. (2020) for warm 2.5% lactic acid. Additional STEC reductions (that were significant at $P \leq 0.05$)

were observed after 18 h of carcass chilling for both the exterior skin-on surface (0.9 log CFU/cm^2) and inside of the carcass cavity (0.5 log reduction) of carcasses sprayed with *l*LA in the present study. A study by Cleyton (2002) reported similar results for 2% lactic acid, with an additional *Salmonella* reduction of 3.2 log CFU/cm^2 after 24 h of chilling, which was greater than the post-chill (T_2) reductions reported for STEC in the current study. These data suggest that the antimicrobial effect of lactic acid is realized after several hours and/or is enhanced by the additional stress that is exerted on pathogens during the chilling process. Van Ba et al. (2019) evaluated *Salmonella enterica* reductions achieved by 2% and 4% lactic acid on non-inoculated pork carcasses and reported that a significantly ($P \leq 0.05$) larger reduction (1.1 log CFU/100 cm^2) was obtained on carcasses sprayed with 4% lactic acid compared to those sprayed with 2% lactic acid (0.5 log CFU/100 cm^2). It is important to note that Van Ba et al. (2019) sampled non-inoculated carcasses for *Salmonella enterica*, and the data are presented as CFU/100 cm^2 versus CFU/cm^2 for STEC-inoculated carcasses in the present study. Therefore, differences in pathogen and the magnitude of reduction must be considered when comparing these data.

Although intervention research is limited for STEC on market hog carcasses, there is a large body of research that reports on the efficacy of antimicrobial intervention technologies on beef carcasses, some of which can serve as a general comparison for the data presented herein. Signorini et al. (2018) reported that 3% lactic acid and hot water (82-87 °C) reduced beef carcasses' prevalence for the *stx* and *eae* genes. The 3% lactic acid reduced the *stx* and *eae* genes by 43.3% and 20%, respectively, and 38 and 18.6%, respectively, when treated with the hot water (82-87 °C) (Signorini et al., 2018). Comparing the prevalence of STEC genes versus the magnitude of STEC population reduction is challenging, particularly when comparing data collected from beef versus market hog carcasses. However, comparison of these data with the data presented herein

suggests that 3% lactic and hot water are effective at reducing STEC on beef and market hog carcasses. This is further supported by a Kalchayanand et al. (2011) study that reported lactic acid, hot water, and acidified sodium chlorite as the most effective (1.5-4.9 log CFU/cm^2 reductions) at reducing non-O157 STEC and *E. coli* O157:H7 on inoculated beef carcasses. Similar to what is reported in the literature for beef carcasses, hot water (71 °C) was one of the more effective treatments in the present study, particularly on the external skin-on carcass surface.

4.4.2 Color evaluation

Even though several studies, including the present study, have tested the efficiency of organic acids for control of pathogens on meat surfaces, quality changes should also be considered when applying these interventions (Mani-López et al., 2012). Color is the most important factor when determining consumer preferences among meat characteristics because it serves as an indicator of the texture, flavor, and taste (Kim et al., 2019). However, there is limited information about the impacts of antimicrobial interventions on pork color compared to beef (Eastwood et al., 2020). The present study addresses this knowledge gap, and color data indicate that the application of intervention technologies did not significantly alter the L*, a*, or b* parameters on any of the carcass tissues studied. Similar results were obtained by Grajales-Lagunes et al. (2012), who reported that the color of pork (*Serratus ventralis* muscle) was not affected (P>0.05) by the application of lactic acid. Similarly, Castelo et al. (2001) did not observe significant differences (P>0.05) between the L*, a* or b* values of commercial pork trim treated with water, followed by a 2% lactic acid wash at 15 °C. There is currently no available information about the impact of antimicrobial interventions on the color of pork carcasses; thus, the results from the current study provide the basis for future research.

4.5 Conclusions

This study used a Chad carcass wash cabinet set to deliver similar high-volume wash or low-volume spray parameters as those currently used in commercial meat processing establishments. All application parameters were set and verified by providers of each intervention technology; therefore, pork processors should be able to utilize these research findings in their commercial operations. The intervention technologies investigated do not negatively impact carcass color and effectively provide STEC reductions, which allows each processor to select the intervention technology that best suits their facility and available resources. More specifically, STEC was reduced by high-volume washes (3.2 to 3.8 log CFU/cm^2) and low-volume sprays (1.6 to 2.0 log CFU/cm^2) on the exterior skin-on surface following chilling. These data provide the information necessary to support decision-making for establishing and managing effective, yet flexible, food safety plans. Further research is needed to properly understand the difference in intervention effectiveness for STEC control on the skin-on exterior surface compared to the lean tissue of the internal carcass cavity.

