SEQUENTIAL APPLICATION OF ε-POLYLYSINE, LAURIC ARGINATE AND ACIDIC CALCIUM SULFATE FOR INACTIVATION OF PATHOGENS ON RAW CHICKEN AND BEEF

A Dissertation

by

HAKAN BENLI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Food Science and Technology

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ABSTRACT

Sequential Application of ε-Polylysine, Lauric Arginate and Acidic Calcium Sulfate for Inactivation of Pathogens on Raw Chicken and Beef. (August 2008)

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Salmonella and *Escherichia coli* O157:H7 (EC) contamination continues to be one of the major concerns for the microbiological safety of raw poultry and beef products. Application of more than one decontamination agent as a multi-hurdle intervention to carcasses in a processing line might produce greater reductions than one treatment alone due to different modes of action of individual antimicrobials. In this study, sequential spray applications of ε-polylysine (EPL), lauric arginate and acidic calcium sulfate (ACS) solutions were evaluated against *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (ST) on artificially inoculated broiler carcasses and against ST and EC on beef rounds and ground beef derived from the rounds.

All possible 2-way combinations and individual applications of 20 % ACS (ACS20), 300 mg/liter EPL (EPL300) and 200 mg/liter LAE (LAE200) were evaluated using a sterile membrane filter model system. The combinations that provided higher *Salmonella* reductions were further evaluated on inoculated chicken carcasses using either response surface methodology (RSM) or in various concentrations applied in a

sequential manner. Sequential spray applications of EPL300 - ACS 30 % (ACS30) or LAE200-ACS30 produced the highest *Salmonella* reductions on inoculated chicken carcasses. In a subsequent experiment, treatment of *Salmonella* inoculated carcasses with EPL300-ACS30 or LAE200-ACS30 combinations were found effective for reducing initial *Salmonella* counts by 1.5 and 1.8 log CFU/ml, respectively, immediately after treatment and by 1.2 and 1.8 log CFU/ml, respectively, following 6 days of storage at 4.4 °C. Evaluation of the resident microflora including aerobic plate counts (APC), *E. coli*, coliforms and psychrotrophs on uninoculated chicken carcasses after treatment with EPL300-ACS30 or LAE200-ACS30 and during storage indicated that these treatments have the potential to increase the shelf-life of poultry carcasses. Furthermore, application of warm (55 °C) EPL300-ACS30 or LAE200-ACS30 onto inoculated beef rounds reduced both ST and EC counts over 6 days of storage at 4.4 °C by 4.5 and 4.3 log CFU/cm^2, respectively. Ground beef manufactured with EPL300-ACS30 or LAE200-ACS30 treated rounds had lower ST and EC counts initially and stayed lower over 4 days of storage at 4.4 °C when compared to control.

DEDICATION

I dedicate this dissertation in honor of my parents Abdullah and Buseyme Benli, my sisters Fatma Deniz, Canan and Derya, my brother Gokhan, my niece Beyza and my nephews Abdullah and Musa Mert who provided the inspiration for pursuit of a further degree, and whose love and encouragement have sustained me throughout my college and graduate education.

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CHAPTER I

INTRODUCTION

The United States Department of Agriculture's Food Safety Inspection Service (USDA-FSIS) issued the Pathogen Reduction: Hazard Analysis and Critical Control Point (PR/HACCP) regulation on July 25, 1996. PR/HACCP established pathogen reduction requirements applicable to meat and poultry establishments to reduce the occurrence and numbers of pathogens in meat and poultry products *(46, 71)*. Foodborne diseases have been estimated to be responsible for approximately 76 million illnesses, 325,000 hospitalizations and 5,000 deaths in the United States each year *(46)*. Furthermore, an estimated 14 million illnesses, 60,000 hospitalizations, and 1,500 deaths have been attributed to known pathogens such as *Salmonella*, *Listeria* and *Toxoplasma (46)*. *Salmonella* is a pathogen of concern in raw meat products, and is one of the most common causes of foodborne illnesses in humans with an estimated 1.4 million cases and 500 deaths annually in the United States *(46, 60)*. In 2006, the USDA-FSIS announced changes in its *Salmonella* verification sampling program for meat and poultry establishments to enhance public health and grouped establishments into one of three categories based on the testing results *(72)*. Establishments grouped into Category 1 showed consistent process control for *Salmonella* reduction, while establishments grouped into Category 3 had highly variable process control for *Salmonella* reduction

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This dissertation follows the style of *Journal of Food Protection*.

and were subjected to more frequent testing. In 2000, the U.S. Department of Health and Human Services (USHHS) set a Healthy People goal of 6.8 *Salmonella* cases per 100,000 persons that was to be achieved by 2010 *(79)*. However, recent data from the Centers for Disease Control and Prevention indicates only a slight decline in the incidence of *Salmonella* from 16.8 cases per 100,000 persons in 1996-98 to 14.9 cases per 100,000 persons in 2007 *(15)*.

POULTRY CARCASS DECONTAMINATION

The USDA-FSIS has estimated that in 2007 poultry products accounted for approximately 60% of the foodborne illnesses originating from *Salmonella (76)*. To meet the Healthy People 2010 goal, USDA-FSIS has set an objective that 90 % of broiler establishments should be in Category 1 by 2010, but as of 2007 only 74 % of the establishments were reported in Category 1 *(74, 76)*. In PR/HACCP, *Salmonella* was selected as the target organism for sampling of poultry carcasses and raw products to verify that establishments met the PR/HACCP standards. Establishments must also conduct testing for generic *Escherichia coli* to verify that their process is under control *(52, 71, 72)*. The USDA-FSIS *Salmonella* verification testing program reported the following percent positive samples for broilers in each respective year: 8.5 %, 2007; 11.4 %, 2006; 16.3 %, 2005; 13.5 %, 2004; 12.8 %, 2003; and 11.5 %, 2002 *(72, 74)*.

During poultry processing various processing steps have been reported to contribute to contamination or cross-contamination of the carcasses including live receiving, immobilization, bleeding, scalding, feather removal, evisceration and chilling *(75, 76)*. The pathogen intervention used most for removing carcass fecal contamination

is an inside-outside cabinet washer which has been reported to slightly reduce aerobic plate counts, as well as *Salmonella*, *Campylobacter*, *E. coli* and coliforms on carcasses *(6, 23, 33, 34, 42, 52, 53, 67, 68).*

A wide variety of immersion or spray intervention methods to reduce or eliminate pathogens on poultry carcasses have been comprehensively reviewed by several authors including Keeton and Eddy *(36)* and Ricke et al. *(60).* Numerous interventions have also been studied. Morris and Fleet *(49)* reported that immersion treatment of inoculated chicken carcasses with hot water (60 °C) reduced *Salmonella* Typhimurium (ST) by 2 logs, and with the addition of 200 ppm chlorine or 2.5 % potassium sorbate to the immersion water, *Salmonella* were reduced by 3 logs. Northcutt et al. *(53)* on the other hand reported that spray washing of chicken carcasses with chlorinated water (50 ppm) and/or increasing water temperature (21.1, 43.3 or 54.4 °C) in an inside-outside bird washer were not effective for reducing total aerobic bacteria, *E. coli* and *Campylobacter* counts. However, they did report a 0.7 to 1.1 log reduction in *Salmonella* levels on contaminated carcasses. Berrang et al. *(3)* found that a second scald applied after defeathering either as an immersion treatment at 60 °C (28 s immediately or 30 min after defeathering) or as a spray treatment at 71-73 °C (20 s immediately or 30 min after defeathering) was not effective for reducing *Campylobacter, E. coli* and coliforms on chicken carcasses. In contrast, chilling with 20 ppm sodium hypochlorite solution reduced coliforms, *Campylocater, E. coli* and *Salmonella* counts by 1.2, 1.3, 1.4 and 0.5 logs, respectively *(51).*

Bourassa et al. *(5)* reported that prechill trisodium phosphate treatment reduced salmonellae-positive samples immediately after chilling or following 7 days of storage on broiler carcasses. Li et al. *(43)* tested 0.85 % sodium chloride, 5 or 10 % trisodium phosphate, 5 or 10 % sodium bisulfate, 0.1 % cetylpyridinium chloride or 1 % lactic acid sprays on prechilled chicken carcasses. Spraying sodium chloride was not found to be effective for *Salmonella* reduction, but they did note reductions of 3.7, 2.4, 1.6 or 1.6 logs of ST with 90 s treatments of 10 % trisodium phosphate, 10 % sodium bisulfate, 0.1 % cetylpyridinium and 1 % lactic acid, respectively. In another study, Mulder et al. *(50)* reported that lactic acid (1 %) and hydrogen peroxide (0.5 %) immersions of chicken carcasses with 5 or 10 min exposure times were highly effective for reducing ST. They observed 4 log reductions of *Salmonella* in pure cultures and on artificially inoculated broiler carcasses in a laboratory setting.

Sakhare et al. *(63)* found that acetic acid (0.5 %) or lactic acid (0.25 %) treatments applied by either dipping or spraying after scalding, defeathering and evisceration of chicken carcasses were more effective than spray washing with water alone to decrease cross contamination and improve microbial quality.

Sinhamahapatra et al. *(66)* tested the effects of hot water (70 °C for 1 min), 2 % lactic acid (30 s), 1200 ppm acidified sodium chlorite (5 s) and 50 ppm chlorine solution (5 min) applied to broiler carcasses as an immersion or spray treatment. The lactic acid dip and hot water dip were the most effective for reducing aerobic plate counts by 1.36 log and 1.28 log /cm^2, respectively, whereas acidified sodium chlorite and a hot water dip reduced presumptive coliforms counts by 1.37 log and 1.34 log/cm^2. In a more recent

study, Mehyar et al. *(47)* evaluated trisodium phosphate (10 %), lactic acid (3 %) with or without sodium chloride (2 %) as well as commercial antimicrobials including acidified sodium chlorite (Sanova®), acidified calcium sulfate ($Safe_2O$®), cetylpyridinium chloride (Cecure®), peroxyacetic acid formulated with hydrogen peroxide, octanoic acid and acetic acid (Inspexx™ 100) dips for improving microbiological quality of chicken drumettes. Trisodium phosphate was found twice as effective as lactic acid for reducing *E. coli* O157:H7 (EC) (> 2 log CFU/g reduction) on chicken skin with and an exposure time of 1 min. They reported that reductions of *Salmonella, C. jejuni* and EC were not significantly different among antimicrobials including trisodium phosphate, Sanova®, $Safe_2O$®, Cecure®, and Inspexx™ 100. It was also noted that all treatments delayed growth of pseudomonads and psychrotrophs on chicken skin when samples were stored aerobically at 7 °C. Del Rio et al. *(16)* reported that 12 % trisodium phosphate, 1200 ppm acidified sodium chlorite, 2 % citric acid and 220 ppm peroxyacid (Inspexx™ 100) immersion solutions were effective on chicken legs for reducing microbial population including mesophilic aerobic counts, psychrotrophs, *Enterobacteriaceae*, coliforms, *Micrococcaceae*, enterococci, *Brochothrix thermosphacta*, pseudomonads, lactic acid bacteria, molds and yeasts during 5 days of storage at 3 °C.

Hinton et al. *(29)* studied the effects of acidic, electrolyzed oxidizing water and chlorinated water sprays on the spoilage microflora of broiler carcasses during 14 days of storage (4 °C). They reported that psychrotrophic bacteria were reduced immediately after spraying with acidic, electrolyzed oxidizing water as well as chlorinated water and that these reductions persisted after 14 days of storage.

BEEF CARCASS DECONTAMINATION

Foodborne Diseases Active Surveillance Network (FoodNet) data for 2007 indicated that no significant reductions in *E. coli* and *Salmonella* infections have occurred in the United States when compared with the 2004-2005 data *(15)*. Contamination of beef carcass surfaces during the slaughter process with EC and *Salmonella* occur due to contact with feces and the hide which are the most likely sources of contamination *(21, 31, 36, 39, 55)*. Beef carcass decontamination methods in the literature that have been found to reduce or eliminate pathogens include: carcass trimming, steam vacuuming, steam pasteurization, cold and hot water rinses, organic acid rinses, ozonated or electrolyzed water, and a variety of chemical rinses including chlorine, chlorine dioxide, trisodium phosphate, peroxyacetic acid, cetylpyridinium chloride, acidified sodium chlorite, or acidic calcium sulfate *(7, 21, 31, 36, 40, 60)*.

Huffman *(31)* noted that hot water applications have the potential of reducing bacterial counts by 1-3 log cycles on beef carcasses. Castillo et al. *(9)* likewise reported that a water wash followed by hot water spray (95 °C) reduced levels of EC, ST, APC and coliforms by 3.7, 3.8, 2.9 and 3.3 log, respectively, on carcass surfaces. Spray-washing (26 °C, 276 kPa followed by 1000 kPa) followed by hot-water rinsing (>77 °C, 138-152 kPa, 2.5 to 8 s) and knife-trimming followed by a second spray-wash also have been shown to be an effective beef carcass decontamination method *(17)*. Hardin et al. *(27)* found that carcass washing followed by warm acid sprays (55 °C) of lactic acid or acetic acid performed better than trimming or washing alone for reducing *Salmonella* and EC and that lactic acid was more effective than acetic acid for EC reduction.

Castillo et al. *(8)* reported that both a water wash and trimming combined with sanitizing treatments of hot water (95 °C) or warm (55 °C) 2 % lactic acid spray or a combination of these two sanitizing methods resulted in reductions of more than 4.0 log CFU/cm^2 for ST and EC on beef carcasses. A 4% L-lactic acid spray at 55 °C prior to fabrication has also been suggested for chilled beef carcasses which were previously subjected to a hot water spray followed by a lactic acid spray (details of prechill decontamination steps were not made available by the authors due to proprietary reasons) prior to chilling *(11)*. In another study, both EC and ST counts were reduced by 3.8 to 3.9 log and 4.5 to 4.6 log with a water wash followed by a phosphoric acid-activated acidified sodium chloride spray or a citric acid-activated acidified sodium chlorite spray, respectively *(10)*.

Ramirez et al. *(59)* tested a water rinse followed by either a 2 % lactic acid (9 s, at 55 °C) or a 12 % trisodium phosphate (60 s, at 55 °C) dip or a combination of these treatments. Both treatments alone or in combination were effective for reducing EC more than 1.6 log/cm^2 on lamb breast tissue.

In a comparative study, King et al. *(38)* reported that a peroxyacetic acid spray was not an effective intervention for EC and ST reduction on chilled beef carcasses when compared to carcasses treated with 2 % L-lactic acid spray before chilling or 4 % L-lactic acid spray after chilling. In another study, Castillo et al. *(13)* reported that aqueous ozone treatment (28 °C; 95 mg/liter) was not found to be effective against EC and ST when sprayed on hot carcass surfaces as compared to a water wash (28 °C) alone.

Dorsa et al. *(20)* tested the effect of 2 % lactic acid, 2 % acetic acid, 12 % trisodium phosphate, and water washes at 72 °C and 32 °C for reducing pathogens and other bacterial populations on beef carcass surfaces and cuts held for up to 21 days (4 °C) under vacuum. They found that lactic acid and acetic acid treatments suppressed or eliminated bacteria on beef carcass surfaces inoculated with low levels (< 2 log) of *Listeria innocua,* ST, EC, and *Clostridium sporogenes* in a bovine fecal cocktail during refrigerated storage. Additionally, Dorsa et al. *(19)* suggested that a 2 % lactic acid or 2 % acetic acid wash during beef carcass processing could lower the bacterial counts in ground beef.

Similarly, Castillo et al. *(12)* reported that pre-chill hot carcass water wash treatments alone or water wash followed by a 2 % lactic acid spray (250 ml, 15 s, 55 °C) produced 3.3 – 5.2 log reductions of EC and ST on contaminated outside rounds. They then applied a post-chill, 4 % lactic acid spray (500 ml, 30 s, 55 °C) that further reduced EC and ST counts by 2.0-2.4 log and 1.6-1.9 log, respectively, when combined with the pre-chill hot carcass treatments. Moreover, significantly lower levels of these pathogens were also observed in ground beef produced from the post-chill decontaminated rounds when compared to ground beef from pre-chill decontaminated rounds alone.

Stivarius et al. *(69)* tested the effect of tumbling inoculated beef trimmings in hot water (82 °C) or 5 % lactic acid prior to grinding to reduce EC and ST in ground beef. They reported that lactic acid was effective for reducing EC counts in ground beef stored refrigerated 7 days, but also noted a reduction in the redness of the ground beef. Conversely, 10 % trisodium phosphate or 0.5 % cetylpyridinium chloride treatment

applied by tumbling significantly reduced EC and ST and improved the redness of ground beef *(58)*. Harris et al. *(28)* reported acidified sodium chlorite (1200 ppm), acetic acid (2 %) and lactic acid (4 %) spray treatments applied to inoculated beef trimmings prior to grinding reduced EC and ST counts by 2.5 log and 1.5 log, respectively, in the ground beef.

DECONTAMINATION AGENTS

Acidic calcium sulfate, also known as acidified calcium sulfate (ACS), is a very acidic (pH 1.0 - 1.5) blend of calcium hydroxide, sulfuric acid and calcium sulfate that has shown minimal corrosive properties to plastics, rubber, stainless steel or human skin *(4, 18, 48, 54, 83)*. Its use in the production of meat and poultry products has been approved as a secondary food additive by USDA-FSIS *(77)*. ACS plus organic acids act as a metabolic inhibitor by disabling the proton pumps in bacterial membranes and affecting bacteria in a different manner than organic acids alone *(35)*. In several studies, ACS's effectiveness for reducing pathogens has been demonstrated on beef or poultry carcass surfaces, RTE meat products such as frankfurters and hams, and in ground beef *(18, 31, 35-37, 45, 54, 83)*. Some claims have indicated that ACS prevents food borne pathogens such as *Escherichia coli, Salmonlla* spp., LM, and *Campylobacter jejeuni* from attaching to food surface and that a prolonged antimicrobial effect remains after application to carcasses *(48)*.

Keeton et al. *(37)* reported that a sequential warm (55 °C) spray application of ACS (20 %) followed by EPL (100 mg/L) at a constant pressure for 15 to 20 s was effective for reducing ST, EC and LM on beef samples. They reported a 4.38 log

Salmonella reduction after 7 days of storage. In the same study, individual applications of ACS (20 %), lactic acid (2.5 %), EPL (100 mg/L) and sterile distilled water spray were not found as effective as the application of ACS followed by EPL. They reported that no single treatment was as effective as the sequential treatment of ACS followed by EPL over the storage period.

Dickens et al. *(18)* found that spraying a higher concentration (1:1 solution of deionized water and $Safe_2O$ Poultry Wash) of ACS (4 ml/wing) increased the shelf life of chicken wings from 7 days to 10 days when compared with deionized water-spray controls. They reported counts of *Pseudomonas* sp., 8.2 and 6.9; *Staphylcoccus* sp., 5.5 and 4.9; LM, 5.2 and 4.6; and psychrotrophs, 8.2 and 6.9 for the water and $Safe_2O$ Poultry Wash treatment, respectively, at the end of 10 days refrigerated storage.

Zhao et al. *(83)* tested the combined effect of freezing and addition of a mixture of 20 % acidic calcium sulfate (0.4 % final concentration in ground beef) and 10 % lactic acid (0.2 % final concentration in ground beef) on thermal sensitivity of EC in ground beef. They found that addition of acidic calcium sulfate and lactic acid addition to ground beef reduced both the temperature and time required to inactivate EC during heating.

Nunez De Gonzalez et al. *(54)* reported that an acidic calcium sulfate with propionic acid and lactic acid (1:2 water) and lactic acid (3.4 % of a 88 % commercially available syrup) dips had bactericidal and bacteriostatic effects, respectively, when used as a post-processing dipping solution to inhibit or control the growth of LM on vacuum-packaged frankfurters stored at 4.5 °C for up to 12 weeks.

ε-Polylysine (EPL) is produced by *Streptomyces albulus* by a natural fermentation process and is a homo-poly-amino acid consisting of 25 to 35 L-lysine molecules connected by a peptide bond between the carboxyl and ε-amino groups. EPL has been shown to have a wide range of antimicrobial activity and is characterized as an edible, water soluble agent that is stable at high temperatures and under acidic and alkaline conditions *(25, 26, 30, 81, 82)*. Antimicrobial activity of EPL has been reported over a pH range of 5-8 and no structural changes have been observed by heat treatment of EPL at pH 3.0 *(81, 82)*. The proposed mechanism for the mode of action of EPL is electrostatic adsorption of EPL into the cell surface of microorganisms due to the molecule's cationic properties which cause further stripping of the outer membrane and distribution in the cytoplasm of microorganisms *(81, 82)*. EPL also has been studied to confirm its safety as a preservative in foods. It has been deemed non-toxic in rats in an acute oral toxicity study (no mortality at levels up to 5g/kg) and was not mutagenic in bacterial reversion assays *(30)*. Additionally, use of EPL as an antimicrobial agent in cooked or sushi rice at levels up to 50 mg/kg rice has been approved as generally recognized as safe (GRAS) by the United States Food and Drug Administration *(80)*.

Geornaras and Sofos *(25)* compared antimicrobial activity of EPL with sodium diacetate, sodium lactate, lactic acid and acetic acid, against different foodborne pathogens including, EC, ST, and LM in a culture broth medium. They concluded that EPL has minimum inhibitory concentrations of 0.02 % for EC and LM and 0.04 % for ST and that EPL inhibited growth of these foodborne pathogens at 24 °C. Enhanced antimicrobial activity has been reported when ELP was combined with 0.25 % sodium

diacetate or 0.1 % acetic acid. However, combining with 3.0 % sodium lactate resulted in a lost of antimicrobial activity of EPL. EPL also has been reported to have enhanced antimicrobial activity when combined with glycine, vinegar, ethanol and thiamine laurylsulfonate *(82)*. Keeton et al. *(37)* reported that sequential sprays of warm solutions (55 °C) of 20 % ACS followed by 100 mg/liter EPL was effective for reducing ST, EC, and LM on beef trimmings. In another study, EPL was found very effective against EC, ST and LM in food extracts with low protein levels such as rice and vegetables. However, lost of activity of the EPL was reported with extended storage of food extracts that contained high protein levels such as beef and bologna extract *(26)*.

Lauramide arginine ethyl ester (LAE), also known as lauric arginate, is an antimicrobial compound derived from lauric acid and arginine with a broad-spectrum antimicrobial activity *(2, 61)*. LAE has been verified to be non-toxic and is metabolized rapidly to naturally occurring amino acids, largely arginine and ornithine, after consumption *(62)*. LAE affects the cyptoplasmic membranes of microorganisms by causing a disruption or instability of the plasma membrane lipid bilayer thus further altering the metabolic process and detaining the cellular cycle *(2)*. LAE was confirmed as GRAS by the USDA-FSIS and is considered a safe and suitable ingredient when used in the production of meat and poultry products *(77)*.

Rodrigues et al. *(61)* exposed ST and *Staphylococcus aureus* to their minimal inhibitory concentrations of 32 and 8 μg/ml of LAE, respectively. They observed alterations mainly in the outer membrane of ST and in the cytoplasm of *S. aureus* after

exposure to LAE. Further, the proportions of damaged cells after 24 h contact time were reported as 97 % and 56.3 % for ST and *S. aureus*, respectively.

LAE has been reported to effectively inhibit the growth of LM in cooked meats during refrigerated storage *(2, 45)*. In addition, LAE and ACS used in combination were found highly effective in a patented "Spray Lethality In Container" (SLIC®) intervention delivery system for reducing LM in ready-to-eat products *(45)*.

Keeton and Eddy *(36)* reviewed chemical methods for decontamination of animal carcasses including chlorine-based derivatives, organic acids, organic and inorganic compounds, bacteriocins and emerging technologies as post-harvest interventions. They stated that no single decontamination method is right for the purpose of completely eliminating pathogens from raw materials due to surface geometries, protected sited of contamination, and inherent inefficiency of specific decontamination processes. Individual interventions in most cases are not as effective for reducing pathogens on poultry and beef carcasses as hurdle technology or a sequential interventions approach *(21, 31, 40, 56, 57, 60)*. Microorganisms operate homeostatically in order to survive under environmental stresses imposed by the interventions applied *(41)*. Application of two or more microbial decontamination treatments appears to produce greater reductions than one treatment alone due to different modes of action of the antimicrobials, thus overcoming the various homeostatic mechanisms of microorganisms.