4.6 Acknowledgements

The authors acknowledge the graduate student workers in the K-State Food Safety and Defense Laboratory, Yashoda Subedi, Mackenzie Mayo, Olivia Harrison, and Gabriela Magossi Boccato for assisting in project preparation and sample analyses. A special note of gratitude to Mr. John Wolf, K-State Meat Lab Manager, for his assistance in swine harvesting and carcass fabrication activities under strict biocontainment protocols. The authors also extend our appreciation for the technical guidance and supplies provided by the antimicrobial technology and equipment companies mentioned in this study. Finally, the assistance of biosafety, project management and

engineering staff at the K-State Biosecurity Research Institute was greatly appreciated. This work was supported in part by the USDA National Institute of Food and Agriculture, Hatch project KS00-0012-HA. This is contribution no. 21-236-J from the Kansas Agricultural Experiment Station. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by Kansas State University.

Funding Statement

This work was supported by the National Pork Board (grant number 19-184). In-kind support of engineering time, new system components, and shipping/installation to upgrade the Chad cabinet system was provided by Chad Company (Lenexa, KS). Additional in-kind funding for intervention technology support equipment, supplies and expert consulting time was received from Arm and Hammer (Passport Solutions), Zee, Birko, Corbion, and Zoetis companies.

4.7 Figure

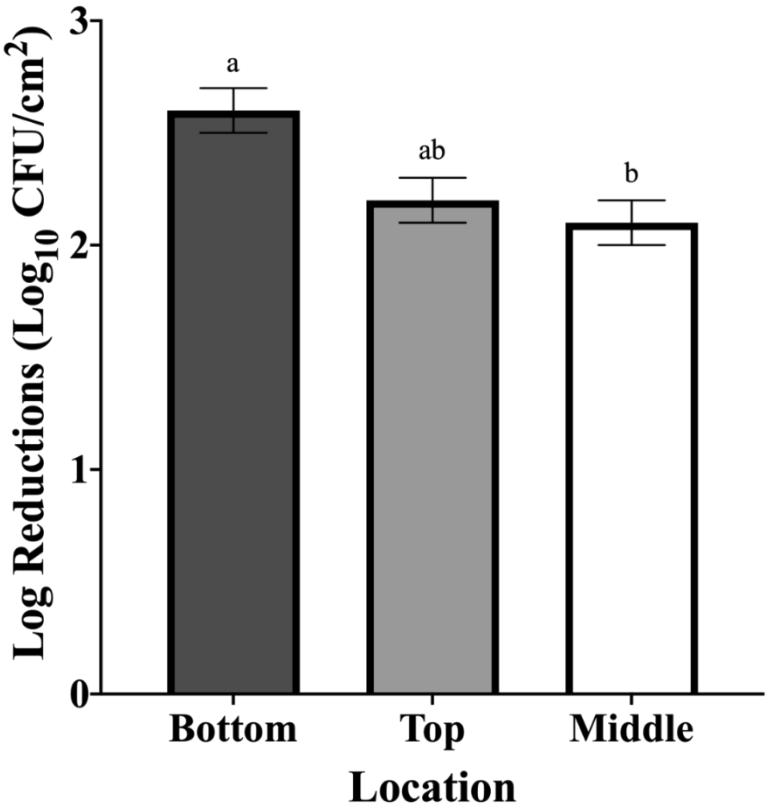

Figure 4.1. STEC population reductions achieved at three anatomical locations on the exterior surface of skin-on pork carcasses. Carcass core samples were excised and enumerated on MacConkey agar with a tryptic soy agar (TSA) overlay to allow for enumeration of injured cells. The main effect of anatomical location was significant (P=0.035).

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Chapter 5 - Conclusions

5.1 Carcass-level comparisons

For the exterior, skin-on carcass surface, the deluge wash treatments *h*PAA, *h*DBDMH$_{600}$, and *h*HW resulted in greater reductions in both *Salmonella* and STEC populations on the pork carcass compared to the *h*AW control wash. With pre-treatment inoculation levels of ca. 5 log CFU/cm^2, these intervention technologies achieved *Salmonella* reductions of 2.8 to 3.1 log CFU/cm^2 (Figure 3.1), and slightly higher STEC population reductions of 3.2 to 3.8 log CFU/cm^2 (Table 4.3). In the *Salmonella* study, the remaining intervention technologies, both low-and high-volume, produced larger *Salmonella* reductions (2.0 to 2.4 log CFU/cm^2) compared to the *h*AW control; however, the reductions were not statistically significant ($P>0.05$). Similarly, in the STEC study, the remaining intervention technologies produced similar ($P>0.05$) STEC reductions (1.6 to 2.0 log CFU/cm^2) compared to the *h*AW control. The *h*DBDMH$_{400}$ treatment achieved a greater ($P\leq0.05$) STEC population reduction (2.4 log CFU/cm^2) compared to the *h*AW control; however, this population reduction was smaller than ($P\leq0.05$) what was achieved by the *h*PAA, *h*DBDMH$_{600}$, and *h*HW applications. Generally, the low-volume intervention applications provided lower (≤2.0-log) STEC reductions compared to the high-volume applications (2.4-3.8-log reductions) by the completion of the 18-h air-chilling period.

In both studies (*Salmonella* and STEC), the intervention technologies applied were generally more effective on the exterior, skin-on carcass surface compared to the lean tissue of the inside body cavity. For the inside cavity of the carcass sides, applying *h*PAA produced a 1.7 log CFU/cm^2 *Salmonella* reduction (Figure 3.2), which was significantly more effective than all other intervention technologies ($P\leq0.05$) except 3% *l*LA and *h*DBDMH$_{600}$, which resulted in statistically

similar (P>0.05) 1.2 and 1.1 log CFU/cm^2 reductions, respectively. The remaining intervention technologies achieved *Salmonella* reductions that ranged from 0.5 to 1.0 log CFU/cm^2, whereas the *h*AW control reduced the *Salmonella* population by only 0.1 log CFU/cm^2. In the STEC study (Table 4.4), the *h*PAA treatment also achieved the largest STEC reduction (post-treatment: 2.1 log CFU/cm^2)*;* however, this reduction was statistically similar (P>0.05) to other treatments, including the *h*AW control (1.0 log CFU/cm^2), except for *h*HW, *l*LA, and *h*DBDMH$_{400}$ that resulted in 0.8, 0.6, and 0.2 log CFU/cm^2 reductions, respectively.

The studies described herein used equipment capable of delivering similar carcass wash/spray parameters to those used in commercial establishments. Therefore, this research can be immediately applied by pork processors. The intervention technologies investigated do not negatively impact carcass color and provide STEC reductions.

5.2 Gaps in pork-related food safety

Even though pork is less associated with foodborne illnesses than other meat sources, it remains significant because it is the most widely consumed meat globally and includes a wide variety of products (Baer et al., 2013). There are still gaps in the control of many foodborne pathogens in pork (Baer et al., 2013). The contamination of pork products with foodborne pathogens starts on the farm, with differences in production practices contributing to potential transmission between animals due to contaminated floor samples and contaminated feed (Baer et al. 2013). Contamination from colonized and non-colonized pigs can also occur during the transportation and holding before slaughter (Baer et al., 2013). However, the slaughterhouse has a crucial role in ensuring food safety because several slaughter process stages can lead to carcass contamination (Baer et al., 2013; Hdaifeh et al., 2020). The use of antimicrobial intervention washes is very common in the beef industry for pathogen control; however, it is not as common in

pork processing facilities. Therefore, there is limited information about the efficacy of these interventions on pork. However, the U.S. Department of Agriculture's (USDA) final rule to modernize swine slaughter inspection has increased pork processors' interest in new intervention technologies (Food Safety and Inspection Services, 2019). The final rule has new requirements for microbial testing for all swine slaughterhouses to demonstrate that they control pathogens throughout the slaughter process (Food Safety and Inspection Services, 2019). Some studies have shown the efficiency of different antimicrobial wash interventions on pork carcasses; however, the use of these antimicrobials has not been demonstrated to be as effective on pork subprimals and trim. Thus, further research is needed on new methods to reduce pathogens on these pork products. Industry efforts should be made throughout the whole production chain, with a higher priority for interventions at the slaughterhouse to improve pork-related food safety (Hdaifeh et al., 2020).