Sequential application of the decontamination agents may result in greater reductions of pathogens because the first decontamination agent inactives some of the pathogens while injuring others; the second decontamination agent acts upon the injured

pathogens by a different mode of action. The purpose of this study was to evaluate combinations of antimicrobials, applied as a hurdle technology or in a sequential intervention approach, to serve as a more effective pathogen intervention strategy for chicken or beef carcasses.

The objectives of this study were to:

1) Determine the efficacy of individually and sequentially applied interventions including ACS, EPL and/or LAE for reducing *S.* Typhimurium and *S.* Enteritidis on a membrane filter model and on commercially processed broiler carcasses. The selection of effective intervention combinations was determined using response surface methodology.
2) Evaluate sequential application of ACS, EPL or LAE on poultry carcasses. By combining these treatments, potential synergistic interactions were expected to improve antimicrobial efficacy due to the different mode of action of individual decontamination agents.
3) Determine the effects of sequential application of EPL or LAE sprays followed by an ACS spray as a multi-hurdle intervention for reducing *Salmonella* on inoculated chicken carcasses stored for up to 6 days under refrigeration. Secondly, further reductions of the resident microflora including aerobic plate counts, *Escherichia coli*, coliforms and psychrotrophs on uninoculated chicken carcasses during processing steps and storage were hypothesized.

4) Determine effectiveness of sequential application of EPL or LAE followed by ACS for reducing EC and ST on inoculated beef surfaces and to determine if these reductions are carried over to ground beef during refrigerated storage.

CHAPTER II

EFFICACY OF ACIDIC CALCIUM SULFATE, ε-POLYLYSINE OR LAURIC ARGINATE APPLIED SEQUENTIALLY FOR *SALMONELLA* REDUCTION ON MEMBRANE FILTERS AND CHICKEN CARCASSES

INTRODUCTION

The United States Department of Agriculture's Food Safety Inspection Service (USDA-FSIS) issued the Pathogen Reduction: Hazard Analysis and Critical Control Point (PR/HACCP) rule on July 25, 1996. PR/HACCP established pathogen reduction requirements applicable to meat establishments to reduce the occurrence and numbers of pathogens in meat and poultry products and to reduce the risk of foodborne disease *(46, 71)*. Foodborne diseases have been estimated to be responsible for approximately 76 million illnesses, 325,000 hospitalizations and 5,000 deaths in the United States each year *(46)*. Furthermore, an estimated 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths were attributed to known pathogens while 1,500 deaths were caused by known pathogens such as *Salmonella*, *Listeria* and *Toxoplasma (46)*. The USDA-FSIS has recently reported a gradual increase in *Salmonella* positive carcass samples in broiler establishments from 11.5% in 2002 to 12.8% in 2003, 13.5% in 2004 and 16.3% in 2005 *(73)*. The Centers for Disease Control and Prevention (CDC) also has noted only a slight decrease in human cases of salmonellosis from 16.8 cases/100,000 persons in 1996-98 to 14.7 cases/100,000 persons in 2004 *(73)*. In addition, the data collected by USDA-FSIS from 2000 through 2005 showed that the annual number of isolates of *Salmonella*

enterica serovar Enteritidis in broiler carcass rinses increased more than four fold and that, the proportion of establishments with *S.* Enteritidis positive samples increased nearly three fold *(1)*.

In order to reduce the prevalence of *Salmonella* spp. in broiler carcasses, the industry has implemented a series of antimicrobial intervention treatments applied at different steps during processing. Some of these interventions include the use of chemical antimicrobials that are approved to be in contact with the carcass surface and can potentially reduce the presence of pathogens. However, more information is needed about the effects of some of these interventions on pathogen survival, and their actual efficacy when applied on the surface of chicken carcasses. Antimicrobials that have been used commercially in processing facilities include: chlorine derivatives, organic acids, and a wide array of agents that need to be validated in a commercial setting.

Acidic calcium sulfate (ACS) is approved by USDA-FSIS as a secondary food additive ingredient for use in the processing of meat and poultry products *(77)*. ACS is a highly acidic (pH ~1.0) blend of calcium hydroxide, sulfuric acid and calcium sulfate that has shown minimal corrosive properties to plastics, rubber, stainless steel or human skin *(4, 18, 48, 54, 83)*. ACS has been reported to be effective for reducing pathogens on the surface of beef and poultry carcasses, in ground beef and on ready-to-eat (RTE) meat and poultry products including frankfurters and hams *(18, 31, 35-37, 45, 54, 83)*. ACS plus organic acids act as a metabolic inhibitor by disabling the proton pumps in bacterial membranes and affecting bacteria in a different manner than organic acids alone *(35)*.

ε-Polylysine (EPL) is produced by *Streptomyces albulus* by a natural fermentation process. It is a homo-poly-amino acid of 25 to 35 L-lysine molecules connected by a peptide bond between the carboxyl and ε-amino groups. EPL has been shown to have a wide range of antimicrobial activity and is characterized as an edible, water soluble agent that is stable at high temperatures and under acidic and alkaline conditions *(25, 26, 30, 81, 82)*. EPL is a broad spectrum antimicrobial that is effective on both Gram-positive and Gram-negative bacteria. The proposed mechanism for the mode of action of EPL is electrostatic adsorption of EPL into the cell surface of microorganisms due to the molecule's cationic properties which cause further distribution of the outer membrane and in the cytoplasm *(81, 82)*. The manufacturers recommended use of EPL as an antimicrobial agent in cooked or sushi rice at levels up to 50 mg/kg of rice is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration *(80)*.

Lauramide arginine ethyl ester (LAE) also known as lauric arginate is a surfactant with broad-spectrum antimicrobial activity. It is derived from lauric acid and arginine *(2, 61)*. LAE is a USDA-FSIS approved ingredient *(77)* for use in the production of meat and poultry products with activity over a wide pH range of 3 to 7 *(2)*. The effects of LAE on Gram-negative and Gram-positive bacteria were studied by Rodriguez et al. *(61)*. They reported LAE to be effective on bacteria due to alterations in the microbial cell membrane, thus preventing their growth.

The objective of this study was to determine the efficacy of individually and sequentially applied interventions of ACS, EPL and/or LAE for reducing *Salmonella*

Typhimurium (ST) and *Salmonella* Enteritidis (SE) on a membrane filter model and on commercially processed broiler carcasses. The selection of effective intervention combinations was to be determined using response surface methodology. By combining various treatments, an improved antimicrobial efficacy as compared to individual treatments was hypothesized. Thus, the sequential application of a combination of ACS, EPL and LAE interventions may be more effective for reducing *Salmonella.*

MATERIALS AND METHODS

***Salmonella* strains and inoculum preparation.** Nalidixic acid and novobiocin resistant *Salmonella enterica* serovars including ST and SE were obtained from Dr. James A. Byrd (USDA-ARS, College Station, Tex.). Isolates were maintained and grown in tryptic soy broth (Difco, Becton Dickinson, Sparks, Md.) at 37 °C supplemented with 20 µg/ml nalidixic acid and 25 µg/ml novobiocin (Sigma, St. Louis, Mo.). A cocktail of 18 h cultures was prepared by placing equal amounts of each strain in a sterile bottle and vortexing to yield approximately 6-7 $\log_{10}$ CFU/ml of inoculum.

Media. Populations of *Salmonella* spp. were plated on Xylose Lysine Tergitol 4 media (XLT4; Difco, Becton Dickson, Sparks, Md.). XLT4 plates were supplemented with 20 mg/liter nalidixic acid and 25 mg/liter novobiocin. Stock solutions of each antibiotic were prepared by dissolving 200 mg nalidixic acid and 250 mg novobiocin (Sigma) in 10 ml of sterile distilled water followed by filter-sterilization. Inoculated plates were incubated at 37 °C for 24 h to recover *Salmonella*. The nalidixic acid and novobiocin resistant *Salmonella* cultures developed black centered colonies on XLT4 plates. The results were converted to units of $\log_{10}$ CFU/ml.

Application of antimicrobial interventions - membrane filter model. All possible sequential combinations of 20 % ACS (Safe$_2$O RTE 01®, Mionix Corporation, Austin, Tex.), 300 mg/liter EPL (50 % powder of ε-polylysine, Chisso America Inc., Rye, N.Y.) and 200 mg/liter LAE (CytoGuard LA®, A&B Ingredients, Fairfield, N.J.) were evaluated using a model system (Fig. 1) described by Thayer et al. *(70).* The system consists of a bench-top 47-mm filter holder (VWR International, LLC., West Chester, Pa.), a vacuum pump (Thomas Industries Inc., Sheboygan, Wis.) and sterile cellulose nitrate 0.45-µm, 47-mm filter papers (Millipore, Bedford, Mass.). After placing the filter paper in the filtering apparatus, 20 ml of sterile distilled water was transferred and filtered to humidify the filter paper surface. One ml of the *Salmonella* cocktail was placed into a tube containing 19 ml sterile buffered peptone water (BPW; Difco, Becton Dickinson, Sparks, Md.) and the contents of this tube were dispensed on the filter surface and then filtered using the vacuum pump. Then, 20 ml of the first antimicrobial solution was transferred to the inoculated filter paper and allowed to stand for different time intervals (20 or 60 s) and then filtered. Similarly, 20 ml of the second antimicrobial solution (for multi-hurdle trials) or sterile distilled water (for individual applications) were applied and allowed to stand for additional time intervals (20 or 60 s) and then filtered. The control filters were treated with sterile distilled water in the same manner as the antimicrobial treatments. The filter papers were rinsed with an additional 20 ml of sterile BPW, aseptically removed and placed in individual stomacher bags (Labplas Inc., Ste-Julie, Canada). After addition of 99 ml of BPW, the bags were hand pummeled for 60 s. The rinses were serially diluted 10-fold in 9 ml tubes of sterile BPW. Plates of

XLT4 media were used for plating of the diluents. Detection limit of *Salmonella* was 1 CFU/ml of rinse liquid. Bacterial log reductions were determined by the differences in cell numbers of *Salmonella* between control and antimicrobial treatment samples per milliliter of the rinse liquid.

Application of antimicrobial interventions to raw chicken carcasses. Fresh, prerigor broiler carcasses were collected immediately post-evisceration from a large broiler processing facility in Bryan, Tex. Eviscerated carcasses were randomly collected from the processing line before entering the inside-outside bird washer and individually placed in 2.5 gal (9.46 liter) Hefty® OneZip bags. The bags were placed in an insulated container and transported to the laboratory in ~20 min. Each bagged carcass then was inoculated by addition of 10 ml of *Salmonella* inoculum and 90 ml BPW (Difco, Becton Dickinson) to the bag. The carcasses were then shaken for 1 min by grasping the carcass in the bag with one hand and the closed top of the bag with the other hand. A rocking motion applied to the bagged carcass ensured that all surfaces were inoculated equally to obtain an inoculum level of approximately 6-7 $\log_{10}$ CFU/ml. Following the inoculation, carcasses were allowed to stand for 10 min to facilitate bacterial attachment and subsequently subjected to the intervention treatments as explained below. After the application of treatments, carcasses were transferred into poultry rinsing bags and a 200 ml aliquot of BPW was added. The carcasses were then rinsed inside and out with a rocking motion for 1 min by grasping the broiler carcass in the bag with one hand and the closed top of the bag with the other hand to assure that all surfaces were rinsed *(78)*. The rinses were serially diluted 10-fold in 9 ml of sterile BPW to be then plated on

XLT4 media for enumeration. Detection limit of *Salmonella* was 100 CFU/ml of rinse liquid.

Response surface methodology for selecting antimicrobial combinations. Sequential spray application of selected decontamination solutions were evaluated using response surface methodology (RSM) in order to predict the highest reduction levels of *Salmonella* and for optimization of the processing parameters on inoculated chicken carcasses. The effects of 3 factors including concentration of first antimicrobial spray, concentration of second antimicrobial spray and time interval between the first and the second antimicrobial sprays were evaluated. Since each factor was measured at three or more points, a quadratic response surface can be estimated by least-squares regression. The resulting response surface can then be plotted to find improved or optimal process combinations *(65)*. The Box-Behnken design which has a total of 15 runs with 3 center points was used in three experiments (Table 1). Each experiment was run in randomized order. Three sequential applications of individual interventions were selected for RSM experiments based on the membrane filter model results. The ranges of the three factors studied in each RSM experiments were (i) EPL-LAE, 20 s spray of EPL (100 to 300 mg/liter) followed by a time interval of 40 s to 120 s, then a 20 s spray of LAE (100 to 200 mg/liter); the experiment was replicated one time; (ii) LAE-EPL, 20 s spray of LAE (100 to 200 mg/liter) followed by a time interval of 40 s to 120 s, then a 20 s spray of EPL (100 to 300 mg/liter); the experiment was replicated one time; (iii) EPL-ACS, 20 s spray of EPL (100 to 300 mg/liter) followed by a time interval of 40 s to 120 s, then a 20 s spray of ACS (20 to 30 %); the experiment was replicated three times.

Spray cabinet, preparation and application of intervention treatments on carcasses. A stainless steel custom-built isolation spray cabinet (CHAD Corporation, Olathe, Kans.) was used to apply all intervention treatment solutions and sterile distilled water washes (Fig. 2 and Fig. 3). Two nozzles were situated inside the cabinet near the top and bottom of the cylindrical spray chamber, through which treatments were delivered. The solutions were poured into spray tanks which have two regulator valves mounted on the top. While one of the valves was connected to the nozzles via a hose the other one was connected to an air compressor. The air compressor was used to pressurize the system to apply a constant spray pressure of 37 psi (255.1 kPa) which delivered 500 – 520 ml of treatment solution during a 20 s application of a treatment.

Treatment solutions were prepared individually in 1 liter bottles at a volume of 900 ml using sterile distilled water. Sprays were applied individually at room temperature for 20 s while rotating the chicken carcass at a constant rate of ~10 revolutions in a uniform spray stream. The orientation of the nozzles (spray angle of 65°) in the spray cabinet allowed delivering the treatment solutions to the internal and external surfaces of the poultry carcasses.

L*a*b* color space values. Color space values for the outer surface of samples were obtained by reflectance using a Minolta Colorimeter (CR-200, Minolta C., Ramsey, N.J.) calibrated using a white standard tile (C $Y = 93.24$, $x = 0.3137$, $y = 0.3196$) set to channel 00. Three readings were taken on the external skin surface breast area of the chicken carcasses. For each measurement, the colorimeter port was covered with clear

Saran Wrap® and random readings were taken at three different locations on the outer surface of the breasts of broiler carcasses.

Statistical analyses. Analysis of variance was performed with SPSS® 12.0.1 for Windows® program using One-Way ANOVA and Univariate procedures and mean separations (Tukey) evaluated at $\alpha = 0.05$.

For the response surface experiments, data were analyzed using response surface design with SAS 9.1 program *(65)*. The following 2nd order polynomial equation was used to develop a predictive model for the inactivation of *Salmonella* by the different combination of antimicrobials:

$$\text{Log reduction} = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_{11}\chi^2_1 + \beta_{22}\chi^2_2 + \beta_{33}\chi^2_3 + \beta_{12}\chi_1\chi_2 + \beta_{13}\chi_1\chi_3 + \beta_{23}\chi_2\chi_3 + \varepsilon$$

where, β_i are constant regression coefficients; χ_1 is concentration of the first antimicrobial spray, χ_2 is concentration of the second antimicrobial spray and χ_3 is the time interval between the first and the second antimicrobial sprays and ε is associated with the random error.

RESULTS AND DISCUSSION

Antimicrobial treatments of *Salmonella* using a membrane filter model. The efficacy of individual and sequentially applied antimicrobial interventions for reducing ST and SE on membrane filters in a model system was evaluated at contact times of either 20 s or 60 s. Mean reductions of *Salmonella* are presented in Table 2. The data were analyzed in a factorial arrangement to examine the interaction between interventions and application times and to examine the effects of the main factors.

Statistical analysis indicated that there were no interactions between interventions and application times. Individually or sequentially applied interventions on log reduction of *Salmonella* were significant ($P < 0.05$); however, contact time (20 and 60 s) on log reduction of *Salmonella* was not different. Mean reductions of *Salmonella* based on pooled main effects are presented in Table 3. Increasing antimicrobial contact time did not produce further reductions in *Salmonella* on membrane filter system. Statistical analysis also indicated that an individual application of an antimicrobial intervention was not as effective as two sequential applications.

Initial levels of ST and SE in control treatments were between 5.8 – 6.9 log CFU/ml. A single application of 20 % ACS reduced the population of *Salmonella* cells on the membrane filter by 3.5 log CFU/ml and 3.7 log CFU/ml for 20 and 60 s, respectively. Similar reduction levels of *Salmonella* were observed for individual applications of ELP or LAE on the membrane filter (Table 2). Both EPL at 300 mg/liter and LAE at 200 mg/liter produced a reduction of 3.5 log CFU/ml irrespective of contact time (Table 3).

Some claims have indicated that ACS prevents food borne pathogens such as *Escherichia coli, Salmonella* spp.*, Listeria monocytogenes,* and *Campylobacter jejuni* from attaching to food surfaces and that it has a prolonged antimicrobial effect on carcasses after application *(48)*. Yoshida and Nagasawa *(82)* reported the minimum inhibitory concentration of EPL to be below 100 μg/ml for bacteria, but that combining with food additives such as glycine, vinegar, ethanol and thiamine laurylsulfonate, the preservative activity of EPL can be greatly enhanced. Geornaras and Sofos *(25)*

compared the antimicrobial effectiveness of EPL with sodium diacetate, sodium lactate, lactic acid and acetic acid, against different foodborne pathogens including *Escherichia coli* O157:H7 (EC), ST, and *Listeria monocytogenes* in a culture broth medium. They concluded that EPL has minimum inhibitory concentrations of 0.02 % for EC and *L.monocytogenes* and 0.04 % for ST, and that EPL inhibited growth of these foodborne pathogens at 24 °C. Enhanced antimicrobial activity has been reported when ELP was combined with 0.25 % sodium diacetate or 0.1 % acetic acid. However, combining with 3.0 % sodium lactate resulted in a lost of antimicrobial activity of EPL. Rodrigues et al. *(61)* exposed ST and *Staphylococcus aureus* to their minimal inhibitory concentrations of 32 and 8 μg/ml of LAE, respectively. They observed alterations mainly in the outer membrane of ST and in the cytoplasm of *S. aureus* after exposure to LAE. Further, the proportions of damaged cells after 24 h contact time were reported to be 97 % and 56.3 % for ST and *S. aureus*, respectively.

The application order of interventions when used sequentially in a multi-hurdle approach may have a significant effect on *Salmonella* reduction. Our results indicated that EPL used as a first intervention in combination with ACS tended to reduce *Salmonella* more when compared with ACS used as a first intervention followed by EPL. ASC is defined as a very acidic (pH 1.0 – 1.5) organic acid-calcium sulfate complex *(36)* and if applied first, may affect the EPL activity. The data tends to indicate that using EPL as a first intervention treatment was more effective than using EPL as a second intervention. Antimicrobial activity of EPL has been reported over a pH range of

5-8, and no structural changes have been observed by heat treatment of EPL at pH 3.0 *(81, 82)*.

A similar trend in *Salmonella* reductions were observed with sequential application of LAE and ACS while numerically the effect was not as notable as the EPL and ACS combination. LAE, a surfactant with broad-spectrum antimicrobial activity, derives its activity by altering the cytoplasmic membranes of microorganisms *(2, 61)*. When LAE was used as a first intervention treatment in combination with EPL, a 5.5 log CFU/ml reduction in *Salmonella* was noted in comparison to a numeric reduction of 4.7 log CFU/ml when EPL was used as the first intervention. However, *Salmonella* reductions with both EPL and LAE combinations were not different ($P > 0.05$).

All possible combinations (Table 3) of sequential applications were evaluated using the Tukey mean separation test ($P < 0.05$) to determine the best combinations. Statistical analysis indicated that the highest *Salmonella* reductions were obtained with following intervention combinations: LAE200-EPL300, 200 mg/liter LAE followed by 300 mg/liter EPL (5.5 log CFU/ml); EPL300-ACS20, 300 mg/liter EPL followed by 20 % ACS (5.1 log CFU/ml); and EPL300-LAE200, 300 mg/liter EPL followed by 200 mg/liter LAE (4.7 log CFU/ml).

To better determine the effectiveness of sequential application of various interventions used in a production environment, tests were conducted using fresh chicken carcasses to determine antimicrobial efficacy of these combinations.

EPL-LAE antimicrobial combinations on raw chicken carcasses - response surface methodology. EPL was applied as an antimicrobial spray (100 to 300 mg/liter),

followed by a LAE (100 to 200 mg/liter) spray with a time interval of 40 to 120 s between applications. The effectiveness of various sequential combinations were tested for their ability to reduce ST and SE on inoculated chicken carcasses using response surface methodology. The results are presented in Table 4. The following mathematical model was developed from inoculation studies to predict the log reduction of *Salmonella* as a function of EPL spray concentration (EPL), time interval (TI), and LAE spray concentration (LAE):

Salmonella reduction = 1.4525 - 0.005287*EPL + 0.0002*LAE – 0.018906*TI + 0.000014*EPL*EPL - 0.000018*EPL*LAE + 0.000034*EPL*TI - 0.000001*LAE*LAE + 0.000079*LAE*TI + 0.00000625*TI*TI.

Eventhough there was no significant lack of fit, the analysis of variance for the response variable indicated that the model, as well as linear, quadratic and interaction effects, were not significant and had a low R^2 (54.86 %) value. The canonical analysis of the response system indicated that the stationary point is a saddle point with no unique minimum or maximum. Therefore, the model may not adequately represent the true relationships among variables, but may be useful for screening purposes in further experiments. To maximize *Salmonella* reduction, the best five predicted values with corresponding standard errors and 95 % prediction intervals as a function of EPL spray concentration, time interval and LAE spray concentration on inoculated chicken carcasses are presented in Table 5. Figure 4 also shows the response surface for the log reductions of *Salmonella* from inoculated chicken carcasses generated using the predictive model for different treatment conditions. The best spray combinations for a

predicted *Salmonella* reduction of 1 log CFU/ml was an initial application of EPL at 300 mg/liter, followed by LAE at 200 mg/liter with a 120 s time interval between treatments.

The response surface reductions in *Salmonella* using the prediction formula suggested that a higher concentration of antimicrobial sprays and a longer time interval between applications may be required to obtain higher reductions of *Salmonella* on poultry carcasses when using EPL as the first intervention and LAE the second intervention. Due to the relatively low predicted *Salmonella* reductions and the longer time intervals required during in-line processing, application of EPL followed by LAE may not be a good choice as a *Salmonella* intervention.

LAE-EPL antimicrobial combinations on raw chicken carcasses - Response surface methodology. LAE was applied as an antimicrobial spray (100 to 200 mg/liter), followed by an EPL (100 to 300 mg/liter) spray with a time interval of 40 to 120 s between applications. The effectiveness of various sequential combinations were tested for their ability to reduce ST and SE on inoculated chicken carcasses using response surface methodology. The results are presented in Table 6. However, a mathematical model could not be developed to predict the log reduction of *Salmonella* as a function of LAE spray concentration (LAE), time interval (TI), and EPL spray concentration (EPL) using the data presented. *Salmonella* reductions on inoculated chicken carcasses were between 0.5 – 2.8 log CFU/ml. When compared to the filter study experiments, LAE-EPL spray combinations used on chicken carcasses produced much lower *Salmonella* reductions and would not be the intervention of choice to decontaminate chicken carcasses.

EPL-ACS antimicrobial combinations on raw chicken carcasses - Response surface methodology. EPL was applied as an antimicrobial spray (100 to 300 mg/liter), followed by an ACS (20 to 30 %) spray with a time interval of 40 to 120 s between applications. The effectiveness of various sequential combinations were tested for their ability to reduce ST and SE on inoculated chicken carcasses using response surface methodology. The results are presented in Table 7. The following mathematical model was developed from inoculation studies to predict the log reduction of *Salmonella* as a function of EPL spray concentration (EPL), time interval (TI) and ACS spray concentration (ACS):

Salmonella reduction = -5.37083 + 0.011108*EPL + 27.075*ACS + 0.06899*TI + 0.000031*EPL*EPL - 0.065*EPL*ACS - 0.000061*EPL*TI + 29.16667*ACS*ACS - 0.222917*ACS*TI - 0.000037*TI*TI.