5.3 Suggested further research

Further research is necessary to determine whether the lower pathogen reductions observed in the current studies on the inside carcass cavity could be due to: a higher propensity and/or strength of microbial attachment to the smooth lean surfaces (e.g., diaphragm region tissue), a difference in physical contact properties (e.g., hydrophobic surface characteristics, buffering capacity of the lean tissue compared to collagen or adipose carcass surfaces) of aqueous-based treatments, or shielding and spray angle limitations of antimicrobial delivery equipment being utilized for body cavity anatomical areas.

Research combining multiple intervention treatments using high-volume 71.1 °C (160 °F) (at the nozzle) water followed by an organic acid rinse, for example, particularly for pre-chill carcass decontamination, could result in better results and ensure *Salmonella* and STEC reductions on the inside carcass cavity. The limited pathogen reductions observed on the chilled pork trim

and subprimals in this study are not surprising. Previous studies have demonstrated that antimicrobial sprays are less effective when applied to chilled meat surfaces (Zhao et al., 2014; Fabrizio and Cutter, 2004; Choi et al., 2009). Therefore, this also substantiates the need for more research on different intervention technologies (or their combination) and application methods to obtain better results. Future investigations should further focus on the validation of intervention technologies that can provide pathogen reductions on pre-rigor skinned pork carcasses used to manufacture fresh pork sausage. The results of previous studies on pre-chill beef carcasses suggest that these types of chemical intervention technologies could effectively reduce pathogens on skinned pork carcass surfaces (Kocharunchitt et al., 2020; Signorini et al., 2018).

Other pathogens of importance for the pork industry include *Staphylococcus aureus* enterotoxin, *Clostridium perfringens, Yersinia enterocolitica, Campylobacter* spp., *Listeria monocytogenes*, and *Bacillus cereus* (Baer et al., 2013; Self et al., 2017). Thus, further research could focus on evaluating antimicrobial intervention technologies' efficacy to reduce the populations of other pathogens on pork carcasses and chilled pork. Particularly, it would be interesting to assess the intervention technologies' effectiveness at reducing pathogenic Gram-positive bacterial populations, as they have a different cellular structure and physiological characteristics.

Moreover, for both the carcass and subprimal level studies, other antimicrobial technologies are, or will be, available for pork processors that the current studies did not evaluate. These technologies should be assessed similarly, particularly where a spray wash technology demonstrates unique characteristics, such as lipophilic properties, a different mode of antimicrobial action, or an extended period of antimicrobial efficacy (e.g., residual antimicrobial efficacy during carcass dripping and chilling).

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Appendix A - Additional tables

Appendix A.1. *Salmonella* reductions on the exterior, skin-on surface of pork carcass sides following antimicrobial spray intervention applications and after 18 h of chilling at 2 °C[a]. Carcass core samples were excised and enumerated on Xylose Lysine Desoxycholate agar (XLD) with a Tryptic Soy Agar (TSA) overlay to allow for enumeration of injured cells.

	LS means ± SE *Salmonella* reductions (log CFU/cm^2)	
Treatments	Post-treatment	Post-chilling
PAA 400 ppm (H)	3.2 ± 0.4	3.0 ± 0.4
DBDMH 600 ppm (H)	3.1 ± 0.4	3.1 ± 0.4
Hot Water (H)	2.4 ± 0.4	3.2 ± 0.4
DBDMH 400 ppm (H)	2.1 ± 0.4	2.7 ± 0.4
Acidified PAA 400 ppm (L)	2.0 ± 0.4	2.8 ± 0.4
PAA 400 ppm (L)	1.8 ± 0.4	2.2 ± 0.4
LAC 3% (L)	1.5 ± 0.4	2.9 ± 0.4
Ambient Water (H)	1.1 ± 0.4	1.8 ± 0.4

[a] The treatment x time interaction was not significant (P=0.185). Table is included for informational purposes only.

Appendix A.2. *Salmonella* reductions on the internal body cavity (lean tissue surface) of pork carcass sides following antimicrobial spray intervention applications and after 18 h of chilling at 2 °C[a]. Carcass core samples were excised and enumerated on Xylose Lysine Desoxycholate agar (XLD) with a Tryptic Soy Agar (TSA) overlay to allow for enumeration of injured cells.

	LS means ± SE *Salmonella* reductions (log CFU/cm^2)	
Treatments	Post-treatment	Post-chilling
PAA 400 ppm (H)	2.0 ± 0.3	1.5 ± 0.3
DBDMH 600 ppm (H)	1.3 ± 0.3	1.0 ± 0.3
LAC 3% (L)	0.8 ± 0.3	1.5 ± 0.3
DBDMH 400 ppm (H)	0.8 ± 0.3	1.1 ± 0.3
PAA 400 ppm (L)	0.8 ± 0.3	0.8 ± 0.3
Acidified PAA 400 ppm (L)	0.7 ± 0.3	1.0 ± 0.3
Hot Water (H)	0.6 ± 0.3	0.4 ± 0.3
Ambient Water (H)	0.1 ± 0.3	0.2 ± 0.3

[a] The treatment x time interaction was not significant (P=0.096). Table is included for informational purposes only.

Appendix A.3. *Salmonella* reductions on chilled pork samples following antimicrobial spray intervention applications and throughout 14 days of vacuum-packaged storage at 2 °C[a]. Butt core samples were excised and enumerated on Xylose Lysine Desoxycholate agar (XLD) with a Tryptic Soy Agar (TSA) overlay to allow for enumeration of injured cells.

	LS means ± SE *Salmonella* reductions (log CFU/cm^2)		
Treatments	Day 0	Day 7	Day 14
Ambient Water	0.0 ± 0.21	0.6 ± 0.21	0.4 ± 0.21
Acidified PAA 400ppm	0.4 ± 0.21	0.9 ± 0.21	1.2 ± 0.21
PAA 400ppm	0.4 ± 0.21	0.7 ± 0.21	1.1 ± 0.21
LAC 2%	0.5 ± 0.21	1.2 ± 0.21	1.1 ± 0.21
DBDMH 400ppm	0.5 ± 0.21	1.0 ± 0.21	0.9 ± 0.21
DBDMH 600ppm	0.6 ± 0.21	0.9 ± 0.21	1.1 ± 0.21

[a] The treatment x time interaction was not significant (P=0.852). Table is included for informational purposes only.

Appendix A.4. *Salmonella* reductions on pork trim samples following antimicrobial intervention applications and throughout 4 days of aerobic storage at 2 °C[a]. Trim samples were enumerated on xylose lysine desoxycholate agar (XLD) with a Tryptic Soy Agar (TSA) overlay to allow for enumeration of injured cells.

	LS means ± SE *Salmonella* reductions (log CFU/g)		
Treatments	Day 0	Day 2	Day 4
Ambient water	0.0 ± 0.12	0.0 ± 0.12	0.2 ± 0.12
Acidified PAA 400 ppm	0.1 ± 0.12	0.3 ± 0.12	0.5 ± 0.12
PAA 400 ppm	0.1 ± 0.12	0.1 ± 0.12	0.2 ± 0.12
LAC 2%	0.2 ± 0.12	0.0 ± 0.12	0.2 ± 0.12
DBDMH 400 ppm	0.1 ± 0.12	0.2 ± 0.12	0.2 ± 0.12
DBDMH 600 ppm	0.0 ± 0.12	0.2 ± 0.12	0.3 ± 0.12

[a] The treatment x time interaction was not significant (P=0.679). Table is included for informational purposes only.

Appendix A. 5. Total aerobic plate counts (APC) on pork trim, pre- and post-antimicrobial spray intervention application, and throughout 4 days of aerobic package storage at 2 °C[a]. Trim samples were enumerated on APC Petrifilm™.