Although there is no significant lack of fit, the analysis of variance for the response variables indicated that the model, as well as linear, quadratic and interaction effects were not significant and had a low R^2 (20.95 %) value. The canonical analysis of the response system indicated that the stationary point is a saddle point with no unique minimum or maximum value. Therefore, the model may not adequately represent the true relationships among variables, but may be useful for screening purposes in further experiments. To maximize *Salmonella* reduction, the best five predicted values with corresponding standard errors and 95 % prediction intervals as a function of EPL spray concentration, time interval and ACS spray concentration on inoculated chicken carcasses are presented in Table 8. Figure 5 also shows the response surface for the log

reductions of *Salmonella* generated from inoculated chicken carcasses using the predictive model for different treatment conditions. The best combinations for *Salmonella* reduction was a spray concentration of 300 mg/liter EPL, a time interval of 40 s between applications and a spray concentration of 30 % ACS. A 4.9 log CFU/ml was the predicted reduction in *Salmonella* with a range of 3.1 – 6.7 log CFU/ml, over a 95 % prediction interval. The results also showed that at the minimum 95 % prediction interval, the best five predictions for reducing *Salmonella* using an EPL-ACS spray combination were all over 3 log CFU/ml which offers promise for further experiments. Keeton et al. *(37)* reported that a sequential warm (55 °C) spray application of ACS (20 %) followed by EPL (100 mg/L) at a constant pressure for 15 to 20 s was effective for reducing ST, EC and LM on beef rounds. They reported a 4.38 log reduction in *Salmonella* after 7 days of storage. In the same study, individual applications of ACS (20 %), lactic acid (2.5 %), EPL (100 mg/L) and sterile distilled water spray were not found as effective as the application of ACS followed by EPL. In another study, Geornaras and Sofos *(25)* reported enhanced antimicrobial activity of EPL when combined with acidic antimicrobials such as sodium diacetate and acetic acid. Similarly, enhanced antimicrobial activity of EPL has also been reported when combined with vinegar *(82)*.

The response surfaces and predicted *Salmonella* reductions calculated using the prediction formula suggested that the time interval between applications should be shorter to obtain higher reductions of *Salmonella* on poultry carcasses when EPL is the first intervention and ACS the second intervention.

An additional experiment was conducted using 300 mg/liter EPL followed by 30 % ACS (EPL300-ACS30) with a 40 s time interval between the first and second antimicrobials to determine the effect of immediate, 1 h, 2 h and 3 h plating of the rinse liquid following a whole bird rinse of the chicken carcasses using 200 ml BPW. Five fresh, prerigor carcasses were treated and rinsed as described previously. Rinse liquids were kept at 4.4 °C for immediate, 1 h, 2 h and 3 h plating. The results are presented in Figure 6. Plating time had a significant ($P < 0.05$) effect on *Salmonella* recovery from the rinse liquids which indicated that 200 ml BPW didn't have enough buffering capacity to neutralize the rinsing liquid. Thus, further experiments were conducted with immediate serial dilution and plating of the rinse liquids to be able to eliminate further *Salmonella* reduction in rinse liquid.

L*a*b* color space value. Color measurements were taken before treatment and after treatment with EPL spray as a first antimicrobial treatment (100 to 300 mg/L), at a time interval of 40 to 120 s and ACS (20 to 30 %) spray as a second antimicrobial treatment. Pooled color values of all treatment combinations were statistically analyzed for differences between "before treatment" and "after treatment" measurements for L*a*b* values. The data taken before treatment and after treatment are presented in Table 9. There were no significant differences before treatment and after treatment for a* values. There were differences ($P < 0.05$) between L* and b* values of before treatment and after treatment samples with mean values of 71.20 and 68.24 for L* and 1.31 and 6.02 for b* respectively. L* values were smaller after treatment which means

that carcasses became darker. Higher b* values also indicated that carcasses became more yellow.

The “after treatment data” were analyzed to determine the effects of EPL spray as a first antimicrobial treatment (100 to 300 mg/L), at a time interval of 40 to 120 s and ACS (20 to 30 %) spray as a second antimicrobial treatment on L*a*b* values using response surface methodology. The mathematical models are presented in Table 10. The models were developed to predict L*a*b* values of inoculated and treated chicken carcasses as a function of EPL spray concentration (EPL), time interval (TI), ACS spray concentration (ACS). Figures 7, 8 and 9 show the L*a*b* value response surface plots derived from chicken carcasses using the predictive models for the various treatment conditions. The response surface analysis of the effects of various concentrations of EPL, ACS and various time intervals on the color of treated chicken carcasses were not significantly different from each other.

Sequential spray applications of ACS, EPL or LAE combinations applied at different time intervals using a membrane filter system were found more effective to reduce *Salmonella* than individual applications of the antimicrobials studied. The most effective combinations were LAE200-EPL300, EPL300-ACS20 and EPL300-LAE200. The data indicated that the order of the application of the combined interventions had a direct effect on *Salmonella* reduction. RSM experiments predicted that an EPL-ACS combination was the most effective spray application on poultry carcasses for reducing *Salmonella* under the conditions evaluated. Further experiments are required to determine and verify the efficiency of EPL300-ACS30 (300 mg/liter EPL, a time

interval of 40 s between applications and a spray concentration of 30 % ACS) combination for reducing pathogens on poultry carcasses. Additionally, various combinations and concentrations of LAE, EPL and ACS should be evaluated to determine the effect of sequential application of these decontamination agents for reducing pathogen on poultry carcasses. Attention should also be given to use ACS as a second intervention application.

CHAPTER III

EFFICACY OF SEQUENTIAL APPLICATION OF ε-POLYLYSINE OR LAURIC ARGINATE FOLLOWED BY ACIDIC CALCIUM SULFATE FOR *SALMONELLA* REDUCTION ON CHICKEN CARCASSES

INTRODUCTION

The United States Department of Agriculture's Food Safety Inspection Service (USDA-FSIS) issued a final regulation on July 25, 1996 establishing pathogen reduction requirements applicable to meat establishments *(71)*. This regulation was designed to reduce the occurrence and numbers of pathogens in meat and poultry products, and thus reduce the risk of foodborne disease *(46)*. The data collected by USDA-FSIS from 2000 through 2005 showed that the annual number of isolates of *Salmonella enterica* serovar Enteritidis (SE) in broiler carcass rinses increased more than four fold and, similarly, the proportion of establishments with SE positive rinses increased nearly three fold *(1)*. In addition, data collected by the Foodborne Diseases Active Surveillance Network (FoodNet) of the Centers for Disease Control and Prevention (CDC) shows that *Salmonella* is the leading cause of foodborne disease, and that the estimated prevalence of *Salmonella* has not changed significantly when compared with baseline. Furthermore, CDC has indicated that chickens are an important source of human SE infections *(14)*.

A wide variety of immersion or spray intervention methods to reduce or eliminate the pathogens have been reported in the literature. Keeton and Eddy *(36)* reviewed chemical methods for decontamination of animal carcasses including chlorine-

based derivatives, organic acids, organic and inorganic compounds, bacteriocins and emerging technologies as post-harvest interventions. They stated that no single decontamination method was right for the purpose of completely eliminating pathogens from raw materials due to surface geometries, protected sited of contamination, and inherent inefficiency of specific decontamination processes. Li et al. *(43)* tested sodium chloride, trisodium phosphate, sodium bisulfate, cetylpyridinium chloride or lactic acid sprays on prechilled chicken carcasses. Spraying sodium chloride was not found to be effective for *Salmonella* reduction. Morris and Fleet *(49)* reported that hot water, chlorine and potassium sorbate immersion treatments reduced *Salmonella* on prechilled chicken carcasses. Immersion of chicken carcasses in lactic acid and hydrogen peroxide was found highly effective for reducing *Salmonella* Typhimurium (ST) with 5 or 10 min exposure times, however, slight color changes on the carcasses were also reported with lactic acid immersion *(50)*. Treatments with acetic acid or lactic acid by either dipping or spraying after scalding, evisceration and defeathering have been claimed to decrease cross contamination and improve the microbial quality of chicken carcasses *(63)*. Northcutt et al. *(53)* reported that spray washing of chicken carcasses with chlorinated water and/or increasing water temperature in an inside-outside bird washer were not effective for decontaminating chicken carcasses. Fabrizio et al. *(22)* suggested the use of electrolyzed oxidizing water as a cost effective alternative as compared to other antimicrobial compounds commonly used in the poultry industry including chlorine, aqueous ozone, acetic acid and trisodium phosphate. In a more recent study, trisodium phosphate, lactic acid with or without sodium chloride as well as commercial

antimicrobials including acidified sodium chlorite (Sanova®), acidified calcium sulfate ($Safe_2O$®), cetylpyridinium chloride (Cecure®), peroxyacetic acid formulated with hydrogen peroxide, octanoic acid and acetic acid (Inspexx™) dips were investigated for improving microbiological quality of chicken carcasses *(47)*.

Limited research has been conducted to evaluate the effects of sequential application of antimicrobial solutions on pathogens. Application of more than one antimicrobial to carcasses in a processing line might produce greater reductions than one treatment alone due to different modes of action of individual antimicrobials. This concept has been reviewed and studied under the names of hurdle technology, combined treatments, multiple hurdle carcass interventions or synergistic effect *(31, 40, 41, 70)*. Acidic calcium sulfate (ACS), also known as acidified calcium sulfate, is a USDA-FSIS approved ingredient for use as a secondary food additive in the production of meat and poultry products *(18, 48, 77)*. ε-Polylysine (EPL) has a wide range of antimicrobial activity and is characterized as an edible, water-soluble agent *(25, 26, 81, 82)*. Lauramide arginine ethyl ester (LAE) also known as lauric arginate is a surfactant with broad-spectrum antimicrobial activity *(2, 61)*. The mode of action of these antimicrobials varies among agents; therefore, one might expect enhanced antimicrobial efficacy when combinations of these antimicrobials are applied sequentially.

The purpose of this study was to evaluate sequential application of ACS, EPL or LAE on poultry carcasses. By combining these treatments, potential synergistic interactions were expected to improve antimicrobial efficacy due to the different modes of action of individual decontamination agents. Thus, the sequential application of

combined interventions could be more effective for reducing *Salmonella* and possibly extending the shelf-life of poultry carcasses as compared to a single antimicrobial application.

MATERIALS AND METHODS

Media. Stock solutions of antibiotics were prepared by dissolving 200 mg nalidixic acid (Sigma, St. Louis, Mo.) and 250 mg novobiocin (Sigma) in 10 ml sterile distilled water and filter-sterilizing. Xylose lysine agar plates supplemented with Tergitol 4 (XLT4; Difco, Becton Dickson, Sparks, Md.) were poured after supplementing 20 mg/liter nalidixic acid (Sigma) and 25 mg/liter novobiocin (Sigma).

Preparation of *Salmonella* inoculum. Nalidixic acid and novobiocin resistant *Salmonella enterica* serovars including ST and SE were obtained from Dr. James A. Byrd (USDA-ARS, College Station, Tex.). Each isolate was maintained and grown in tryptic soy broth (Bacto, Becton Dickinson, Sparks, Md.) at 37 °C supplemented with 20 µg/ml nalidixic acid (Sigma) and 25 µg/ml novobiocin (Sigma). The cultures were transferred on three consecutive days before use in experiments. On the experiment day, a cocktail of 18 h cultures was prepared by placing and mixing equal amounts of each serovar in a sterile bottle and the cocktail was used for inoculation of the chicken carcasses. The prepared inoculum was used within 3 h and kept at room temperature during the experiments.

Sample collection and inoculation. Fresh, prerigor broiler carcasses were obtained immediately post-evisceration from a poultry processor located in Bryan, Tex. Eviscerated broiler chicken carcasses were randomly collected from the processing line

before entering the inside-outside bird washer and individually placed in 2.5 gal (9.46 liter) Hefty® OneZip bags. The bags were placed in an insulated container and transported to the laboratory with in 20 min. Each bagged carcass then was inoculated by addition of 10 ml of *Salmonella* inoculum and 90 ml BPW (Difco, Becton Dickinson) into the bag. The carcasses were then shaken for 1 min by grasping the carcass in the bag with one hand and the closed top of the bag with the other hand to make sure that all surfaces were inoculated equally and to obtain an inoculum level of approximately 6-7 $\log_{10}$ CFU/ml. Following the inoculation, carcasses were allowed to stand for 10 min for bacterial attachment and then subjected to appropriate intervention treatments.

Application of intervention treatments. A stainless steel, custom-built isolation spray cabinet (CHAD Corporation, Olathe, Kans.) was used to apply all intervention treatment solutions and sterile distilled water (Fig. 2). Two VeeJet® spray nozzles (model H1/8VV-SS65015, Spraying Systems Co., Wheaton, Ill.) were situated inside the cabinet near the top and bottom of the cylindrical spray chamber, through which treatments were delivered. The solutions were poured into spray tanks with two regulator valves mounted top. While one of the valves was connected to the nozzles via a hose the other one was connected to an air compressor (Campbell Hausfeld, South Pasadena, Calif.). The air compressor was used to pressurize the system to apply a constant spraying pressure of 37 psi (255.1 kPa) which delivered a 500 – 520 ml of treatment solutions during 20 s a spray application for all treatments.

Treatment solutions were prepared individually in 1 liter bottles at a volume of 900 ml using sterile distilled water, transferred to individual tanks and sprayed at room

temperature for 20 s while rotating the carcasses at constant rate of ~10 revolutions per 20 s in a uniform spray stream. The orientation of the nozzles (spray angle of 65°) in the spray cabinet allowed delivery of each treatment solution to the internal and external surfaces of the poultry carcasses. The carcasses were attached to a single set of stainless steel hooks, the hooks were then hung on a hanger attached to a turn-style inside of the spray cabinet lid (Fig. 3). The carcasses hung onto the lid were positioned into the cabinet by placing the fitted lid onto the cabinet frame and sealing. A turn-style handle connected to the hanger was used to rotate the carcasses in the spray cabinet during application of the treatments.

Sequential application of intervention treatments. Experiments were performed to determine *Salmonella* reductions on inoculated chicken carcasses after 20 s spray applications of various concentrations of EPL, LAE and ACS applied sequentially with a 40 s time interval between first and second intervention. After reviewing the results of membrane filter model study and RSM studies**,** sequential interventions for this study were selected. The combinations to be evaluated included: (i) 300 mg/liter EPL followed by 30 % ACS (EPL300-ACS30) (ii) 100 mg/liter EPL followed by 30 % ACS (EPL100-ACS30) (iii) 100 mg/liter EPL followed by 10 % ACS (EPL100-ACS10) (iv) 300 mg/liter EPL followed by 10 % ACS (EPL300-ACS10) (v) 200 mg/liter LAE followed by 30 % ACS (LAE200-ACS30) (vi) 200 mg/liter LAE followed by 10 % ACS (LAE200-ACS10) (vii) 100 mg/liter LAE followed by 10 % ACS (LAE100-ACS10).

Individual application of intervention treatments. Effects of an individual 20 s spray of 30 % ACS (ACS30), 10 % ACS (ACS10), 300 mg/liter ELP (EPL300) or 200

mg/liter LAE (LAE200) solutions on *Salmonella* reduction were determined on inoculated chicken carcasses. In this experiment, interventions were sprayed individually on chicken carcasses as described above.

Sampling and microbiological analysis. After the application of treatments, carcasses were transferred into poultry rinsing bags (Nasco, Fort Atkinson, Wis.) and 200 ml of sterile BPW (Difco, Becton Dickinson) added. The carcasses were then rinsed inside and out with a rocking motion for 1 min by grasping the broiler carcass in the bag with one hand and the closed top of the bag with the other hand to assure that all surfaces were rinsed *(78)*. Counts of nalidixic acid and novobiocin-resistant ST and SE were determined by an immediate dilution of the rinses with sterile BPW (Difco, Becton Dickinson) and plating the appropriate dilutions onto the plates of the selective XLT4 agar compounded as described above. The plates were incubated at 37 °C for 24 h before enumeration. Detection limit of *Salmonella* was 100 CFU/ml of rinse liquid. The nalidixic acid and novobiocin-resistant *Salmonella* developed black centered colonies when viewed on media.

L*a*b* color space value. Color space values for the outer skin surfaces of samples were obtained by reflectance using a Minolta Colorimeter (CR-200, Minolta C., Ramsey, N.J.) calibrated to a white standard tile (C $Y = 93.24$, $x = 0.3137$, $y = 0.3196$) set to channel 00 after the colorimeter port was covered with clear Reynolds® Foodservice film. Three readings were taken on the breast area of all chicken carcasses. Positive L* values indicate the degree of lightness while negative L* values indicate darkness. Positive a* values quantify the degree of redness whereas negative values

indicate greenness. Positive b* values indicate yellowness while negative values indicate blueness. For each measurement, the colorimeter port was covered with clear Reynolds® Foodservice film and random readings were taken at three locations on the outer surface of the breasts of broiler carcasses.

Statistical analyses. The average number of colonies from the duplicate plates was recorded for each chicken carcass sample. The results were converted to units of $\log_{10}$ CFU/ml of rinse. The reduction values were the difference in the cell number of *Salmonella* of a treatment and untreated controls. The data were collected in four experiment days and the numbers of replications (chicken carcasses) for each sequential intervention treatment are presented in Table 11. The data for the individual interventions were generated using 6 carcasses per treatment solution. Statistical analyses of the data were performed with SAS 9.1 software (SAS Institute, Cary, N.C.). Analysis of variance (ANOVA) procedures were performed using PROC GLM procedure and Tukey's multiple comparison test was used to determine which treatments were significantly different *(65)*.

RESULTS AND DISCUSSION

Sequential application of intervention treatments. A total of 64 whole poultry carcasses were taken from the processing line before entering the inside-outside bird washer. Following *Salmonella* inoculation, the carcasses were subjected to sequential spray application of various concentrations of antimicrobial solutions (Table 12). The average initial *Salmonella* count on control samples after inoculation was 6.9 log CFU/ml. Six interventions were effective in significantly ($P < 0.05$) reducing *Salmonella*

numbers on inoculated chicken carcasses. The most effective treatments were EPL300-ACS30 and LAE200-ACS30 which reduced *Salmonella* counts by 2.1 and 2.2 log CFU/ml, respectively (Fig. 10). *Salmonella* reduction levels for EPL100-ACS30, EPL100-ACS10 and EPL300-ACS10 treatments were observed to be between 1.1 – 1.4 log CFU/ml. The water spray resulted in only a 0.3 log CFU/ml reduction in *Salmonella*, and the LAE200-ACS10 and LAE100-ACS10 treatments (0.5 and 0.4 log CFU/ml, respectively) were not different from the water spray treatment. Similarly, spray washing poultry carcasses with water alone has been reported ineffective for reducing either *Salmonella* or the total bacterial load on carcasses in several studies *(32, 43, 47, 52, 53, 63)*. Furthermore, Lillard *(44)* proposed that water immersion of poultry carcasses during processing forms cervices on the skin in which bacteria lodge and are protected from effects of saline, and other solutions of varying ionic strength or surfactants. This hypothesis was suggested to explain the persistence of salmonellae on poultry carcasses and the ineffectiveness of some antimicrobial applications for reducing salmonellae. ACS claims that it prevents pathogenic bacteria including *Salmonella* from attaching to the poultry skin surface and that its antimicrobial effect after application is due to lowering the pH and disabling the proton pumps in the bacterial membrane *(18, 48, 60, 83)*. Yoshida and Nagasawa *(82)* reported enhanced antimicrobial activity of EPL when combined with glycine, vinegar, ethanol or thiamine laurylsulfonate. In this study, all concentrations of EPL followed by ACS resulted in more than a 1 log CFU/ml reduction of *Salmonella* and at higher concentrations over a 2 log CFU/ml reduction was obtained. Similar results also reported by Keeton et al. *(37)* who noted sequential application of

warm solutions (55 °C) of 20 % ACS followed by 100 mg/liter EPL was effective for reducing ST, *E. coli* O157:H7 (EC), and *Listeria monocytogenes* (LM) on beef trimmings. They reported a *Salmonella* reduction of 4.38 log after 7 days of refrigerated storage. LAE has been shown to effectively inhibit the growth of LM on cooked meats during refrigerated storage *(2, 45)*. In addition, when used in combination with ACS, LAE has been effective in a patented "Spray Lethality In Container" (SLIC®) intervention delivery system for ready-to-eat products *(45)*. This study showed that spraying LAE followed by ACS at high concentration levels of both antimicrobials were required to obtain higher *Salmonella* reductions.

Color measurements were taken before inoculation, after inoculation and after a 20 s treatment sequential application of EPL or LAE and ACS, with a 40 s time interval between treatments. Data were statistically analyzed to determine if there were differences among "before inoculation", "after inoculation" and "after treatment" measurements for each intervention (Table 13). There were no significant differences among mean L*, a* and b* values before inoculation, after inoculation and after treatment with distilled water spray. L* values were significantly ($P < 0.05$) smaller (darker) after treatment for all sequential interventions when compared to before inoculation except for the EPL300-ACS30 and LAE100-ACS10 treatments (P values of 0.051 and 0.072, respectively). However, lower L* values were observed after treatment for EPL300-ACS30 and LAE100-ACS10 (mean values of 66.67 and 68.42, respectively) when compared to before inoculation and after inoculation mean values (68.46 and 69.58 for EPL300-ACS30 and 72.04 and 70.55 for LAE100-ACS10, respectively).

Nonetheless, observed differences between mean values of before inoculation and after treatment L* values ranged from 1.79 to 5.30 indicating that the effect of sequential interventions were relatively smaller on L* values of the chicken carcasses. Mean a* values (redness) before inoculation, after inoculation and after treatment across all treatments were not different. Conversely, after treatment, b* values were significantly ($P < 0.05$) higher (more yellow) than before inoculation and after inoculation values on inoculated and sprayed chicken carcasses for all sequential treatments. Thus, all treatments increased b* values (yellowness) by 3 to 6 units which might be of sufficient magnitude to be noted by consumers.

Decontamination of *Salmonella* on raw chicken carcasses with individual antimicrobials. The effect of individual applications of antimicrobial treatments on ST and SE inoculated onto chicken carcasses was evaluated. Recoveries and reductions of *Salmonella* are presented in Table 14 and Figure 11, respectively. Individual applications of antimicrobial solutions were significantly different ($P < 0.05$) for reducing *Salmonella* and ranged 0.5 to 2.4 log CFU/ml. Application of 30 % ACS was found to be more effective for reducing *Salmonella* on inoculated chicken carcasses (2.4 log CFU/ml reduction). Applications of ACS 10 %, EPL 300 mg/liter and LAE 200 mg/liter were not as effective as 30 % ACS spray for reducing *Salmonella* but were more effective than the water spray only. An individual treatment of 30 % ACS, when compared to sequential applications was not different from EPL300-ACS30 or LAE200-ACS30 for reducing *Salmonella* on inoculated chicken carcasses (Table 15). Similarly, Keeton et al. *(37)* reported no differences among the spray application (55 °C) of ACS (20 %), lactic

acid (2.5 %), EPL (100 µl/liter), water and ACS (20 %) followed by EPL (100 µl/liter) for reducing ST on inoculated beef trimmings. Nevertheless, in their study ACS followed by EPL treatment reduced levels of ST, EC and LM significantly after 7 days of refrigerated storage. They reported that no single treatment was as effective as the sequential treatment of ACS followed by EPL over the storage period. In addition, Dickens et al. *(18)* found that spraying a higher concentration (1:1 solution of deionized water and $Safe_2O$ Poultry Wash) of ACS (4 ml/wing) increased the shelf-life of chicken wings from 7 days to 10 days when compared with deionized water-spray controls. In contrast, EPL was found very effective against EC, ST and LM in food extracts with low protein levels such as rice and vegetables. However, lost of activity of the EPL was reported with extended storage of food extracts that contained high protein levels such as beef and bologna extract *(26)*.