	LS means ± SE APC counts (log CFU/g)			
Treatments	Day 0 Pre-treatment	Day 0 Post-treatment	Day 2	Day 4
Ambient water	4.1 ± 0.4	3.7 ± 0.4	4.0 ± 0.4	3.6 ± 0.3
Acidified PAA 400 ppm	3.7 ± 0.4	3.8 ± 0.4	3.8 ± 0.4	3.6 ± 0.3
PAA 400 ppm	3.5 ± 0.4	2.8 ± 0.4	3.2 ± 0.4	2.6 ± 0.3
LAC 2%	3.4 ± 0.4	3.4 ± 0.4	3.9 ± 0.4	3.2 ± 0.3
DBDMH 400 ppm	3.2 ± 0.4	3.3 ± 0.4	3.7 ± 0.4	3.1 ± 0.3
DBDMH 600 ppm	3.2 ± 0.4	3.2 ± 0.4	3.2 ± 0.4	2.8 ± 0.3

[a] The treatment x time interaction was not significant (P=0.975). Table is included for informational purposes only.

Appendix A.6. Lactic acid bacteria (LAB) populations on pork loin samples pre- and post-antimicrobial spray intervention applications, and throughout 28 days of vacuum-packaged storage at 2 °C[a]. Loin core samples were excised and enumerated on APC Petrifilm™ using DeMan, Rogosa and Sharpe (MRS) broth as a diluent.

	LS means ± SE Lactic acid bacteria counts (log CFU/cm^2)					
Treatments	Day 0 Pre-treatment	Day 0 Post-treatment	Day 7	Day 14	Day 21	Day 28
Ambient Water	2.5 ± 0.4	2.5 ± 0.4	2.4 ± 0.4	2.0 ± 0.4	1.8 ± 0.4	1.7 ± 0.4
Acidified PAA 400 ppm	2.2 ± 0.4	1.8 ± 0.4	1.9 ± 0.4	2.1 ± 0.4	1.2 ± 0.4	1.3 ± 0.4
LAC 2%	2.1 ± 0.4	1.8 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	1.4 ± 0.4	1.6 ± 0.4
DBDMH 600ppm	2.1 ± 0.4	1.8 ± 0.4	1.0 ± 0.4	1.3 ± 0.4	1.5 ± 0.4	1.0 ± 0.4
DBDMH 400ppm	2.0 ± 0.4	1.8 ± 0.4	2.1 ± 0.4	1.5 ± 0.4	1.6 ± 0.4	2.1 ± 0.4
PAA 400ppm	1.3 ± 0.4	1.9 ± 0.4	1.0 ± 0.4	1.4 ± 0.4	1.0 ± 0.4	1.1 ± 0.4

[a] The treatment x time interaction was not significant (P=0.284). Table is included for informational purposes only.

Appendix A.7. Pork loin TBARS values following antimicrobial intervention applications and throughout 28 days of vacuum-packaged storage at 2 °C[a]. TBARS values were determined using a spectrophotometer and compared to a standard curve.

	LS means ± SE mg MDA/kg of meat				
Treatments	Day 0	Day 7	Day 14	Day 21	Day 28
Ambient Water	0.55 ± 0.12	0.61 ± 0.12	0.44 ± 0.12	0.33 ± 0.12	0.33 ± 0.12
Acidified PAA 400 ppm	0.37 ± 0.12	0.31 ± 0.12	0.62 ± 0.12	0.74 ± 0.12	0.32 ± 0.12
PAA 400 ppm	0.49 ± 0.12	0.30 ± 0.12	0.54 ± 0.12	0.51 ± 0.12	0.41 ± 0.12
LAC 2%	0.42 ± 0.12	0.41 ± 0.12	0.39 ± 0.12	0.39 ± 0.12	0.37 ± 0.12
DBDMH 400 ppm	0.55 ± 0.12	0.42 ± 0.12	0.41 ± 0.12	0.42 ± 0.12	0.30 ± 0.12
DBDMH 600 ppm	0.46 ± 0.12	0.44 ± 0.12	0.57 ± 0.12	0.46 ± 0.12	0.34 ± 0.12

[a] The treatment x time interaction was not significant (P=0.765). Table is included for informational purposes only.

Appendix A.8. Trim TBARS values following antimicrobial intervention application and throughout 4 days of aerobic-packaged storage at 2 °C[a]. TBARS values were determined using a spectrophotometer and compared to a standard curve.

	LS means ± SE mg MDA/kg of meat		
Treatments	Day 0	Day 2	Day 4
Ambient water	0.35 ± 0.22	0.70 ± 0.22	0.40 ± 0.22
Acidified PAA 400 ppm	0.33 ± 0.22	0.47 ± 0.22	0.78 ± 0.22
PAA 400 ppm	0.37 ± 0.22	0.36 ± 0.22	0.72 ± 0.22
LAC 2%	0.48 ± 0.22	0.54 ± 0.22	1.02 ± 0.22
DBDMH 400 ppm	0.30 ± 0.22	0.82 ± 0.22	0.92 ± 0.22
DBDMH 600 ppm	0.39 ± 0.22	0.34 ± 0.22	0.76 ± 0.22

[a] The treatment x time interaction was not significant (P=0.902). Table is included for informational purposes only.

Appendix A.9. Pork loin L*, a*, and b* color values following antimicrobial intervention applications and throughout 28 days of vacuum-packaged storage at 2 °C[1]. L*, a*, and b* values were quantified using a handheld colorimeter.

Treatments	LS means ± SE Color (L* a* and b* values)								
	Day 0 pre-treatment			Day 0 post-treatment			Day 7		
	L*	a*	b*	L*	a*	b*	L*	a*	b*
Ambient Water	54.3 ± 2.1	10.5± 0.9	17.8± 0.6	56.3± 1.8	8.7± 0.8	16.7± 1.1	57.6± 1.3	7.3± 0.8	14.6± 0.7
Acidified PAA 400 ppm	52.8± 2.1	9.3± 0.9	17.0± 0.6	53.0± 1.8	10.0± 0.8	16.9± 1.1	56.3± 1.3	7.5± 0.8	14.6± 0.7
PAA 400 ppm	56.4± 2.1	8.1± 0.9	16.7± 0.6	58.3± 1.8	7.5± 0.8	16.6± 1.1	59.9± 1.3	6.0± 0.8	13.6± 0.7
LAC 2%	55.4± 2.1	9.5± 0.9	17.6± 0.6	56.8± 1.8	8.1± 0.8	15.8± 1.1	58.7± 1.3	8.3± 0.8	15.8± 0.7
DBDMH 400 ppm	56.1± 2.1	9.3± 0.9	17.0± 0.6	56.5± 1.8	9.2± 0.8	16.0± 1.1	57.5± 1.3	7.4± 0.8	14.8± 0.7
DBDMH 600 ppm	55.3± 2.1	7.9± 0.9	16.2± 0.6	56.1± 1.8	8.3± 0.8	15.9± 1.1	59.6± 1.3	6.9± 0.8	14.3± 0.7

[1] The treatment x time interaction was not significant for L*(P= 0.604), a*(P=0.739), and b* (P=0.865). Table is included for informational purposes only.

Appendix A.9 continued

Treatments	LS means ± SE Color (L* a* and b* values)								
	Day 14			Day 21			Day 28		
	L*	a*	b*	L*	a*	b*	L*	a*	b*
Ambient Water	59.0± 1.0	7.8± 0.6	16.7± 1.2	60.4± 0.8	6.8± 0.6	15.5± 0.9	58.0± 1.3	8.2± 0.6	16.0± 0.9
Acidified PAA 400 ppm	60.1± 1.0	7.0± 0.6	15.0± 1.2	59.9± 0.8	7.5± 0.6	15.2± 0.9	62.8± 1.3	6.8± 0.6	14.7± 0.9
PAA 400 ppm	59.6± 1.0	7.7± 0.6	15.7± 1.2	59.6± 0.8	6.9± 0.6	16.3± 0.9	60.5± 1.3	7.5± 0.6	15.9± 0.9
LAC 2%	59.5± 1.0	7.6± 0.6	16.0± 1.2	60.3± 0.8	7.1± 0.6	14.7± 0.9	59.9± 1.3	7.5± 0.6	16.1± 0.9
DBDMH 400 ppm	58.6± 1.0	7.3± 0.6	16.1± 1.2	60.5± 0.8	7.1± 0.6	15.7± 0.9	61.1± 1.3	7.0± 0.6	14.6± 0.9[£]
DBDMH 600 ppm	63.0± 1.0	6.8± 0.6	15.0± 1.2	60.3± 0.8	7.3± 0.6	15.4± 0.9	62.1± 1.3	6.5± 0.6	13.3± 0.9

[1] The treatment x time interaction was not significant for L*(P= 0.604), a*(P=0.739), and b* (P=0.865). Table is included for informational purposes only.