Color measurements were taken before inoculation, after inoculation and after treatment for individual applications of ACS, EPL and LAE for 20 s. Data were statistically analyzed to determine differences among "before inoculation", "after inoculation" and "after treatment" measurements for each intervention (Table 16). There was no significant differences among mean values of before inoculation, after inoculation and after treatment of distilled water spray for L*, a* and b* values. The differences among L* values before inoculation, after inoculation and after treatment were different only ($P < 0.05$) for the 30 % ACS spray. This observation also indicated that the effect of ACS on the L* value of chicken carcasses was relatively small, but might be detrimental with a higher concentration of ACS. As was noted for the

sequential applications, there were no significant differences among before inoculation, after inoculation and after treatment of a* values for all treatments. This indicated that applications of ACS, EPL and LAE did not alter the redness values of chicken carcasses. The higher b* values (yellowness) observed were mostly caused by the ACS spray treatments regardless of concentration or application (sequential or individual).

Treatment with EPL300-ACS30 and LAE200-ACS30 resulted in the highest *Salmonella* reductions on inoculated chicken carcasses. Statistical comparison of the data obtained from sequential spray application of interventions and individual spray applications showed that the 30 % ACS application was as effective as sequential application of EPL and LAE followed by ACS on initial counts of *Salmonella* on chicken carcasses. However, further experiments are needed to determine effects of sequential applications over an extended storage period.

CHAPTER IV

SEQUENTIAL APPLICATION OF ε-POLYLYSINE OR LAURIC ARGINATE FOLLOWED BY ACIDIC CALCIUM SULFATE FOR REDUCING *SALMONELLA* AND OTHER MICROFLORA ON CHICKEN CARCASSES DURING PROCESSING AND STORAGE

INTRODUCTION

Salmonella is a pathogen of concern on raw meat products, and is one of the most common causes of foodborne illnesses in humans with an estimated 1.4 million cases and 500 deaths annually in the United States *(46, 60)*. The United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) has estimated that in 2007 poultry products accounted for approximately 60 % of the foodborne illnesses originating from *Salmonella (76)*. In 2006, the USDA-FSIS announced changes in its *Salmonella* verification sampling program for meat and poultry establishments to enhance public health and grouped establishments into one of three categories based on the testing results *(72)*. Establishments grouped into Category 1 showed consistent process control for *Salmonella* reduction, while establishments grouped into Category 3 had highly variable process control for *Salmonella* reduction and were subjected to more frequent testing. The USDA-FSIS *Salmonella* verification testing program reported the following percent positive samples for broilers: 8.5 %, 2007; 11.4 %, 2006; 16.3 %, 2005; 13.5 %, 2004; 12.8 %, 2003; 11.5 %, 2002; *(72, 74)*. In 2000, the U.S. Department of Health and Human Services (USHHS) set a Healthy People goal of 6.8 *Salmonella*

cases per 100,000 persons that was to be achieved by 2010 *(79)*. However recent data from the Centers for Disease Control and Prevention indicates only a slight decline in the incidence of *Salmonella* from 16.8 cases per 100,000 persons in 1996-98 to 14.9 cases per 100,000 persons in 2007 *(15)*. To meet the Healthy People 2010 goal, USDA-FSIS has set an objective that 90 % of broiler establishments should be in Category 1 by 2010, but as of 2007 only 74 % of the establishments were reported in Category 1 *(74, 76)*.

During poultry processing, various steps have been reported to contribute to contamination or cross-contamination of carcasses including live receiving, immobilization, bleeding, scalding, feather removal, evisceration and chilling *(75, 76)*. In 1996, USDA-FSIS published the Hazard Analysis and Critical Control Points (HACCP) regulation to establish consistent process control procedures in poultry and meat establishments to prevent, eliminate or reduce contamination of raw meat and poultry products with pathogens *(71)*. *Salmonella* was selected as the target organism for sampling of poultry carcasses and raw products to verify that establishments met the *Salmonella* standards. Establishments must also conduct testing for generic *Escherichia coli* to verify that their process is under control *(52, 71, 72)*. The pathogen intervention used most for removing carcass fecal contamination is an inside-outside cabinet washer which has been reported to slightly reduce aerobic plate counts, as well as *E. coli* and coliforms on carcasses *(42, 52, 67, 68)*.

To achieve the USHHS goal of 6.8 cases of *Salmonella* per 100,000 persons and the USDA-FSIS goal of 90 % broiler processors in Category 1, enhanced measures are

needed to further reduce pathogens and the total bacterial load on poultry carcasses. One approach would be to spray carcasses sequentially with more than one decontamination agent to take advantage of their different modes of action for inactivating pathogens *(31, 40, 41, 70)*, this approach is also known as the multi-hurdle interventions concept.

A blend of organic acid-calcium sulfate, known as acidic calcium sulfate (ACS), is a very acidic (pH 1.0 -1.5) decontamination agent for meat and poultry products that is approved by USDA-FSIS *(36, 77)*. A combination of ACS plus organic acids have been reported to disable the proton pumps in bacterial membranes and thus serve as a metabolic inhibitor *(35)*. The effectiveness of ACS as a surface decontamination agent for reducing pathogens on beef or poultry carcasses or RTE meat products has been reported in several studies *(18, 31, 35-37, 45, 54, 83)*. Another antimicrobial, ε-polylysine (EPL), is a cationic homopolymer of 25 to 35 L-lysine residues connected at the ε-amino and α-carboxyl group juncture *(26)*. EPL is an edible, water soluble agent with a wide range of antimicrobial activity that includes both Gram-positive and Gram-negative bacteria *(25, 26, 81, 82)*. EPL has been reported to be non-toxic in an acute oral toxicity study in rats with no mortality at concentrations up to 5 g/kg body weight. It was not observed to be mutagenic in bacterial reversion assays and is confirmed safe as a food preservative *(30)*. EPL is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration as an antimicrobial agent for use in cooked or sushi rice at levels up to 50 mg/kg rice *(80)*. Lauramide arginine ethyl ester (LAE), also known as lauric arginate, is an antimicrobial compound derived from lauric acid and arginine with a broad-spectrum of antimicrobial activity *(2, 61)*. LAE has been verified to be non-toxic

and is metabolized rapidly to naturally occurring amino acids, largely arginine and ornithine after consumption *(62)*. LAE affects the cyptoplasmic membranes of microorganisms by causing a disruption or instability of the plasma membrane lipid bilayer thus further altering the metabolic process and detaining the cellular cycle *(2)*. LAE was confirmed as GRAS by the USDA-FSIS and is considered a safe and suitable ingredient when used in the production of meat and poultry products *(77)*.

The objectives of this study were to determine the effects of sequential application of EPL or LAE sprays followed by an ACS spray as a multi-hurdle intervention for reducing *Salmonella* on inoculated chicken carcasses stored for up to 6 days under refrigeration. Secondly, further reductions of the resident microflora on uninoculated chicken carcasses during processing steps and storage were hypothesized.

MATERIALS AND METHODS

Media. Xylose lysine agar plates supplemented with Tergitol 4 (XLT4; Difco, Becton Dickson, Sparks, Md.) were poured after supplementing 20 mg/liter nalidixic acid and 25 mg/liter novobiocin (Sigma, St. Louis, Mo.). Stock solutions of each antibiotic were prepared by dissolving 200 mg nalidixic acid and 250 mg novobiocin in 10 ml sterile distilled water followed by filter-sterilization. Coliforms and *Escherichia coli* counts were enumerated using 3M Petrifilm™ *E. coli /* Coliforms Count Plates (3M Microbiology, St. Paul, Minn.). Aerobic plate counts (APC) and psychrotrophs were conducted using standard plate count agar (PCA; Difco, Becton Dickson, Sparks, Md.).

Sample collection. Fresh, prerigor broiler carcasses were obtained immediately post-evisceration from a poultry processor located in Bryan, Tex. Eviscerated broiler

carcasses were randomly collected from the processing line before entering the inside-outside bird washer and individually placed in 2.5 gal (9.46 liter) Hefty® OneZip bags. The bags were then placed in an insulated container and transported to the laboratory within 20 min.

Application of decontamination sprays. Broiler carcasses were placed in a stainless steel, custom-built isolation spray cabinet (CHAD Corporation, Olathe, Kans.) and intervention treatment solutions applied singly (Fig. 2). A sterile distilled water solution served test the spray effect alone. Two VeeJet® spray nozzles (model H1/8VV-SS65015, Spraying Systems Co., Wheaton, Ill.) were situated inside the cabinet near the top and bottom of the cylindrical spray chamber, through which treatments were delivered. Treatment solutions were poured into individual spray tanks which had two regulator valves mounted on top. One valve was connected to the nozzles via a hose the other was connected to an air compressor (Campbell Hausfeld, South Pasadena, Calif.) by another hose. The air compressor was used to pressurize the system and apply a constant spray pressure of 37 psi (255.1 kPa) which delivered 500 – 520 ml a treatment solution over a 20 s spray interval.

Treatment solutions (900 ml) were prepared individually in 1 liter bottles using sterile distilled water, and transferred to individual tanks. Once the spray system was pressurized a 20 s spray cycle was applied while rotating the contaminated chicken carcass at constant rate for ~10 revolutions in a uniform spray stream. The orientation of the nozzles (spray angle of 65°) in the spray cabinet allowed delivery of each treatment solution to the internal and external surfaces of the poultry carcasses. The carcasses were

attached to a single set of stainless steel hooks, and the hook set was then suspended on a hanger attached to a turn-style inside the spray cabinet lid (Fig. 3). The lid with carcasses attached was then placed into the cabinet by sealing the fitted lid onto the cabinet frame. A turn-style handle connected to the hanger through the lid was used to rotate the carcasses in the spray stream to apply the treatments.

Sequential interventions for reducing *Salmonella* on inoculated chicken carcasses. Nalidixic acid and novobiocin resistant *Salmonella enterica* serovars including *Salmonella* Typhimurium (ST) and *Salmonella* Enteritidis (SE) were obtained from Dr. James A. Byrd (USDA-ARS, College Station, Tex.). Each isolate was maintained and grown in tryptic soy broth (Bacto, Becton Dickinson, Sparks, Md.) at 37 °C supplemented with 20 μg/ml nalidixic acid and 25 μg/ml novobiocin. The cultures were transferred on three consecutive days before use in experiments. On the experiment day, a cocktail of 18 h cultures was prepared by placing and mixing equal amounts of each serovar in a sterile bottle and the cocktail was used for inoculation of the chicken carcasses. The prepared inoculum was used within 3 h and kept at room temperature during the experiments.

After transporting to the laboratory, each bagged carcass then was inoculated by addition of 10 ml of *Salmonella* inoculum and 90 ml buffered peptone water (BPW) into the bag. The carcasses were then shaken for 1 min by grasping the carcass in the bag with one hand and the closed top of the bag with the other hand to make sure that all surfaces were inoculated equally to obtain an inoculum level of approximately 6-7 $\log_{10}$ CFU/ml. Following the inoculation, carcasses were allowed to stand for 10 min to

facilitate bacterial attachment and subsequently subjected to the intervention treatments as described above.

Experiments were performed to determine antibiotic-resistant *Salmonella* reductions on inoculated chicken carcasses after a 20 s spray application of EPL or LAE followed by ACS with a 40 s time interval between the first and second intervention. Carcasses were sampled and then stored at 4.4 °C for up to 6 days. Sequential interventions to be evaluated included: (i) 300 mg/liter EPL followed by 30 % ACS (EPL300-ACS30) (ii) 200 mg/liter LAE followed by 30 % ACS (LAE200-ACS30) and (iii) sterile distilled water sprayed on carcasses 2x (iv) a control without spray. After application of the treatments, the carcasses were randomly assigned to a 0, 3 and 6 day storage period and placed in labeled poultry rinsing bags (Nasco, Fort Atkinson, Wis.). The bag labeled 0 day was subjected to an immediate rinse as described below while the bags labeled for 3 and 6 days were tied and held at 4.4 °C for further analyses. Detection limit of *Salmonella* was 100 CFU/ml of rinse liquid.

Sequential interventions for reducing APC, *E. coli*, coliforms, psychrotrophs on uninoculated chicken carcasses. Experiments were performed to determine reductions in APC, *E. coli*, coliforms, and psychrotrophs on uninoculated chicken carcasses. Carcasses received a 20 s spray of EPL or LAE followed by ACS after a 40 s time interval between first and second intervention. After transporting to the laboratory, each bagged carcass then subjected to the appropriate intervention treatment as described above. Carcasses were randomly picked for decontamination treatments, chilling and refrigerated storage for up to 10 days. Following the spray application of the assigned

treatments, carcasses were transferred into the poultry rinsing bags for an immediate rinse of BPW as described below while the remaining carcasses were transferred into iced water to chill for 1 h. Carcasses were then transferred into poultry rinsing bags for either an immediate rinse to perform microbiological analyses or held for up to 10 days 4.4 °C. The APC and *E. coli* detection limit was 1 CFU/ml of rinse liquid while coliforms and psychrotrophs detection limit was 10 CFU/ml of rinse liquid.

Sampling and microbiological analyses. Carcasses on which microbiological analyses to be performed were transferred into poultry rinsing bags (Nasco, Fort Atkinson, Wis.) and 400 ml of sterile BPW added. The carcasses were then rinsed inside and out with a rocking motion for 1 min by grasping the broiler carcass in the bag with one hand and the closed top of the bag with the other hand to assure that all surfaces were rinsed *(78)*. Following immediate serial dilutions of the rinses with sterile BPW, appropriate dilutions were used for duplicate plating of each microbiological analysis.

Counts of nalidixic acid and novobiocin-resistant ST and SE were determined by plating 0.1 ml of appropriate diluents onto the selective XLT4 agar as described above. The plates were incubated at 37 °C for 24 h before enumeration of characteristic colonies. The nalidixic acid and novobiocin-resistant *Salmonella* developed black centered colonies when viewed on the XLT4 media. Coliforms and *E. coli* counts were enumerated after spread plating 1 ml of appropriate diluents onto 3M Petrifilm™ *E. coli* /Coliforms Count Plates (3M Microbiology, St. Paul, MN) and incubating at 37 °C for 24 h (Coliforms, red colonies closely associated with entrapped gas) and 48 h (*E. coli*, blue colonies closely associated with entrapped gas) according to the manufacturer's

instructions. APC were determined by plating 0.1 ml of appropriate diluents onto the plate count agar (PCA, Difco, Becton Dickinson) and incubated at 37 °C for 48 h while counts of psychrotrophs were determined after plating on PCA and incubating at 4 °C for 10 days before enumeration.

L*a*b* color space value. Color space values for the outer skin surfaces were taken by reflectance using a Minolta Colorimeter (CR-200, Minolta C., Ramsey, N.J.) calibrated to a white standard tile (C $Y = 93.24$, $x = 0.3137$, $y = 0.3196$) set to channel 00 after the colorimeter port was covered with clear Reynolds® Foodservice film. Three readings were taken on the external skin surface of the breast.

Statistical analyses. The average number of colonies from the duplicate plates was recorded for each sample and the results converted $\log_{10}$ CFU/ml of rinse liquid. Three whole chicken carcasses were used per treatment on each storage day to determine *Salmonella* reduction on inoculated chicken carcasses. Three chicken carcasses were also used per treatment on each processing step to determine reductions in APC, *E. coli*, coliforms, and psychrotrophs on uninoculated chicken carcasses. The simple effect of treatments within each storage day and the simple effect of storage days within each treatment were determined with analysis of variance (ANOVA) procedures for *Salmonella* reduction on inoculated chicken carcasses. In addition, the simple effect of treatments within each processing step and simple effect of processing steps within each treatment were determined with ANOVA procedures for APC, *E.coli*, coliforms, and psychrotrophs on uninoculated chicken carcasses. Statistical analyses of data were performed with SAS 9.1 software (SAS Institute, Cary, N.C.). ANOVA procedures were

performed using PROC GLM procedure and Tukey multiple comparison test was used to determine the significant differences *(65)*.

RESULTS AND DISCUSSION

Sequential interventions for reducing *Salmonella* on inoculated chicken carcasses. Mean counts of *Salmonella* on control chicken carcasses and chicken carcasses sprayed with distilled water, EPL300-ACS30 or LAE200-ACS30 and stored at 4.4°C for up to 6 days are presented in Table 17 and Figure 12. The initial inoculation level of *Salmonella* was 6.2 log CFU/ml on the surfaces of inoculated, untreated control carcasses. There were no significant differences ($P > 0.05$) among *Salmonella* counts on control carcasses over 6 days of storage. *Salmonella* counts on the distilled water sprayed carcasses were 6.0, 6.1 and 5.9 log CFU/ml on day 0, 3 and 6 of storage, respectively, but these were not different. *Salmonella* counts of 4.7, 5.2 and 5.1 log CFU/ml were observed for the EPL300-ACS30 treatment on 0, 3 and 6 days of storage, respectively. EPL300-ACS30 reduced *Salmonella* counts significantly ($P < 0.05$) by 1.5 and 1.2 log CFU/ml on storage day 0 and 6 when compared to the control. LAE200-ACS30 likewise, produced significantly ($P < 0.05$) lower *Salmonella* counts on each storage day when compared to the control with *Salmonella* reductions of 1.8, 1.4 and 1.8 on 0, 3 and 6 days of storage, respectively.

Similar to our findings with the distilled water spray, bird washing systems with water alone have been reported not to be effective interventions for reducing attached pathogens such as *Salmonella*, *Campylobacter*, *E. coli* and APC or coliforms *(6, 23, 33, 34, 52, 53)*. However, bird washers are effective for removing loose material from

carcass surfaces during evisceration *(6, 23, 33)*. EPL has been reported to have enhanced antimicrobial activity when combined with glycine, vinegar, ethanol and thiamine laurylsulfonate *(82)*. Keeton et al. *(37)* reported that sequential sprays of warm solutions (55 °C) of 20 % ACS followed by 100 mg/liter EPL was effective for reducing ST, *E. coli* O157:H7 (EC), and *Listeria monocytogenes* (LM) on beef rounds. In addition, LAE has been shown to effectively inhibit growth of LM on cooked meats during refrigerated storage *(2, 45)*. LAE and ACS, when used in combination, were found to be highly effective in a patented "Spray Lethality In Container" (SLIC®) intervention delivery system for reducing *L. monocytogenes* on ready-to-eat products *(45)*. Further, the data in Figure 12 indicates that none of the treatments produced incremental reductions in *Salmonella* numbers over storage.

Color measurements were taken before and after inoculation and after treatment with EPL300-ACS30, LAE200-ACS30 and distilled water on the external skin surface of the breast. L* a* b* values were statistically analyzed to determine differences between "before inoculation" "after inoculation" and "after treatment" measurements for each intervention (Table 18). L* a* b* mean values before inoculation, after inoculation and after treatment were not different with distilled water spray. EPL300-ACS30 produced a significantly ($P < 0.05$) lower L* value and higher b* value after treatment. Similarly, LAE200-ACS30 caused a significantly ($P < 0.05$) lower L* value and higher b* value after treatment. None of the decontamination treatments affected a* values of chicken carcasses regardless of the time of measurement. The color data indicated that surface of the chicken carcass became just slightly darker and more yellow after

application of EPL300-ACS30 and LAE200-ACS30 treatments, and that these changes might be detected by consumers.

Sequential interventions for reducing APC, *E. coli*, coliforms, psychrotrophs on uninoculated chicken carcasses. The effects of sequential application of EPL300-ACS30, LAE200-ACS30 or distilled water for reducing APC, *E. coli,* coliforms and psychrotrophs on uninoculated chicken carcasses after treatment, after chilling and after storage (10 day at 4°C) were evaluated. The initial APC count on uninoculated, control chicken carcasses was 4.7 log CFU/ml (Table 19 and Fig. 13). APC counts after treatment with EPL300-ACS30, LAE200-ACS30 and distilled water were not different with corresponding counts of 3.6, 3.8 and 5.0 log CFU/ml, respectively. Similar trends were observed after chilling and after storage for APCs of treatments (Table 19). The effects of processing steps on APCs for each decontamination treatment are presented in Figure 13. APCs were lower (3.9 log CFU/ml) ($P < 0.05$) after chilling on distilled water sprayed chicken carcasses when compared to counts immediately after treatment and storage. This same trend was noted in the control, but not the other samples. APCs were not different after treatment, chilling and storage for the EPL300-ACS30 and LAE200-ACS30 treatments (Fig. 13). In contrast, Sinhamahapatra et al. *(66)* tested the effects of hot water (70 °C for 1 min), 2 % lactic acid for (30 s), and 1200 ppm acidified sodium chlorite (5 s) and 50 ppm chlorine solution (5 min) applied to broiler carcasses as an immersion or spray treatment. They found the lactic acid dip and hot water dip were the most effective for reducing APCs by 1.36 log and 1.28 log/cm^2, respectively, whereas

acidified sodium chlorite and a hot water dip reduced presumptive coliform counts by 1.37 log and 1.34 log/cm^2.

The average initial count of *E. coli* on uninoculated chicken carcasses was 4.0 log CFU/ml (Table 20). Distilled water treatment was not effective for reducing *E. coli* counts on uninoculated chicken carcasses. However, immediately after treatment EPL300-ACS30 and LAE200-ACS30 both reduced *E. coli* counts significantly by 2.6 and 2.9 log CFU/ml, respectively. But, following chilling and storage, counts of *E. coli* were not different for any of the treatments. *E. coli* counts at different intervals for each decontamination treatment are presented in Figure 14. *E. coli* counts decreased ($P < 0.05$) incrementally on control and distilled water sprayed chicken carcasses from 4.0 to 1.8 log CFU/ml and 4.0 to 1.9 log CFU/ml, respectively, after chilling and storage (Fig. 14). EPL300-ACS30 and LAE200-ACS30 *E. coli* counts were both lower ($P < 0.05$) immediately after treatment compared to the control and distilled water spray (Table 20). Like the control and distilled water spray, *E. coli* counts for the other treatments decreased after 10 days storage. Unlike the EPL300-ACS30, the LAE200-ACS30 counts increased ($P < 0.05$) after chilling but then declined to levels similar to EPL300-ACS30 after 10 days storage at 4.4 °C. *E. coli* counts on all treatments were just above the detection limit at the end of the storage. Berrang et al. *(3)* also found that a second scald applied after defeathering either as an immersion treatment at 60 °C (28 s immediately or 30 min after defeathering) or as a spray treatment at 71-73 °C (20 s immediately or 30 min after defeathering) was effective for reducing *Campylobacter, E. coli* and coliforms on chicken carcasses. In contrast, chilling carcasses in a 20 ppm sodium hypochlorite

solution has also been shown to reduce coliforms, *Campylobacter, E. coli* and *Salmonella* counts by 1.2, 1.3, 1.4 and 0.5 log respectively *(51)*.

Coliform counts on control and treated poultry carcasses were almost the same as *E. coli* counts and followed the same pattern of reduction after treatment, chilling and storage (Table 21, Fig. 15). Fluckey et al. *(24)* investigated the microbiological profile of air-chilled poultry from the farm through the processing plant. They found a positive correlation between contamination of ceca with *Salmonella* on the farm and the presence of the *Salmonella* on carcasses at the plant collected before evisceration, after evisceration, and after chilling. However, no reduction in *Salmonella* numbers was observed during processing. Coliforms counts were 3.91, 3.27 and 2.59 log CFU/ml before evisceration, after evisceration and after chilling respectively, while generic *E. coli* counts were 3.74, 3.08 and 2.20 log CFU/ml.

The initial psychrotroph counts on uninoculated chicken carcasses were low at 1.2 log CFU/ml (Table 22) while distilled water treatment had a similar count of 1.5 log CFU/ml. Eventhough EPL300-ACS30 and LAE200-ACS30 psychrotroph counts were under the detection limit, there were no significant differences among treatments (Table 22). Over a 10 day storage period, EPL300-ACS30 and LAE200-ACS30 produced lower psychrotrophic counts at each interval (after treatment, chilling, and storage) when compared to the control and distilled water treatments (Fig. 16). All psychrotrophs increased significantly ($P < 0.05$) with 10 days of storage at 4.4°C for all treatments. Nevertheless, sequential application of EPL300-ACS30 and LAE200-ACS30 reduced psychrotrophic counts more than 1 log CFU/ml after 10 days of storage at 4.4 °C when

compared to the control and distilled water treatments (Table 22). Dickens et al. *(18)* found that spraying a higher concentration (1:1 solution of deionized water and $Safe_2O$ Poultry Wash) of ACS (4 ml/wing) increased the shelf life of chicken wings from 7 days to 10 days when compared with deionized water-spray controls. For the water and $Safe_2O$ Poultry Wash treatment, they reported the following counts: *Pseudomonas* sp., 8.2 and 6.9; *Staphylcoccus* sp., 5.5 and 4.9; *L. monocytogenes*, 5.2 and 4.6; and psychrotrophs, 8.2 and 6.9 respectively, at the end of 10 days storage.

Sanchez et al. *(64)* compared immersion chilling and air chilling for reducing microbiological loads and the incidence of *Salmonella* spp. and *Campylobacter* spp. on broiler carcasses. They found no significant differences between immersion chilling and air chilling for total aerobic counts (3.38 log and 3.31 log CFU/ml, respectively), generic *E. coli* (1.17 log and 1.43 log CFU/ml, respectively) or coliforms (1.72 log and 1.97 log CFU/ml, respectively). Counts of psychrotrophs were significantly higher for immersion chilled carcasses than air-chill carcasses (3.20 log and 1.91 log CFU/ml, respectively). The incidence of *Salmonella* spp. and *Campylobacter* spp. were reported lower in air-chilled broilers due to a higher prevalence of cross-contamination among immersion-chilled broilers. Hinton et al. *(29)* studied the effects of acidic, electrolyzed oxidizing water and chlorinated water sprays on the spoilage microflora of broiler carcasses during 14 days of storage (4 °C). They reported that psychrotrophic bacteria was reduced immediately after spraying with acidic, electrolyzed oxidizing water and chlorinated water and after 14 days of storage when compared to carcasses sprayed with tap water.

In conclusion, sequential treatment of *Salmonella* inoculated carcasses with an EPL300-ACS30 combination reduced *Salmonella* counts initially by 1.5 and by 1.2 log CFU/ml following 6 days of storage at 4.4 °C when compared to control samples. Likewise, LAE200-ACS30 treatment reduced initial *Salmonella* counts on poultry carcasses by 1.8 log, 1.4 log and 1.8 log CFU/ml, respectively, after 0, 3 and 6 days storage. Color data indicated that surface of the poultry carcasses became slightly darker and more yellow following both EPL300-ACS30 and LAE200-ACS30 treatments. EPL300-ACS30 treatment was slightly more effective on APC counts than LAE200-ACS30. Both treatments produced lower numerical APCs after treatment and after 10 days of storage. APC counts decreased with chilling immediately after spraying on the control and distilled water treatment, but APC counts increased after 10 days of storage for both control and distilled water samples. APC counts did not change over 10 days after treatment with EPL300-ACS30 or LAE200-ACS30. EPL300-ACS30 and LAE200-ACS30 reduced *E. coli* counts significantly by 2.6 and 2.9 log CFU/ml, respectively, but the distilled water counts were not reduced. Following chilling and storage, *E. coli* counts were not different among the control and other treatments. Similar results were also observed for coliform counts. Psychrotroph counts for the control and distilled water treatment were barely detectable after spraying and EPL300-ACS30 and LAE200-ACS30 were just under detection limit. Psychrotrophs increased significantly with 10 days of storage at 4.4 °C for all treatments, but sequential applications of EPL300-ACS30 and LAE200-ASC30 were effective in lowering counts of psychrotrophs by 1 log CFU/ml when compared to the control and distilled water treatments. Reductions in

psychrotrophic counts of EPL300-ACS30 and LAE200-ACS30 treatments after 10 days storage at 4.4 °C indicate that these treatments have the potential to increase the shelf-life of poultry carcasses.

CHAPTER V

REDUCTION OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* TYPHIMURIUM ON BEEF SURFACES AND IN GROUND BEEF USING SEQUENTIAL SPRAY APPLICATION OF ε-POLYLYSINE OR LAURIC ARGINATE FOLLOWED BY ACIDIC CALCIUM SULFATE

INTRODUCTION

The United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) issued a final regulation on July 25, 1996 establishing pathogen reduction requirements applicable to meat establishments *(71)*. These were designed to reduce the occurrence and numbers of pathogens in meat and poultry products, thus reducing the risk of food-borne disease *(46)*. Although the incidence of certain foodborne pathogens has declined significantly since 1996, this decline occurred before 2004. Foodborne Diseases Active Surveillance Network (FoodNet) data for 2007 indicate that no significant reductions in *Escherichia coli* and *Salmonella* infections have occurred in the United States when compared with the 2004-2005 reports *(15)*. In the 2007 report, the incidence of *Salmonella* infections was 14.92 cases per 100,000 persons and not on track to meet the national target of 6.8 cases per 100,000 persons by 2010.

Contamination of beef carcass surfaces during the slaughter process with *E. coli* O157:H7 (EC) and *Salmonella* occur due to contact with feces and the hide which are the most likely sources of contamination *(21, 31, 36, 39, 55)*. Carcass decontamination methods widely reviewed in the literature to reduce or eliminate pathogens include

trimming, steam vacuuming, steam pasteurization, cold and hot water rinses, organic acid rinses, ozonated or electrolyzed water, and a variety of chemical rinses including chlorine, chlorine dioxide, trisodium phosphate, peroxyacetic acid, cetylpyridinium chloride, acidified sodium chlorite, or acidic calcium sulfate *(21, 31, 36, 40, 60)*. Huffman *(31)* noted that hot water applications have the potential of reducing bacterial counts by 1-3 log cycles on beef carcasses. Castillo et al. *(9)* likewise reported that a water wash followed by hot water spray (95 °C) reduced levels of EC, *Salmonella* Typhimurium (ST), APC and coliforms by 3.7, 3.8, 2.9 and 3.3 log, respectively, on carcass surfaces. Spray-washing (26 °C, 276 kPa followed by 1000 kPa) followed by hot-water rinsing (>77 °C, 138-152 kPa, 2.5 to 8 s) and knife-trimming followed by a second spray-wash also have been shown to be an effective beef carcass decontamination method *(17)*. Hardin et al. *(27)* found that carcass washing followed by warm acid sprays (55 °C) of lactic acid or acetic acid performed better than trimming or washing alone for reducing *Salmonella* and EC and that lactic acid was more effective than acetic acid for EC reduction. Castillo et al. *(8)* reported that both a water wash and trimming combined with sanitizing treatments of hot water (95 °C) or warm (55 °C) 2 % lactic acid spray or a combination of these two sanitizing methods resulted in reductions of more than 4.0 log CFU/cm^2 for ST and EC on beef carcasses. In another study, both EC and ST counts were reduced by 3.8 to 3.9 log and 4.5 to 4.6 log with a water wash followed by a phosphoric acid-activated acidified sodium chloride spray or a citric acid-activated acidified sodium chlorite spray, respectively *(10)*. A 4% L-lactic acid spray at 55 °C prior to fabrication has also been suggested for chilled beef carcasses which were

previously subjected to a hot water spray followed by a lactic acid spray (details of prechill decontamination steps were not made available by the authors due to proprietary reasons) prior to chilling *(11)*. Ramirez et al. *(59)* tested a water rinse followed by either a 2 % lactic acid (9 s, at 55 °C) or a 12 % trisodium phosphate (60 s, at 55 °C) dip or a combination of these treatments. Both treatments alone or in combination were effective for reducing EC by more than 1.6 log/cm^2 on lamb breast tissue. In a comparative study, King et al. *(38)* on the other hand reported that a peroxyacetic acid spray was not an effective intervention for EC and ST reduction on chilled beef carcasses when compared to carcasses treated with 2 % L-lactic acid spray before chilling or 4 % L-lactic acid spray after chilling. In another study, aqueous ozone treatment (28 °C; 95 mg/liter) was not found to be effective against EC and ST when sprayed on hot carcass surfaces as compared to a water wash (28 °C) alone *(13)*. Dorsa et al. *(20)* tested the effect of 2 % lactic acid, 2 % acetic acid, 12 % trisodium phosphate, and water washes at 72 °C and 32 °C for reducing pathogens and other bacterial populations on beef carcass surfaces and cuts held for up to 21 days (4 °C) under vacuum. They found that lactic acid and acetic acid treatments suppressed or eliminated bacteria on beef carcass surfaces inoculated with low levels (< 2 log) of *Listeria innocua,* ST, EC, and *Clostridium sporogenes* in a bovine fecal cocktail during refrigerated storage. Additionally, Dorsa et al. *(19)* suggested that a 2 % lactic acid or 2 % acetic acid wash during beef processing beef could suppress pathogen proliferation in ground beef during long-term refrigerated storage or short-term abusive temperature storage. Similarly, Castillo et al. *(12)* reported that pre-chill hot carcass water wash treatments alone or water wash followed by a 2 %

lactic acid spray (250 ml, 15 s, 55 °C) produced 3.3 – 5.2 log reductions of EC and ST on contaminated outside rounds. They then applied a post-chill, 4 % lactic acid spray (500 ml, 30 s, 55 °C) that further reduced EC and ST counts by 2.0-2.4 log and 1.6-1.9 log, respectively when combined with the pre-chill hot carcass treatments. Moreover, significantly lower levels of these pathogens were also observed in ground beef produced from the post-chill decontaminated rounds when compared to ground beef from pre-chill decontaminated rounds alone. Stivarius et al. *(69)* tested the effect of tumbling inoculated beef trimmings in hot water (82 °C) or 5 % lactic acid prior to grinding to reduce EC and ST in ground beef. They reported that lactic acid was effective for reducing EC counts in ground beef stored refrigerated 7 days, but also noted a reduction in the redness of the ground beef. Conversely, 10 % trisodium phosphate or 0.5 % cetylpyridinium chloride treatment applied by tumbling significantly reduced EC and ST and improved the redness of ground beef *(58)*. Harris et al. *(28)* reported acidified sodium chlorite (1200 ppm), acetic acid (2 %) and lactic acid (4 %) spray treatments applied to inoculated beef trimmings prior to grinding reduced EC and ST counts by 2.5 log and 1.5 log, respectively in the ground beef.

Individual interventions in most cases are not as effective for reducing pathogens on beef carcasses as hurdle technology or a sequential interventions approach *(21, 31, 40, 56, 57, 60)*. Application of two or more microbial decontamination treatments appears to produce greater reductions than one treatment alone due to different modes of action of the antimicrobials. Acidic calcium sulfate, also known as acidified calcium sulfate (ACS), is a very acidic (pH 1.0 - 1.5) organic acid-calcium sulfate complex *(36)*.

Its use in the production of meat and poultry products has been approved as a secondary food additive by USDA-FSIS *(77)*. In several studies and reviews, ACS's effectiveness has been demonstrated to reduce pathogens on beef or poultry carcass surfaces, RTE meat products including frankfurters and hams, and in ground beef *(18, 31, 35-37, 45, 54, 83)*. ε-Polylysine (EPL) is a homo-poly-amino acid of 25 to 35 L-lysine molecules connected by the peptide bond between the carboxyl and ε-amino groups. As an edible and water soluble agent, EPL has a wide range of antimicrobial activity including Gram-positive and Gram-negative bacteria *(25, 26, 81, 82)*. EPL has been studied to confirm its safety as a preservative in foods and deemed non-toxic in rats in an acute oral toxicity study (no mortality at levels up to 5g/kg), and it was not mutagenic in bacterial reversion assays *(30)*. Additionally, use of EPL as an antimicrobial agent in cooked or sushi rice at levels up to 50 mg/kg rice has been approved as generally recognized as safe (GRAS) by the United States Food and Drug Administration *(80)*. Lauramide arginine ethyl ester (LAE), also known as lauric arginate, is a derivative of lauric acid and arginine with broad-spectrum antimicrobial activity *(2, 61)*. LAE has been demonstrated to be non-toxic and rapidly metabolized to naturally occurring amino acids, mainly arginine and ornithine, following consumption *(62)*. LAE is affirmed as a GRAS ingredient and is allowed in a wide range of food products, including poultry and meat products *(77)*.

ACS plus organic acids have been reported to disable proton pumps in bacterial membranes and thus act as metabolic inhibitors *(35)*. EPL on the other hand is adsorbed into the cell surface of microorganisms as a result of its cationic properties and strips the outer membrane and distributes the cytoplasm *(81, 82)*. LAE in comparison prevents

bacterial growth by altering the cell membrane structure of microorganisms *(61)*. Thus, sequential application of two of these decontamination agents in combination might result in greater pathogen reductions than any single application of these agents on beef sample due to their differing mode of action. The objectives of this study were to determine effectiveness of sequential application of EPL or LAE followed by ACS for reducing EC and ST on inoculated beef surfaces and to determine if these reductions carried over to ground beef during refrigerated storage.

MATERIALS AND METHODS

Media. Lactose-sulfite-phenol red-rifampicin (LSPR) is a selective, differential medium developed by Castillo et al. *(9)*. A modified LSPR was prepared and used in this study *(37)*. The medium consisted of following ingredients per liter: tryptic soy agar (TSA; Difco, Becton Dickson, Sparks, Md.) 40 g, yeast extract (Difco, Becton Dickinson) 3 g, beef extract (Difco, Becton Dickinson) 3 g, lactose 5 g (Difco, Becton Dickinson), sodium sulfite (Sigma, St. Louis, Mo.) 2.5 g, ferrous sulfate (Sigma) 0.3 g, phenol red (Sigma) 25 mg, and rifampicin (Sigma) 0.1 g. Two ml of 0.1 N NaOH was used to dissolve phenol red before adding to the medium. The medium was autoclaved at 121 °C for 15 min and cooled to 50 °C without rifampicin. Rifampicin was prepared by dissolving in 5 ml methanol, filter-sterilized and added to the sterile medium prior to pouring into petri plates. The medium allowed simultaneous enumeration of rifampicin-resistant EC and ST. Yellow colonies were produced by rifampicin-resistant EC on the medium while rifampicin-resistant ST formed black centered colonies surrounded by pink halos.

Bacterial cultures and inoculum preparation. Rifampicin-resistant mutants of *Salmonella* Typhimurium (ST) and *Escherichia coli* O157:H7 (EC) ATCC 43895 were obtained from Dr. Alejandro Castillo (Texas A&M University, College Station, Tex.). Each mutant was maintained on TSA at 4 °C. The cultures were transferred in tryptic soy broth (TSB; Bacto, Becton Dickinson, Sparks, Md.) on three consecutive days at 37 °C before use in the experiments. Rifampicin-resistance was confirmed by streaking the cultures onto LSPR plates and incubating at 37 °C. On the night before each experiment day, fecal samples were collected at the Texas A&M Beef Center from randomly selected cattle after defecation and transported to the laboratory. The fecal samples were kneaded by hand in a stomacher bag (Labplas Inc., Ste-Julie, Canada) for 1 min and then 10 g portions transferred into individual stomacher bags and stored at 4 °C until the next day. Eighteen-hour cultures of rifampicin-resistant ST and EC (5 ml each) were transferred into stomacher bags and hand-kneaded for 1 min to prepare the fecal inoculum *(9, 13)*. A fecal sample without the marked pathogens was used to confirm that no rifampicin-resistant organisms were present among the background flora of the fecal samples by plating onto LSPR agar. Inoculums were used within 3 h after preparation and kept at room temperature during the experiments.

Sample collection and inoculation. Sample collection and inoculation were performed according to methods described by Castillo et al. *(9)* and Keeton et al. *(37)*. Fresh, prerigor beef round samples were collected from a local abattoir. Immediately after skinning, a 15 x 25 x 5 cm^3 round sample was excised from the dorsal side of a carcass prior to evisceration. Each round was individually placed in a 2.5 gal (9.46 liter)

Hefty® OneZip bag. The bags were then placed in insulated containers and transported to the laboratory.

A 10 x 15 cm^2 area on the hide surface of a beef round was outlined with metallic pins and inoculated with equal amounts of the fecal inoculums (≈ 7 g) prepared as described above. The inoculums were allowed to stand for 10 min for bacterial attachment. Following inoculation, the samples were subjected to the appropriate pathogen intervention treatment.

For the ground beef experiments, the entire outer surface of a beef round was inoculated with equal amounts of the fecal inoculums (≈ 12 g) and allowed to stand for 10 min for bacterial attachment. Following inoculation, the samples were then subjected to appropriate pathogen intervention treatments.

Application of intervention treatments. Beef rounds were placed in a stainless steel custom-built isolation spray cabinet (CHAD Corporation, Olathe, Kans.) and intervention treatment solutions applied singly (Fig. 2). A sterile distilled water solution served test the spray effect alone. Two VeeJet® spray nozzles (model H1/8VV-SS65015, Spraying Systems Co., Wheaton, Ill.) were situated (spray angle of 65°) inside the cabinet near the top and bottom of the cylindrical spray chamber, through which treatments were delivered. Treatment solutions were poured into individual spray tanks which had two regulator valves mounted on top. While one of the valves was connected to the nozzles via a hose, the other one was connected to an air compressor (Campbell Hausfeld, South Pasadena, Calif.). The air compressor was used to pressurize the system

and apply a constant spray pressure of 37 psi (255.1 kPa) which delivered 500 – 520 ml of treatment solution over a 20 s spray interval.

Treatment solutions (900 ml) were prepared individually in 1 liter bottles using sterile distilled water and kept in a water bath to maintain a solution temperature of 55 °C. Warm decontamination solutions were then transferred to individual tanks, attached to the cabinet and sprayed for 20 s while rotating the contaminated beef rounds at constant rate for ~10 revolutions in a uniform spray stream. Prior to spraying, an inoculated round was suspended on set of stainless steel hooks, with a single rod support that allowed attachment to a turn-style inside the center of the spray cabinet lid (Fig. 3). After the sample was attached to the lid, it was positioned into the central cabinet cavity by placing the fitted lid onto the top of the cabinet frame and sealing. A turn-style handle projecting through the lid was connected to the hanger and used to rotate the inoculated samples in the spray stream during application of the treatments.

Sequential spray application of interventions to the beef round surface. Two pathogen interventions were tested with rifampicin-resistant ST and EC to determine reductions on inoculated round surfaces after a 20 s spray application. EPL or LAE were applied first followed by ACS with a 40 s time interval between the first and second intervention to allow for some drainage of the first treatment. The sequential interventions evaluated were: (i) 300 mg/liter EPL followed by 30 % ACS (EPL300-ACS30) (ii) 200 mg/liter LAE followed by 30 % ACS (LAE200-ACS30) and (iii) sterile distilled water sprayed on carcasses 2x (iv) a control without spray.

Following treatment application, the outlined area on the round surface was aseptically removed and cut into 3 equal pieces (50 cm^2 each) using a sterile scalpel and forceps. Individual samples were excised 2-3 mm deep, randomly transferred into stomacher bags (low density polyethylene 0.93 g/cm^3 with polymer film permeability of 7 for N_2, 20 for O_2, 100 for CO_2 and 1 cm^2/m^2/day/bars for water vapor) and held for 0, 3 or 6 days at 4.4 °C. The stomacher bags labeled for storage day 3 or day 6 were tied and held at 4.4 °C to evaluate the effect of the storage on the growth of rifampicin-resistant ST and EC.

Sequential spray application of EPL or LAE and ACS for ground beef. EPL (300 mg/liter) or LAE (200 mg/liter) followed sequentially by 30 % ACS were applied to prerigor beef rounds inoculated with rifampicin-resistant ST and EC. Following the application of previously described decontamination treatments; beef rounds were individually placed in 2.5 gal (9.46 liter) Hefty® OneZip bags and stored for 2 days at 4.4 °C before being processed into ground beef. Eighteen sterile cast iron manual meat grinders (model 10HC, Admiral Craft Equipment Corp., Hicksville, N.Y.) were used to grind each sample separately through a 3/8" (9.53 mm) plate. Each sample was passed through a hand grinder two times and hand kneaded using sterile technique for 1 min between the first and the second grind to obtain a homogeneous distribution of the pathogens in the ground beef. Ground samples were then divided into 3 groups (300 – 400 g each) and randomly placed on white styrofoam meat trays, over-wrapped with Reynolds® Foodservice film and held for 0, 2 and 4 days storage at 4.4 °C.

Sampling and microbiological analysis on surfaces of beef rounds. Sterile buffered peptone water (100 ml) (BPW; Difco, Becton Dickson, Sparks, Md.) was used for pathogen recovery on each storage day (0, 3, 6). To facilitate recovery stomacher bags containing an excised beef sample and BPW were pummeled for 1 min in a Stomacher 400. Numbers of rifampicin-resistant ST and EC were simultaneously enumerated from these samples by preparing dilutions of the homogenate with sterile BPW and plating the appropriate 10-fold dilutions onto prepoured and dried LSPR agar plates. The plates were then incubated at 37 °C for 24 h, enumerated and the results reported as $\log_{10}$ CFU/cm^2 of rifampicin-resistant ST and EC. Detection limit for ST and EC was 20 CFU/cm^2 of round surface.

Sampling and microbiological analysis of ground beef. Two separate 25 g samples were collected randomly from each tray for microbiological analysis on each storage day. The samples were then transferred into individual stomacher bags, 225 ml of sterile BPW was added and the bags were pummeled for 1 min in a Stomacher 400. Numbers of rifampicin-resistant ST and EC were simultaneously enumerated from these samples by preparing dilutions of the homogenate with sterile BPW and plating the appropriate 10-fold dilutions onto prepoured and dried LSPR agar plates. The plates were then incubated at 37 °C for 24 h, enumerated and the results were reported $\log_{10}$ CFU/g of rifampicin-resistant ST and EC. Detection limit for ST and EC was 10 CFU/g of ground beef.

L*a*b* color space value. Color space values of beef round surfaces were taken by reflectance using a Minolta Colorimeter (CR-200, Minolta C., Ramsey, N.J.)

calibrated to a white standard tile (C $Y = 93.24$, $x = 0.3137$, $y = 0.3196$). The colorimeter was set to channel 00 after the colorimeter port was covered with clear Reynolds® Foodservice film. Three random readings were taken outside the inoculated area on each round surface before and after decontamination. Positive L* values indicate the degree of lightness while negative L* values indicate darkness. Positive a* values quantify the degree of redness whereas negative values indicate greenness. Positive b* values indicate yellowness while negative values indicate blueness.

Statistical analyses. The average number of colonies on duplicate plates for the beef round surface experiment and duplicate samples for the ground beef experiment were recorded for each sample. Reduction values for each pathogen were the differences in cell numbers between untreated-control samples and treated samples. The beef round surface experiment data and the ground beef experiment data were generated by performing 6 replications (6 samples per treatment) of the treatments described previously. Statistical analyses of the data were performed with SAS 9.1 software (SAS Institute, Cary, N.C.). Simple effect of treatments within each storage day and simple effect of storage days within each treatment were determined with analysis of variance (ANOVA) procedures. ANOVA procedures were performed using the PROC GLM procedure and Tukey's multiple comparison test was used to determine which means were significantly different at $P < 0.05$ significance level *(65)*.

RESULTS AND DISCUSSION

Pathogen reductions on beef round surfaces. Rifampicin-resistant ST counts on control beef rounds and those sprayed with distilled water, EPL300-ACS30 or

LAE200-ACS30 at 55°C and stored at 4.4°C for up to 6 days are presented in Table 23. At day 0, the mean initial inoculation level of ST on the surfaces of untreated control samples was 6.4 log CFU/cm^2. There were no significant differences ($P > 0.05$) among the counts of ST on control samples over 6 days of storage. Distilled water spray produced ST counts of 5.4, 5.2 and 4.9 log CFU/cm^2 on 0, 3 and 6 days of storage, respectively; however, no significant differences were observed between the control and distilled water samples within each storage day. ST reductions on beef rounds sprayed with distilled water, EPL300-ACS30 or LAE200-ACS30 at 55 °C and stored at 4.4 °C for up to 6 days are presented in Figure 17. The reductions were the differences between ST counts of untreated controls and ST counts of the treatments on each storage day. The data in Figure 17 indicate no further reductions with a ST distilled water spray over 6 days of storage. Both EPL300-ACS30 and LAE200-ACS30 produced significantly ($P<0.05$) lower ST counts on each storage day when compared to the control and distilled water treatment (Table 23). An initial count of 4.1 log CFU/cm^2 for EPL300-ACS30 was observed with a corresponding 2.3 log CFU/cm^2 reduction. EPL300-ACS30 further reduced the counts of ST to 2.8 log CFU/cm^2 on storage day 3 and 1.6 log CFU/cm^2 on storage day 6 with corresponding reductions of 3.1 and 4.5 log CFU/cm^2, respectively. Thus, an EPL300-ACS30 combination was effective ($P < 0.05$) for reducing counts of ST initially and caused further reductions of ~ 2 logs by the 6th storage day (Fig. 17). LAE200-ACS30 applied to the surface of beef rounds resulted in an initial count of 3.9 log CFU/cm^2 with a corresponding reduction of 2.5 log CFU/cm^2 on day 0 (Table 23). Although LAE200-ACS30 reduced ST counts by 2.9 and 3.2 log

CFU/cm^2 on storage days 3 and 6, respectively, there were no ($P > 0.05$) differences among ST counts over the 6 days of storage (Fig. 17).

Rifampicin-resistant EC counts on control meat samples and meat samples sprayed with distilled water, EPL300-ACS30 or LAE200-ACS30 at 55°C and stored at 4.4°C for up to 6 days are presented in Table 24. Results similar to those observed for ST were obtained. The initial mean inoculation level of EC was 6.5 log CFU/cm^2 on the surfaces of untreated control samples. There were no differences ($P > 0.05$) among the counts of EC on control samples over 6 days of storage. Distilled water spray resulted in EC counts of 5.5, 5.4 and 5.1 log CFU/cm^2 on 0, 3 and 6 days of storage, respectively; however, no significant differences were observed between the control and distilled water samples for each storage day. EC reductions on round samples sprayed with distilled water, EPL300-ACS30 or LAE200-ACS30 at 55 °C and stored at 4.4 °C for up to 6 days are presented in Figure 18. The reductions were the differences between the EC counts of untreated controls and the EC counts of treatments on each storage day. The reduction data in Figure 18 indicate that no further reduction was obtained with distilled water spray over 6 days of storage. Both EPL300-ACS30 and LAE200-ACS30 produced significantly ($P < 0.05$) lower EC counts on each storage day when compared to control and distilled water treatments (Table 24). An initial EC count of 4.2 log CFU/cm^2 for EPL300-ACS30 was observed with a subsequent reduction of 2.3 log CFU/cm^2. EPL300-ACS30 further reduced counts of EC to 2.9 log CFU/cm^2 on storage day 3 and 1.9 log CFU/cm^2 on storage day 6 with corresponding reductions of 3.0 and 4.3 log CFU/cm^2, respectively. EPL300-ACS30 significantly ($P < 0.05$) reduced counts

of EC on days 0 and 3, but an even greater reduction was noted after 6 days of storage (Fig. 18). LAE200-ACS30 produced an initial count of 4.2 log CFU/cm^2 with a corresponding 2.3 log CFU/cm^2 reduction on day 0 which was the same as the EPL300-ACS30. Although LAE200-ACS30 reductions of EC were not different across storage days, the EPL300-ACS30 treatment resulted in a 4.3 log CFU/cm^2 reduction on day 6 as compared to a 3.5 log CFU/cm^2 with LAE200-ACS30. There were no differences among EC reductions over 6 days of storage with LAE200-ACS30. A sequential warm (55 °C) spray application of ACS (20 %) followed by EPL (100 mg/liter) at a constant pressure for 15 to 20 s was found effective for reducing ST, ET and *Listeria monocytogenes* (LM) on beef carcass surfaces by Keeton et al. *(37)*. In fact, they reported a *Salmonella* reduction of 4.38 log observed after 7 days of storage. Individual applications of ACS (20 %), lactic acid (2.5 %), and EPL (100 mg/liter) were not as effective as the application of ACS followed by EPL. In another study, Geornaras and Sofos *(25)* reported that acidic antimicrobials such as sodium diacetate and acetic acid, when combined with EPL, enhanced the antimicrobial activity of EPL. Similarly, the antimicrobial activity of EPL has been shown to be enhanced when combined with glycine, vinegar, ethanol and thiamine laurylsulfonate *(82)*. LAE has been reported to effectively inhibit the growth of LM in cooked meats during refrigerated storage *(2, 45)*. In addition, LAE and ACS used in combination were found highly effective in a patented “Spray Lethality In Container” (SLIC®) intervention delivery system for reducing LM in ready-to-eat products *(45)*.

Color measurements were taken before and after decontamination treatments with EPL300-ACS30, LAE200-ACS30 and distilled water (Table 25). There were no significant differences among mean values before or after treatment with distilled water for L*, a* and b* values (Table 25). The only significant ($P < 0.05$) difference between before and after treatments was for the L* values (61.26 and 48.80, respectively) of the EPL300-ACS30 treatment. Whether these differences would affect a consumer's perception of the treated surface is not known at this time. a* and b* values were significantly lower ($P < 0.05$) after treatment with LAE200-ACS30 when compared to the before treatment values. However, these values were rather small and may not be perceived by consumers. Treatment with LAE200-ACS30 had no effect on L* value before and after treatment. L*a*b* color values indicated that beef carcass surfaces become darker following treatment with EPL300-ACS30 while they become less red and less yellow with the LAE200-ACS30 treatment.

Pathogen reductions in ground beef. Rifampicin-resistant ST counts of ground beef samples stored 0, 2, and 4 days (4.4 °C) after grinding are presented in Table 26. Non-treated control and treated beef rounds sprayed with EPL300-ACS30 or LAE200-ACS30 were stored 2 days (4.4 °C) prior to grinding. The initial inoculation level of ST on control ground samples was 5.7 log CFU/g. There were no significant differences ($P > 0.05$) among ST counts on control samples over 4 days of storage. Initial ST counts of 4.1 and 3.8 log CFU/g for EPL300-ACS30 and LAE200-ACS30 were observed with 1.6 and 1.9 log CFU/g reductions, respectively (Fig. 19), and stayed significantly ($P < 0.05$) lower on each day of storage when compared to the ground control beef (Table 26).

However, neither EPL300-ACS30 nor LAE200-ACS30 reduced ST further in ground beef over the 4 day storage period at 4.4 °C (Fig. 19).

Rifampicin-resistant EC counts of ground beef samples stored 0, 2, and 4 days (4.4 °C) after grinding are presented in Table 27. Non-treated control samples and meat samples sprayed with EPL300-ACS30 or LAE200-ACS30 were stored 2 days (4.4 °C) prior to grinding. Their initial inoculation level of EC on the control ground samples was 6.7 log CFU/g. There were no significant differences ($P > 0.05$) among EC counts on control samples over 4 days of storage. Initial EC counts of 5.1 and 4.7 log CFU/g for EPL300-ACS30 and LAE200-ACS30 were observed with a 1.6 and 2.0 log CFU/g reductions, respectively (Fig. 20), and stayed significantly ($P < 0.05$) lower on each day of storage when compared to the ground control beef samples (Table 27). Like the ST results, neither EPL300-ACS30 nor LAE200-ACS30 reduced EC over 4 days of storage at 4.4 °C (Fig. 20). Zhao et al. *(83)* tested the combined effect of freezing and a mixture of 20 % acidic calcium sulfate (0.4 % final concentration in ground beef) and 10 % lactic acid (0.2 % final concentration in ground beef) on the thermal sensitivity of EC in ground beef. They found that addition of acidic calcium sulfate and lactic acid to ground beef reduced the temperature and time required to inactivate EC during heating. Nunez De Gonzalez et al. *(54)* reported that an acidic calcium sulfate with propionic acid and lactic acid (1:2 water) and lactic acid (3.4 % of a 88 % commercially available syrup) dips had bactericidal and bacteriostatic effects, respectively when used as a post-processing dipping solution to inhibit or control the growth of LM on vacuum-packaged frankfurters stored at 4.5 °C for up to 12 weeks.

Both warm (55 °C) EPL or LAE applied sequential by ACS onto inoculated beef round surfaces significantly ($P < 0.05$) reduced both ST and EC counts over 0, 3 and 6 days of storage at 4.4 °C by 2.3 to 4.5 log CFU/cm^2 and 2.3 to 4.3 log CFU/cm^2, respectively. A spray application of EPL300-ACS30 resulted in an even greater reduction after 6 days of storage for both ST and EC. EPL or LAE followed by ACS were applied as a spray to inoculated beef rounds and were stored 2 days (4.4 °C) prior to grinding. Ground beef manufactured from these rounds had lower ($P < 0.05$) ST and EC counts initially and stayed lower over 4 days of storage at 4.4 °C. Reductions in counts averaged from 1.6 to 2.4 log CFU/g for ST and 1.6 to 2.0 log CFU/g for EC. Overall, these results confirmed that sequential, multi-hurdle interventions were effective for reducing ST and EC on beef round surfaces as well as in ground beef produced from treated beef round tissues.

CHAPTER VI

CONCLUSIONS

Application of more than one antimicrobial to meat and poultry carcasses on a processing line might produce greater reductions than one treatment alone due to different modes of action of individual antimicrobials. This concept has been reviewed and studied under the names of hurdle technology, combined treatments, multiple hurdle carcass interventions or synergistic effect *(31, 40, 41, 70)*. Limited research has been conducted to evaluate the effects of sequential application of acidic calcium sulfate (ACS), ε-polylysine (EPL) and lauramide arginine ethyl ester (LAE) on pathogens. In this study, a broad approach was used to evaluate multi-hurdle interventions for reducing pathogens on both poultry carcasses and beef rounds starting with a membrane filter model, and concluding with optimization and storage studies carried out on poultry carcasses, beef rounds and ground beef.

In the first phase of the study, the efficacy of individually and sequentially applied interventions including ACS, EPL and/or LAE for reducing *Salmonella* Typhimurium (ST) and *Salmonella* Enteritidis (SE) on a membrane filter model were evaluated. Further, the selection of effective intervention combinations was determined using response surface methodology (RSM) on inoculated chicken carcasses. By combining these treatments, improved antimicrobial efficacy, as compared to individual treatments, was observed. Sequential spray applications of combinations of ACS, EPL or LAE applied at different time intervals using a membrane filter system were found more

effective than individual applications of the antimicrobials studied to reduce *Salmonella*. The most effective combinations were LAE200-EPL300, EPL300-ACS20 and EPL300-LAE200. The data indicated that the order of the application of the combined interventions had a direct effect on *Salmonella* reduction. RSM experiments predicted that an EPL300-ACS20 combination was the most effective spray application on poultry carcasses for reducing *Salmonella* under the conditions evaluated in the experiments.

In the second phase of the study, individual or sequential application of ACS, EPL or LAE at various concentrations on poultry carcasses were evaluated. The results of previous phase were taken into consideration to determine the application order of sequential interventions, application concentrations and time interval between the first and second intervention. EPL300-ACS30 and LAE200-ACS30 sequential spray applications produced the highest *Salmonella* reductions on inoculated chicken carcasses. Statistical comparison of the data obtained from sequential spray application of interventions and individual spray application of interventions showed that a 30 % ACS application was as effective as sequential application of EPL or LAE followed by ACS for reducing the initial counts of *Salmonella* inoculated onto chicken carcasses. However, in the third phase of the study, sequential applications of EPL300-ACS30 or LAE200-ACS30 as a multi-hurdle intervention for reducing *Salmonella* on inoculated chicken carcasses were performed and carcasses held under refrigeration for up to 6 days. In addition, further reductions of the resident microflora on uninoculated chicken carcasses during processing and storage were also determined. Sequential treatment of *Salmonella* inoculated carcasses with EPL300-ACS30 or the LAE200-ACS30

combination were effective for reducing *Salmonella* counts initially by 1.5 and 1.8 log CFU/ml, respectively, immediately after treatment, and by 1.2 and 1.8 log CFU/ml, respectively, following 6 days of storage at 4.4 °C. The surface of the poultry carcasses became slightly darker and more yellow following both EPL300-ACS30 and LAE200-ACS30 treatments. Both EPL300-ACS30 and LAE200-ACS30 treatments produced lower numerical aerobic plate counts after treatment and after 10 days of storage. EPL300-ACS30 and LAE200-ACS30 reduced *Escherichia coli* counts significantly by 2.6 and 2.9 log CFU/ml, respectively, but the distilled water counts were not reduced. However, following chilling and storage, *E. coli* counts were not different among the control and other treatments. Similar results were also observed for coliform counts. Psychrotrophs increased significantly after 10 days of storage at 4.4 °C for all treatments, but sequential applications of EPL300-ACS30 and LAE200-ASC30 were effective in lowering psychrotrophs counts by 1 log CFU/ml on day 10 when compared to the control and distilled water treatments. Reductions in psychrotrophic counts of EPL300-ACS30 and LAE200-ACS30 treatments after 10 days storage at 4.4 °C indicate that these treatments have the potential to increase the shelf-life of poultry carcasses.

In the fourth phase of the study, effectiveness of sequential application of EPL300-ACS30 or LAE200-ACS30 were investigated for reducing ST and EC on inoculated beef rounds, and to determine if these reductions carried over to ground beef during refrigerated storage. Both warm (55 °C) EPL or LAE applied sequential by ACS onto inoculated beef rounds reduced both ST and EC counts over 0, 3 and 6 days of storage at 4.4 °C by 2.3 to 4.5 log CFU/cm^2 and 2.3 to 4.3 log CFU/cm^2, respectively. A

spray application of EPL300-ACS30 resulted in an even greater reduction after 6 days of storage for both ST and EC. Ground beef manufactured from these rounds had lower ($P < 0.05$) ST and EC counts initially and stayed lower over 4 days of storage at 4.4 °C. Reductions in counts averaged from 1.6 to 2.4 log CFU/g for ST and 1.6 to 2.0 log CFU/g for EC. Overall, these results confirmed that sequential, multi-hurdle interventions were effective for reducing ST and EC on beef round surfaces as well as in ground beef produced from treated beef round tissues.

This research demonstrates that multi-hurdle interventions including EPL300-ACS30 or LAE200-ACS30 treatments can be effectively used for pathogen reduction and possibly for increasing shelf-life of fresh poultry and beef products. Further investigations may include in-plant verification of multi-hurdle interventions with comparisons to the individual interventions for successful adoption of this technology by the poultry and meat industry.

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APPENDIX A

FIGURES

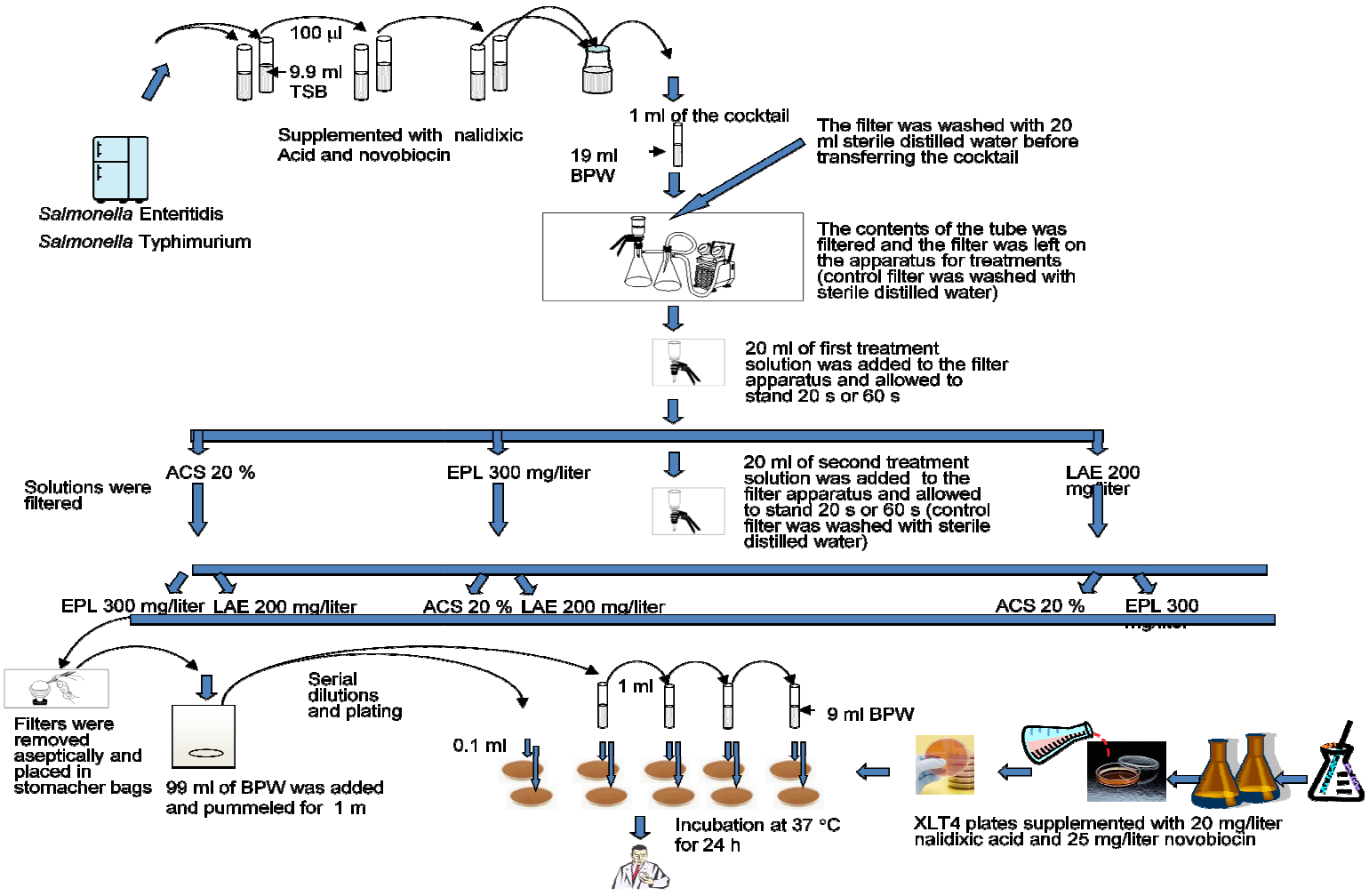

FIGURE 1. *Schema of application of antimicrobial interventions using a membrane filter model system.*

FIGURE 2. *A custom built isolation spray cabinet designed by CHAD Corporation, showing containment chamber, three stainless steel reservoirs for application of treatments, white plastic receptacle for application of cleaning solutions, and pressure gauges.*

FIGURE 3. *A custom built isolation spray cabinet designed by CHAD Corporation, showing rotating stainless steel hooks for suspension of chicken carcasses and beef rounds.*

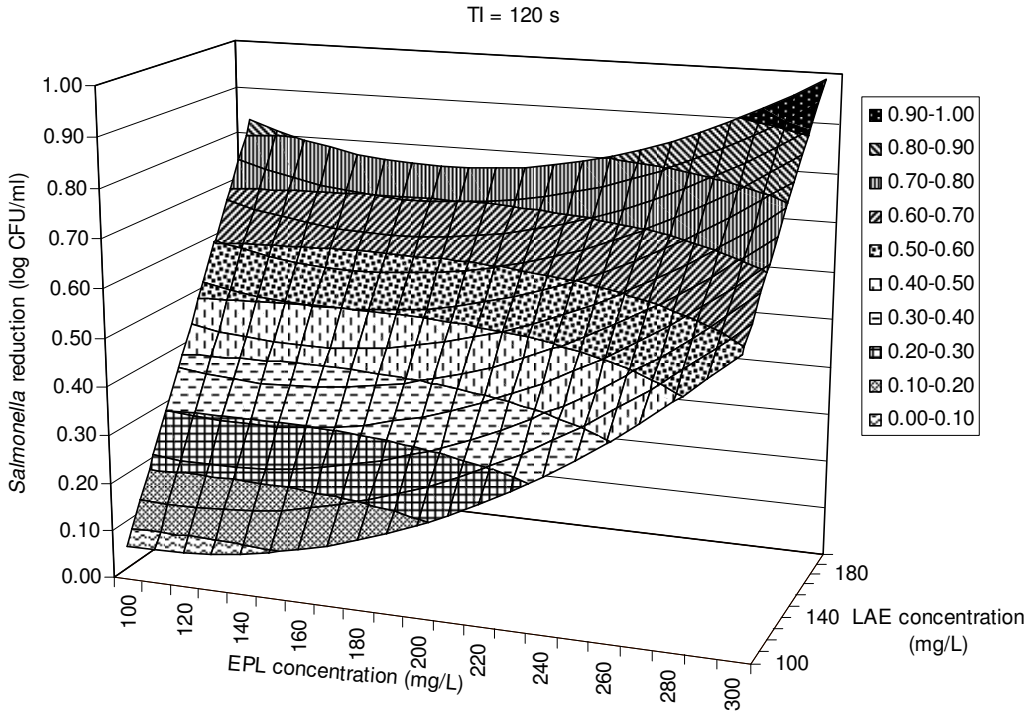

FIGURE 4. *Response surface plot of reduction of* Salmonella *inoculated onto chicken carcasses and treated with EPL and LAE sequentially for 20 s using RSM in Box-Behnken design at a 120 s time interval between applications.*

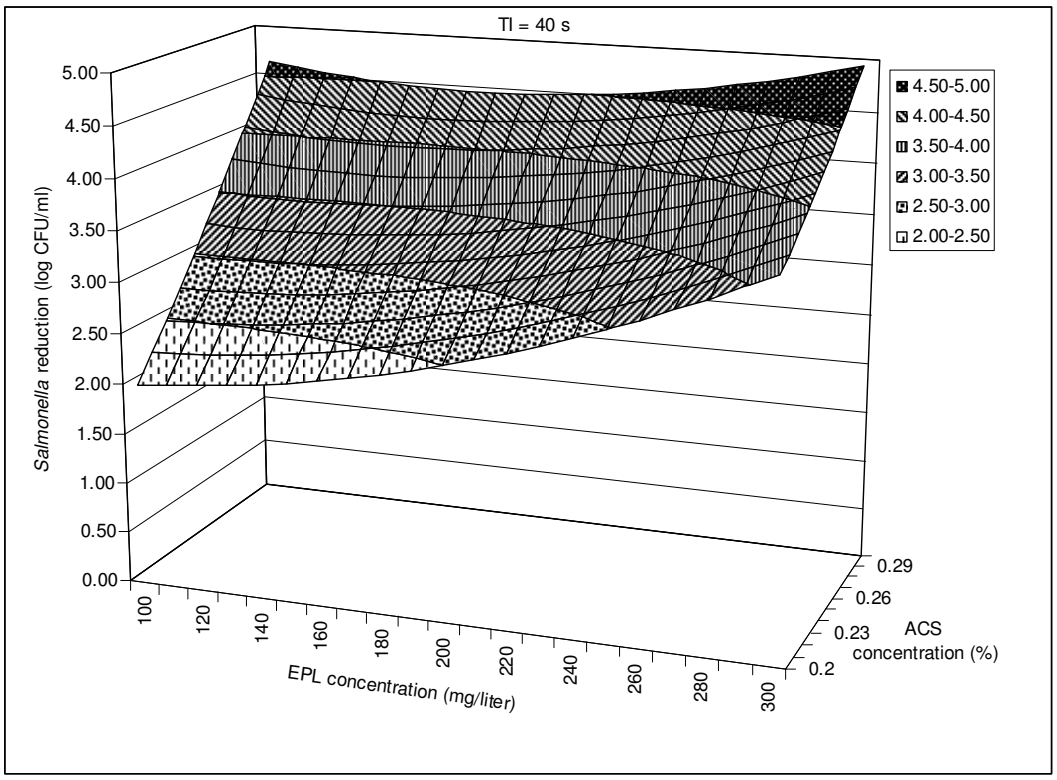

FIGURE 5. *Response surface plot of reduction of* Salmonella *inoculated onto chicken carcasses and treated with EPL and ACS sequentially for 20 s using RSM in Box-Behnken design at a 40 s time interval between applications.*

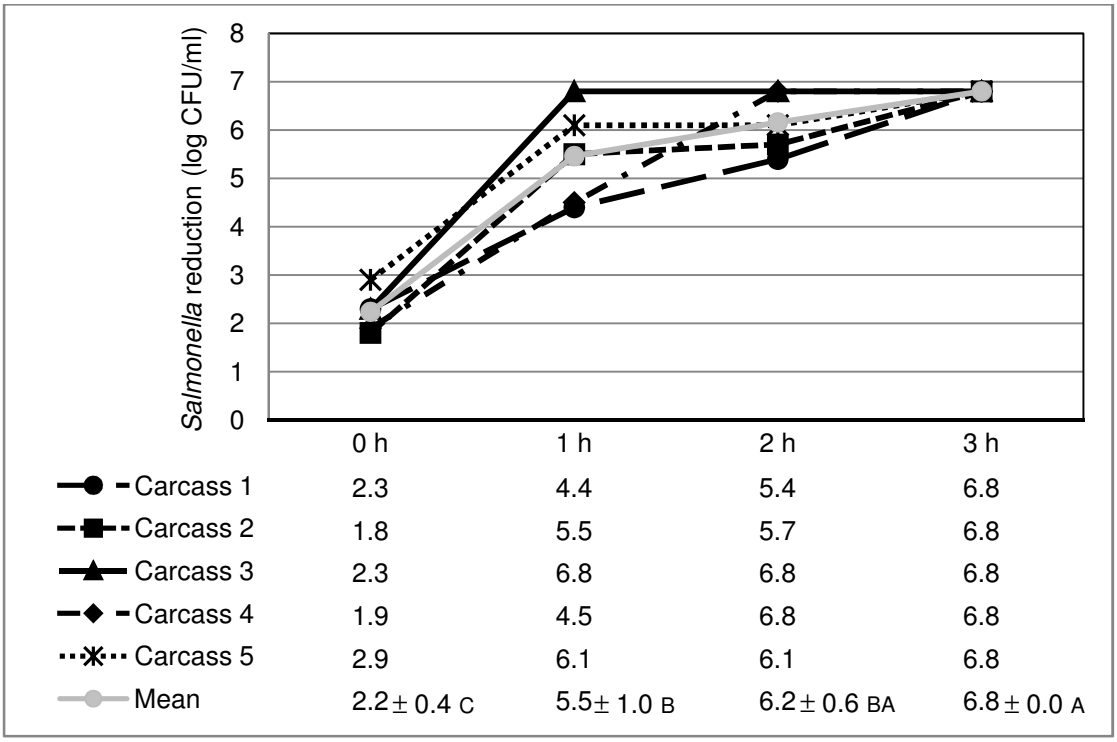

FIGURE 6. E*ffect of immediate, 1 h, 2 h and 3 h plating of the rinse liquid following a whole bird rinse of the chicken carcasses treated with 300 mg/liter EPL followed by 30 % ACS (EPL300-ACS30) with a 40 s time interval between the first and second antimicrobial. Means with different capital letters are significantly different (*P < *0.05).*

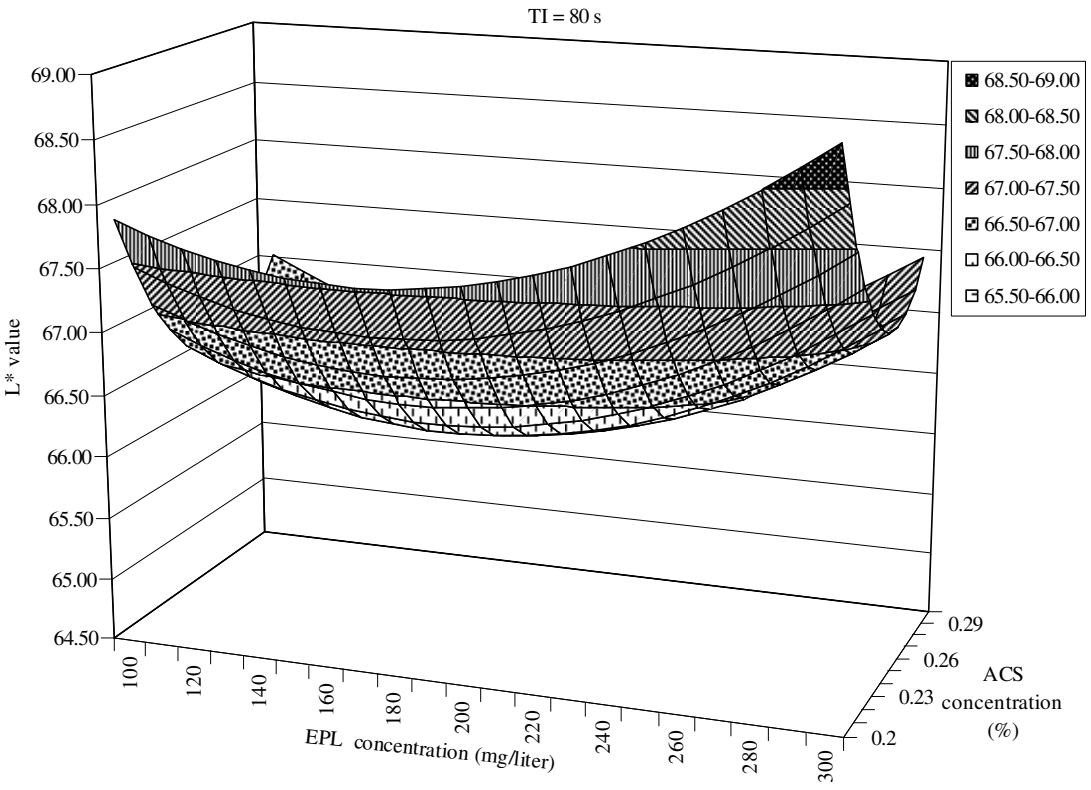

FIGURE 7. *Response surface plot of L* values of chicken carcasses treated with EPL and ACS sequentially for 20 s using RSM in Box-Behnken design for 80 s time interval.*

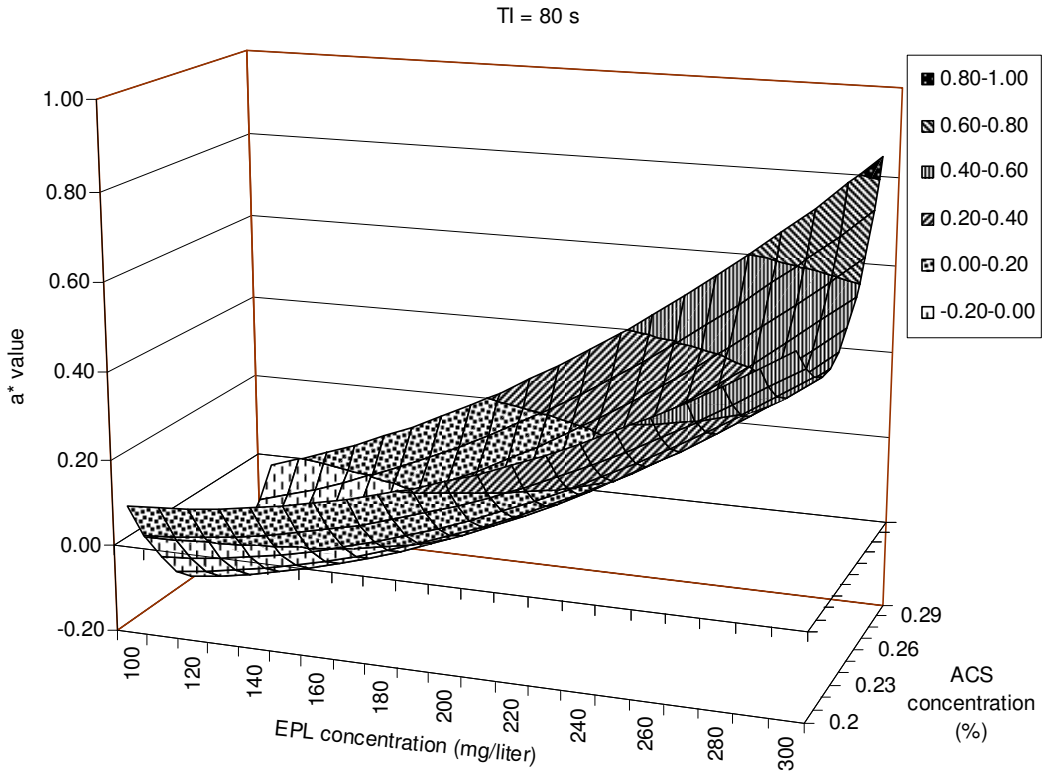

FIGURE 8. *Response surface plot of a* values of chicken carcasses treated with EPL and ACS sequentially for 20 s using RSM in Box-Behnken design for 80 s time interval.*

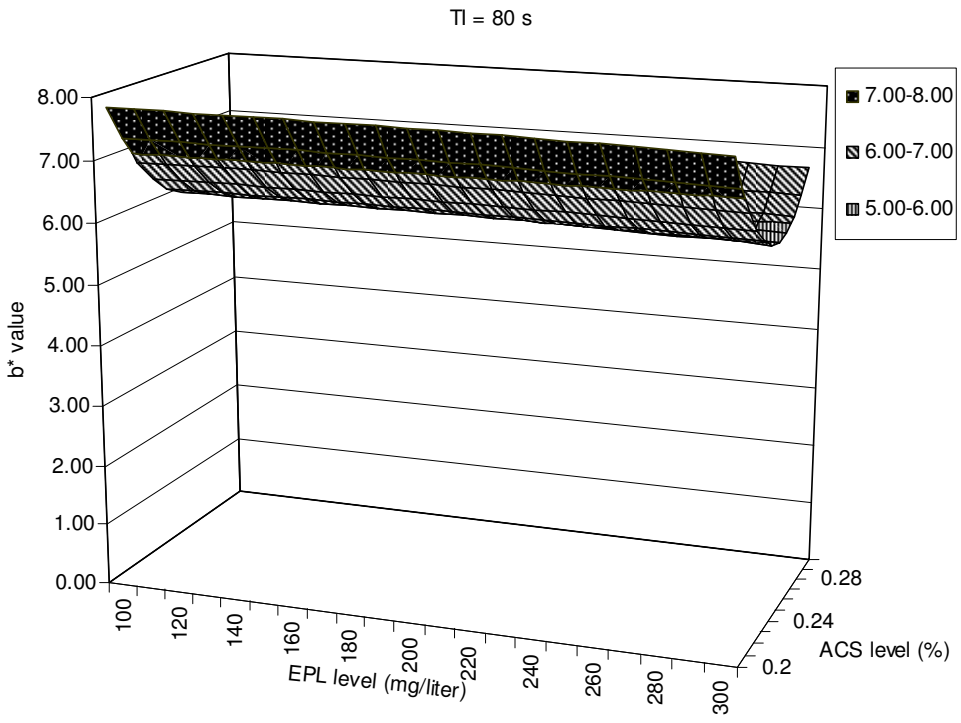

FIGURE 9. *Response surface plot of b* values of chicken carcasses treated with EPL and ACS sequentially for 20 s using RSM in Box-Behnken design for 80 s time interval.*

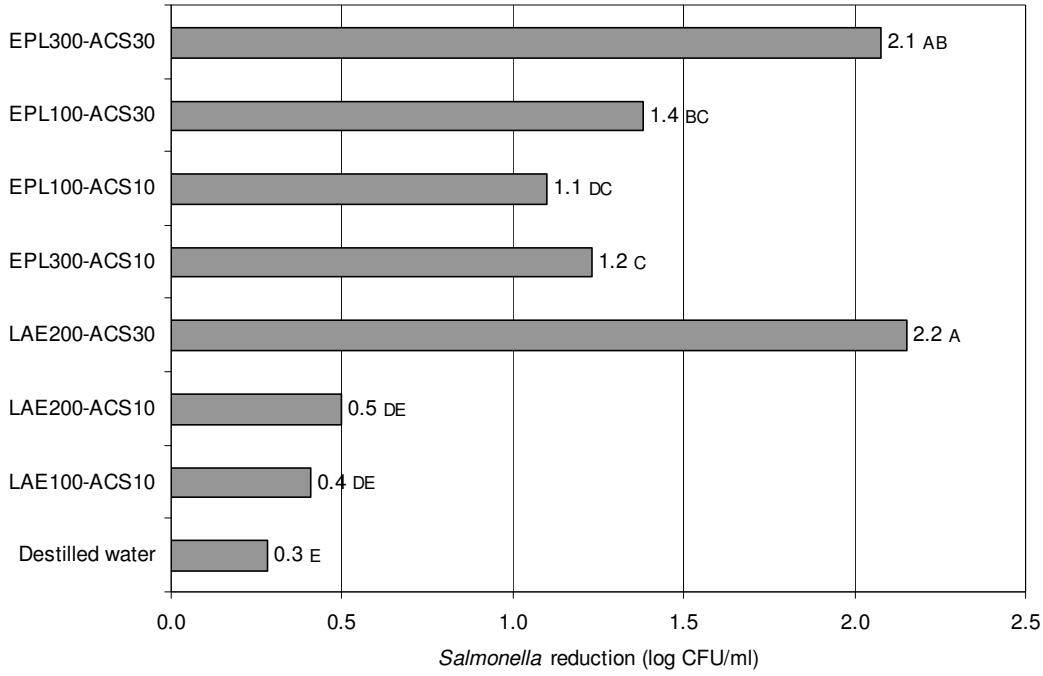

FIGURE 10. *Mean* Salmonella *reductions on inoculated chicken carcasses after sequential spray applications of EPL or LAE followed by ACS. Means with different capital letters are significantly different (*P < *0.05). EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS30, EPL 100 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS10, EPL 100 mg/liter followed by ACS 10% solution with a 40 s time interval; EPL300-ACS10, EPL 300 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS10, LAE 200 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE100-ACS10, LAE 100 mg/liter followed by ACS 10% solution with a 40 s time interval.*

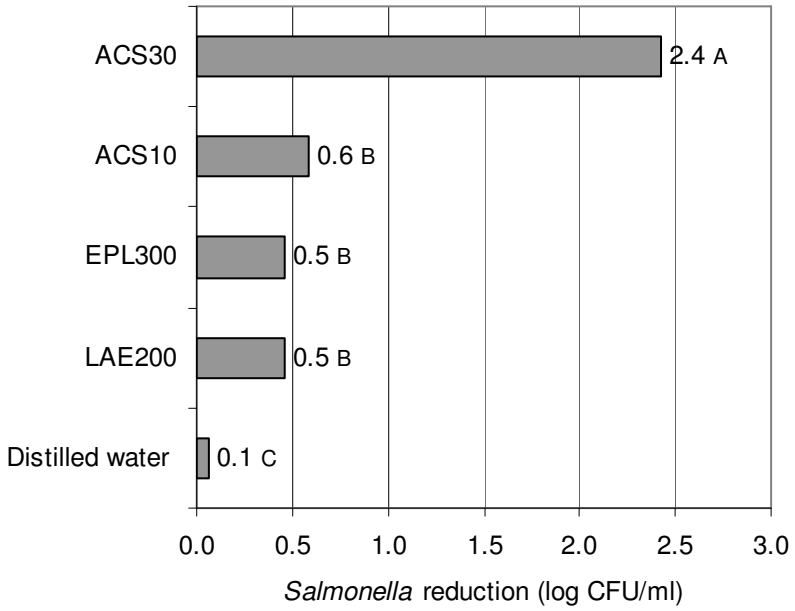

FIGURE 11. *Mean* Salmonella *reductions on inoculated chicken carcasses after individual spray applications of ACS, EPL and LAE. Means with different capital letters are significantly different (*P<*0.05). ACS30, ACS 30%; ACS10, ACS 10%; EPL300, EPL 300 mg/liter; LAE200, LAE 200 mg/liter.*

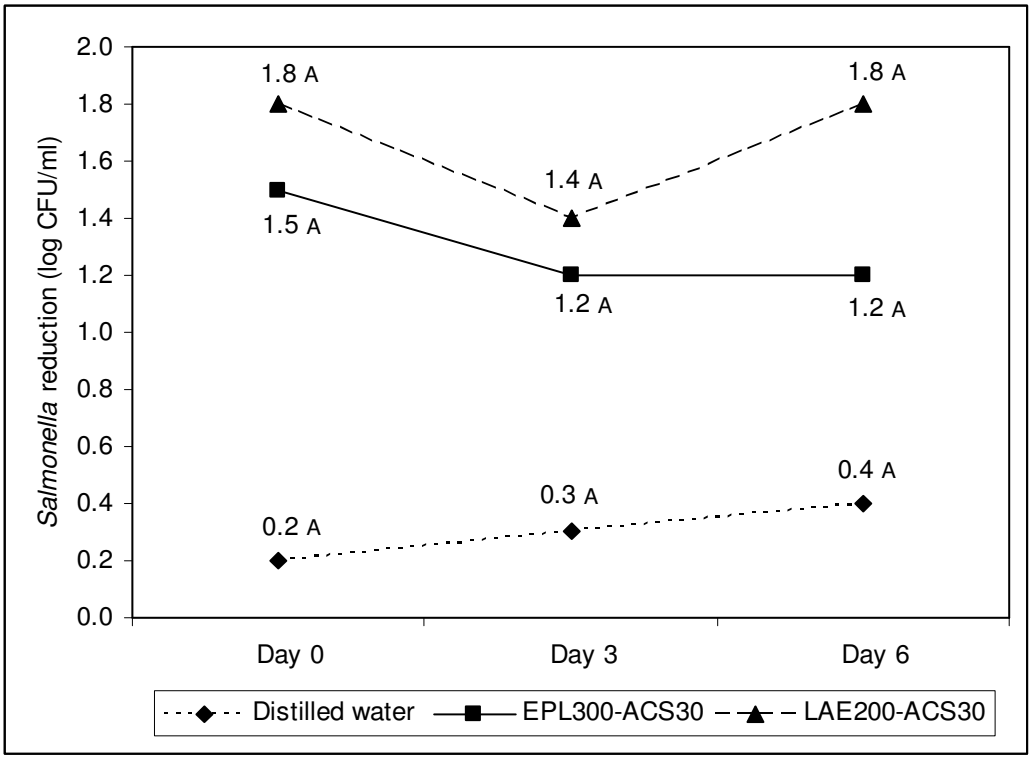

FIGURE 12. *Mean reduction of* Salmonella *on chicken carcasses sprayed with distilled water, EPL300-ACS30 or LAE200-ACS30 and stored at 4.4°C for up to 6 days. Within each intervention, means with different capital letters are significantly different (*$P < 0.05$*). EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; Day 0, Day 3 and Day 6, storage days at 4.4 °C.*

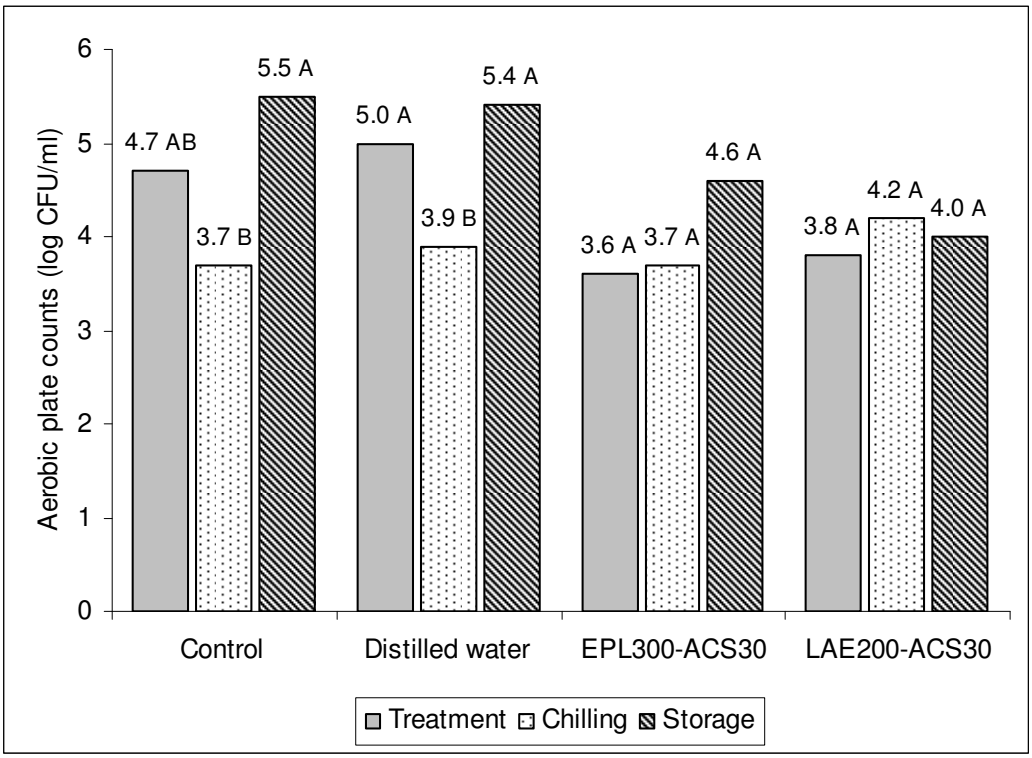

FIGURE 13. *Mean aerobic plate counts on chicken carcasses after spray treatment with distilled water, EPL300-ACS30 or LAE200-ACS30, after chilling and after 10 days of storage at 4.4℃. Within each intervention, m*eans *with different capital letters are significantly different (*$P < 0.05$*). EPL300-ACS30, spray treatment of EPL 300 mg/liter followed by ACS 30 % solution with a 40 s time interval; LAE200-ACS30, spray treatment of LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.*

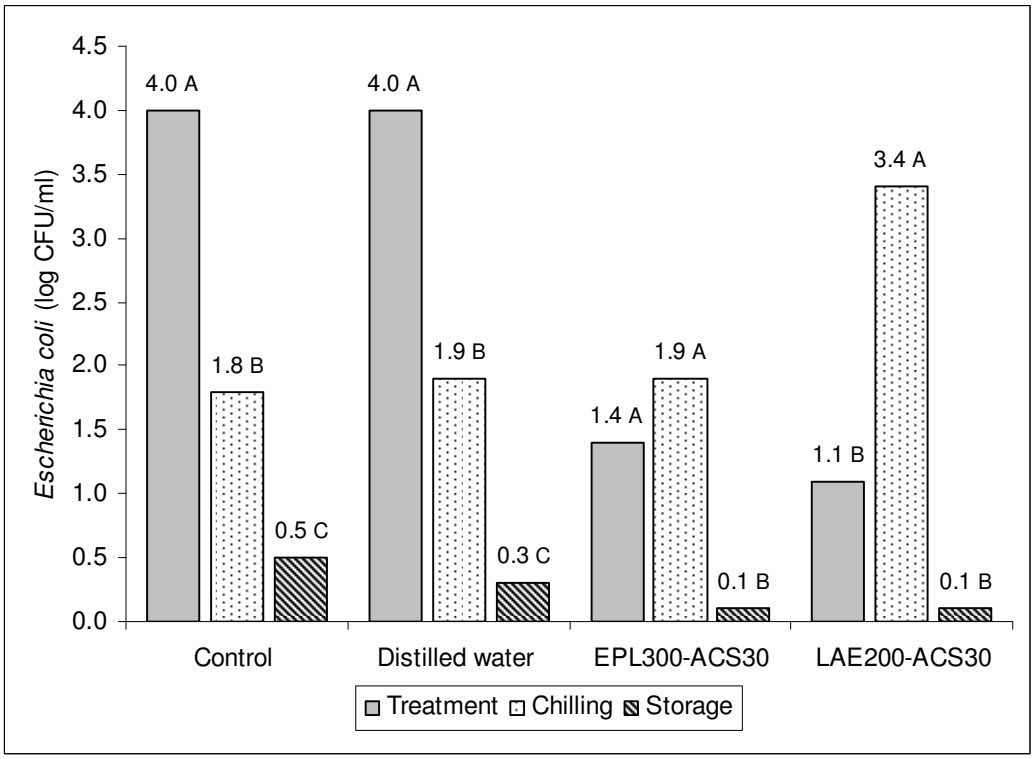

FIGURE 14. *Mean counts of* Escherichia coli *on chicken carcasses after spray treatment with distilled water, EPL300-ACS30 or LAE200-ACS30, after chilling and after 10 days of storage at 4.4°C. Within each intervention, means with different capital letters are significantly different (*$P < 0.05$*). EPL300-ACS30, spray treatment of EPL 300 mg/liter followed by ACS 30 % solution with a 40 s time interval; LAE200-ACS30, spray treatment of LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.*

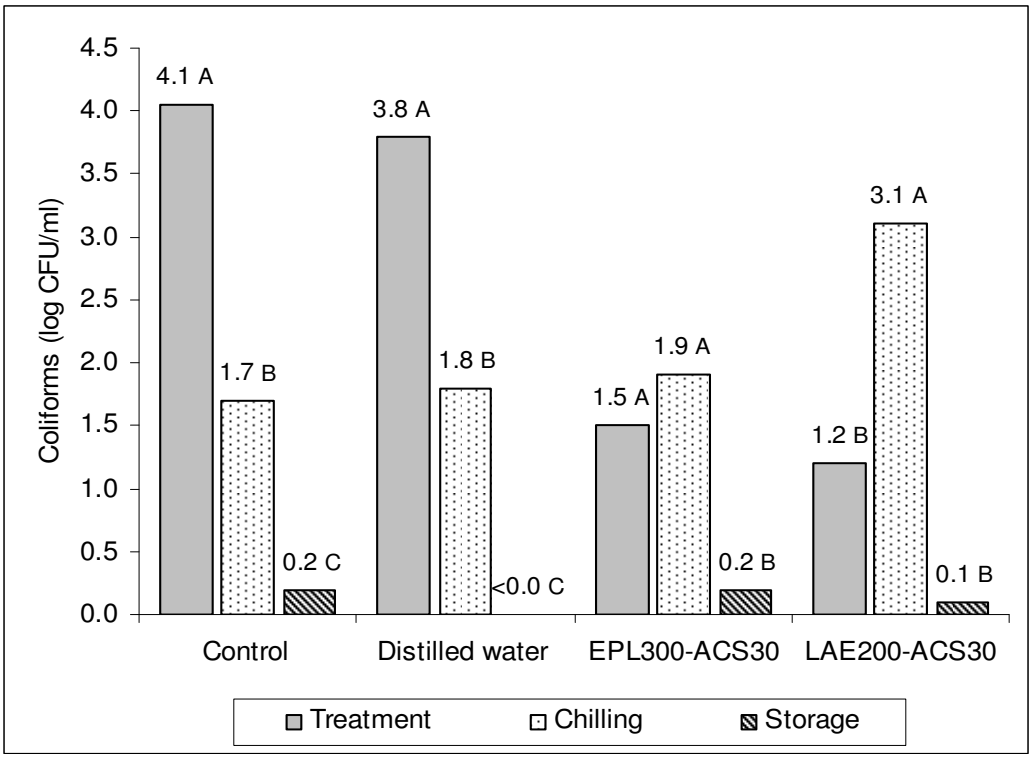

FIGURE 15. *Mean counts of coliforms on chicken carcasses after spray treatment with distilled water, EPL300-ACS30 or LAE200-ACS30, after chilling and after 10 days of storage at 4.4℃. Within each intervention, means with different capital letters are significantly different (*$P < 0.05$*). EPL300-ACS30, spray treatment of EPL 300 mg/liter followed by ACS 30 % solution with a 40 s time interval; LAE200-ACS30, spray treatment of LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.*

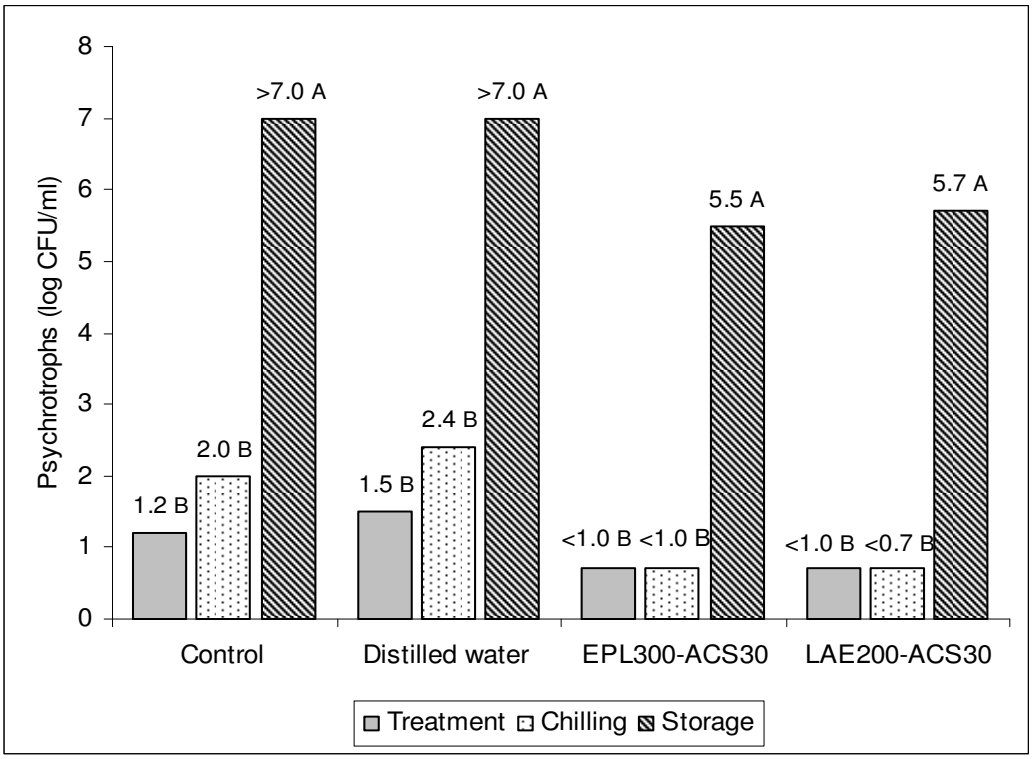

FIGURE 16. *Mean counts of psychrotrophs on chicken carcasses after spray treatment with distilled water, EPL300-ACS30 or LAE200-ACS30, after chilling and after 10 days of storage at 4.4°C. Within each intervention, means with different capital letters are significantly different (*$P < 0.05$*). EPL300-ACS30, spray treatment of EPL 300 mg/liter followed by ACS 30 % solution with a 40 s time interval; LAE200-ACS30, spray treatment of LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.*

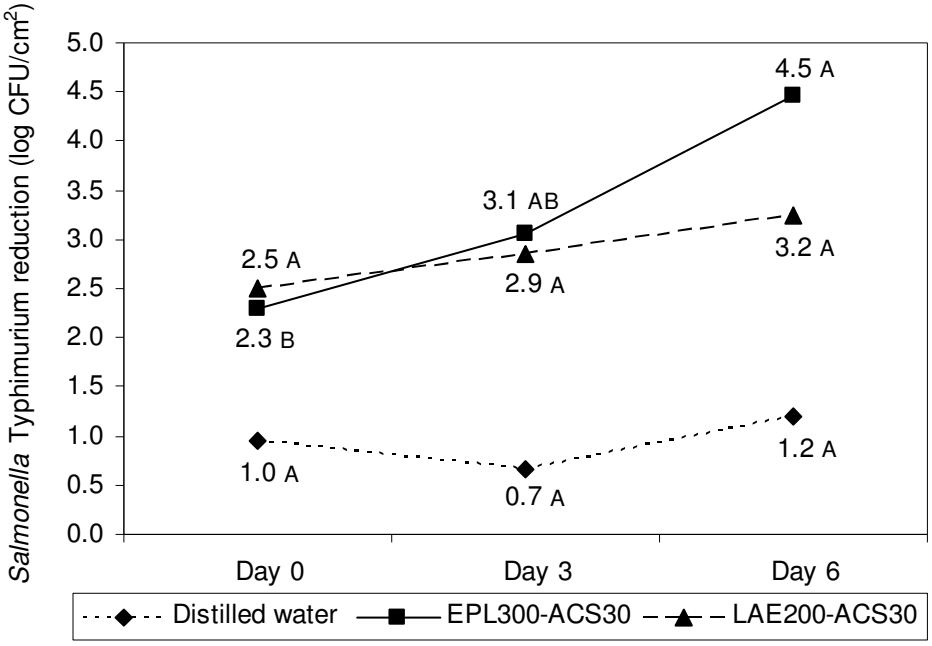

FIGURE 17. Salmonella *Typhimurium reductions on the surface of beef rounds sprayed with distilled water, EPL300-ACS30 or LAE200-ACS30 at 55℃ and stored at 4.4℃ for up to 6 days. Within each intervention, means with different capital letters are significantly different (*P < *0.05). EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; Day 0, Day 3 and Day 6, storage days at 4.4 ℃.*

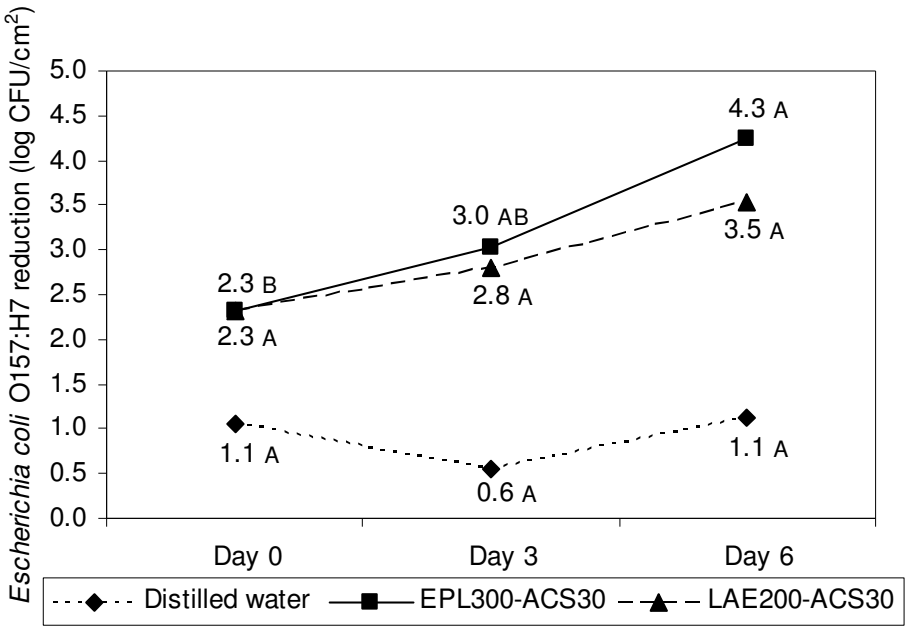

FIGURE 18. Escherichia coli *O157:H7 reductions on the surface of beef rounds sprayed with distilled water, EPL300-ACS30 or LAE200-ACS30 at 55℃ and stored at 4.4℃ for up to 6 days. Within each intervention, means with different capital letters are significantly different (*$P < 0.05$*). EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; Day 0, Day 3 and Day 6, storage days at 4.4 ℃.*

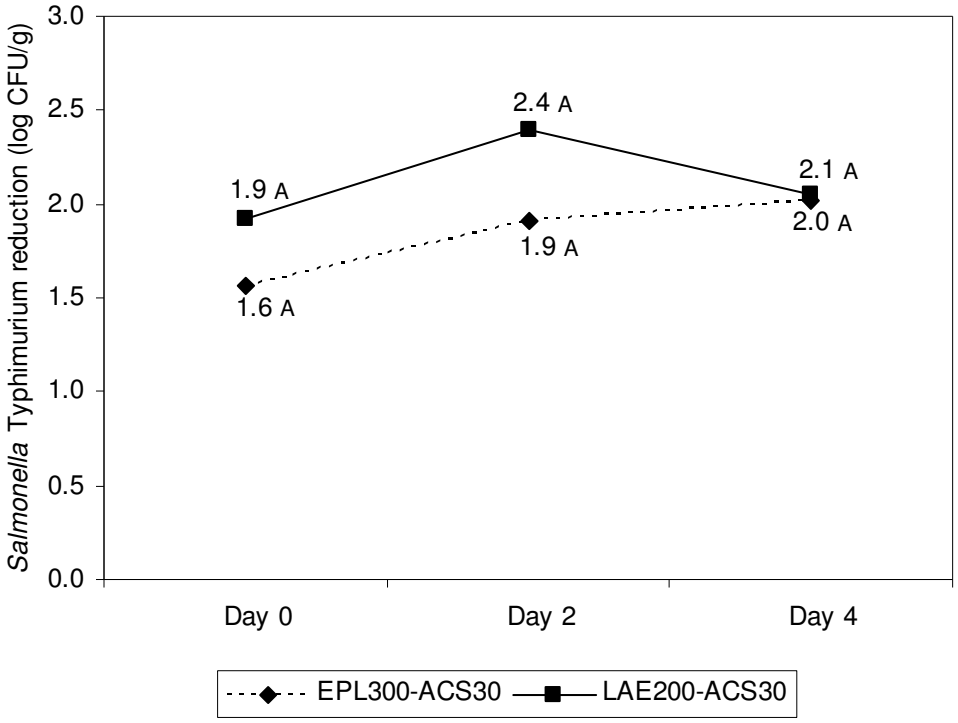

FIGURE 19. Salmonella *Typhimurium reduction of ground beef manufactured from beef rounds treated with EPL300-ACS30 or LAE200-ACS30 at 55℃ and stored at 4.4℃ for 0, 2 and 4 days. Within each intervention, means with different capital letters are significantly different (*P < *0.05). EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; Day 0, Day 2 and Day 4, storage days at 4.4 ℃.*

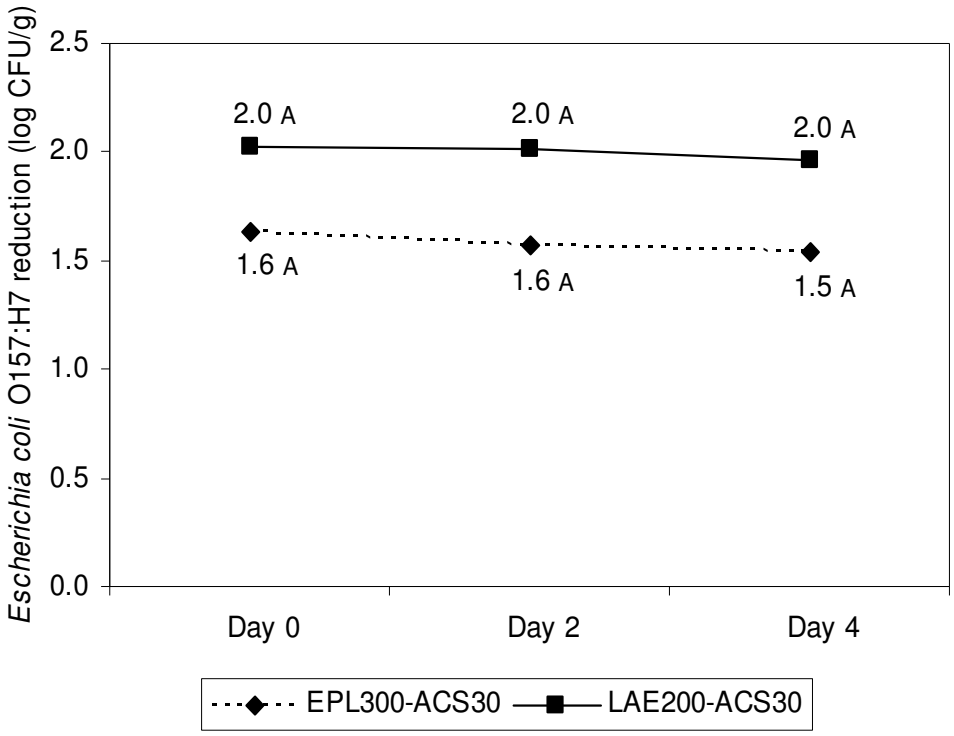

FIGURE 20. Escherichia coli *O157:H7 reduction of ground beef manufactured from beef rounds treated with EPL300-ACS30 or LAE200-ACS30 at 55℃ and stored at 4.4℃ for 0, 2 and 4 days. Within each intervention, means with different capital letters are significantly different (*P < *0.05). EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; Day 0, Day 2 and Day 4, storage days at 4.4 ℃.*

APPENDIX B

TABLES

TABLE 1. *Structural settings for Box-Behnken design for three factors*

Replications	Factor 1	Factor 2	Factor 3
1	-1	-1	0
1	1	-1	0
1	-1	1	0
1	1	1	0
1	-1	0	-1
1	1	0	-1
1	-1	0	1
1	1	0	1
1	0	-1	-1
1	0	1	-1
1	0	-1	1
1	0	1	1
3	0	0	0

TABLE 2. *Mean reductions of* Salmonella *inoculated onto sterile filter paper and treated with ACS, EPL and LAE or paired combinations of these antimicrobials applied for 20 or 60 s*

	Salmonella reduction ± SD (log CFU/ml)[a]	
Treatments[b]	20 s	60 s
ACS20	3.5 ± 0.7 B	3.7 ± 1.0 B
ACS20-EPL300	4.0 ± 0.5 AB	4.1 ± 0.1 AB
ACS20-LAE200	3.5 ± 0.6 B	4.0 ± 0.3 AB
EPL300	3.5 ± 0.5 B	3.5 ± 0.5 B
EPL300-ACS20	5.4 ± 0.6 A	4.6 ± 0.4 AB
EPL300-LAE200	4.3 ± 0.8 AB	5.0 ± 0.8 AB
LAE200	3.7 ± 0.7 AB	3.3 ± 0.2 B
LAE200-ACS20	3.7 ± 0.9 AB	4.6 ± 0.8 AB
LAE200-EPL300	5.2 ± 0.8 AB	5.7 ± 0.1 A

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$).

[b]ACS20, ACS 20 %; ACS20-EPL300, ACS 20 % followed by EPL 300 mg/liter; ACS20-LAE200, ACS 20 % followed by LAE 200 mg/liter; EPL300, EPL 300 mg/liter; EPL300-ACS20, EPL 300 mg/liter followed by ACS 20 %; EPL300-LAE200, EPL 300 mg/liter followed by LAE 200 mg/liter; LAE200, LAE 200 mg/liter; LAE200-ACS20, LAE 200 mg/liter followed by ACS 20 %; LAE200-EPL300, LAE 200 mg/liter followed by EPL 300 mg/liter.

TABLE 3. *Main effect means for reduction of* Salmonella *inoculated on sterile filter paper and treated with ACS, EPL and LAE or paired combinations of these antimicrobials for 20 or 60 s*

Main effects		*Salmonella* reduction (log CFU/ml)[a]
Treatments[b]	ACS20	3.6 C
	ACS20-EPL300	4.0 BC
	ACS20-LAE200	3.8 C
	EPL300	3.5 C
	EPL300-ACS20	5.1 AB
	EPL300-LAE200	4.7 ABC
	LAE200	3.5 C
	LAE200-ACS20	4.2 BC
	LAE200-EPL300	5.5 A
Contact time (s)	20	4.1 A
	60	4.3 A

[a] Means with different capital letters are significantly different ($P < 0.05$) within each main effect.

[b]ACS20, ACS 20 %; ACS20-EPL300, ACS 20 % followed by EPL 300 mg/liter; ACS20-LAE200, ACS 20 % followed by LAE 200 mg/liter; EPL300, EPL 300 mg/liter; EPL300-ACS20, EPL 300 mg/liter followed by ACS 20 %; EPL300-LAE200, EPL 300 mg/liter followed by LAE 200 mg/liter; LAE200, LAE 200 mg/liter; LAE200-ACS20, LAE 200 mg/liter followed by ACS 20 %; LAE200-EPL300, LAE 200 mg/liter followed by EPL 300 mg/liter.

TABLE 4. Mean *reductions of* Salmonella *inoculated onto fresh chicken carcasses and treated with combinations of EPL and LAE sequentially for 20 s using RSM in Box-Behnken design*

EPL (mg/liter)	Time interval (s)	LAE (mg/liter)	*Salmonella* reduction (log CFU/ml)
100	80	100	0.4
100	80	200	0.5
300	80	100	0.9
300	80	200	0.7
200	40	100	0.3
200	120	100	0.0
200	40	200	0.6
200	120	200	0.9
100	40	150	0.8
300	40	150	0.3
100	120	150	0.6
300	120	150	0.7
200	80	150	0.5
200	80	150	0.4
200	80	150	0.5

TABLE 5. *Predicted reductions of* Salmonella *inoculated onto chicken carcasses and treated with EPL and LAE sequentially for 20 s using RSM in Box-Behnken design*

EPL (mg/liter)	Time interval (s) (Between applications)	LAE (mg/liter)	Predicted *Salmonella* reduction ± SD (log CFU/ml)	95% prediction interval
300	120	200	1.0 ± 0.3[a]	0.2 – 1.8
300	120	175	0.9 ± 0.2	0.3 – 1.5
250	120	200	0.9 ± 0.2	0.2 – 1.5
300	100	200	0.8 ± 0.2	0.2 – 1.5
100	120	200	0.8 ± 0.3	0.0 – 1.6

[a] Standard error of predicted *Salmonella* reduction.

TABLE 6. Mean *reductions of* Salmonella *onto fresh chicken carcasses and treated with combinations of LAE and EPL sequentially for 20 s using RSM in Box-Behnken design*

LAE (mg/liter)	Time interval (s)	EPL (mg/liter)	*Salmonella* reduction (log CFU/ml)
100	80	100	0.6
100	80	300	1.3
200	80	100	1.3
200	80	300	0.7
150	40	100	0.5
150	120	100	1.2
150	40	300	2.3
150	120	300	0.6
100	40	200	1.1
200	40	200	2.8
100	120	200	0.7
200	120	200	1.3
150	80	200	1.5
150	80	200	1.8
150	80	200	1.1

TABLE 7. *Mean reductions of* Salmonella *inoculated onto fresh chicken carcasses and treated with combinations of EPL and ACS sequentially for 20 s using RSM in Box-Behnken design*

EPL (mg/liter)	Time interval (s)	ACS (%)	*Salmonella* reduction (log CFU/ml)
100	80	20	2.6
100	80	30	4.2
300	80	20	3.8
300	80	30	4.1
200	40	20	2.1
200	120	20	3.3
200	40	30	4.2
200	120	30	3.6
100	40	25	3.6
300	40	25	4.5
100	120	25	3.1
300	120	25	3.0
200	80	25	2.9
200	80	25	3.2
200	80	25	3.7

TABLE 8. *Predicted reductions of* Salmonella *inoculated onto chicken carcasses and treated with EPL and ACS sequentially for 20 s using RSM in Box-Behnken design*

EPL (mg/liter)	Time interval(s) (Between applications)	ACS (%)	Predicted *Salmonella* reduction ± SD (log CFU/ml)	95% prediction interval
300	40	30	4.9 ± 0.9[a]	3.2 - 6.7
250	40	30	4.6 ± 0.7	3.3 - 6.0
100	40	30	4.6 ± 0.9	2.9 - 6.3
300	40	27.5	4.6 ± 0.8	3.2 - 5.9
300	80	30	4.1 ± 0.6	2.8 - 5.4

[a] Standard error of predicted *Salmonella* reduction.

TABLE 9. *Mean L*a*b* color values of chicken carcasses after inoculation and after treatment with EPL and ACS for 20 s using RSM in Box-Behnken design*

EPL (mg/liter)	Time interval (s)	ACS (%)	Before treatment L* ± SD	After treatment L* ± SD	Before treatment a* ± SD	After treatment a* ± SD	Before treatment b* ± SD	After treatment b* ± SD
Distilled water			71.08 ± 3.21	71.45 ± 2.14	-0.09 ± 0.98	-0.19 ± 0.74	1.10 ± 2.04	-0.22 ± 0.98
100	80	20	72.51 ± 2.25	68.10 ± 1.34	-0.17 ± 1.03	0.01 ± 0.76	2.40 ± 2.34	7.35 ± 1.37
100	80	30	69.71 ± 2.32	68.47 ± 2.07	-1.04 ± 0.96	-0.54 ± 2.02	2.16 ± 2.44	6.61 ± 2.60
300	80	20	70.38 ± 1.86	67.38 ± 2.69	0.40 ± 1.69	1.08 ± 3.13	1.42 ± 3.74	7.92 ± 4.39
300	80	30	70.11 ± 2.83	67.22 ± 2.52	-0.03 ± 1.10	0.92 ± 1.78	2.91 ± 1.76	7.16 ± 4.33
200	40	20	71.65 ± 4.36	69.94 ± 4.35	-0.34 ± 0.84	0.09 ± 0.98	1.40 ± 3.55	9.21 ± 3.75
200	120	20	71.43 ± 4.65	68.94 ± 2.78	0.26 ± 0.98	0.26 ± 0.96	2.17 ± 2.66	8.24 ± 2.27
200	40	30	70.35 ± 4.00	67.93 ± 3.70	0.17 ± 1.50	0.47 ± 1.68	1.92 ± 3.70	7.59 ± 4.28
200	120	30	70.26 ± 2.10	66.12 ± 4.07	0.30 ± 0.62	0.85 ± 1.29	0.59 ± 1.08	6.99 ± 0.92
100	40	25	71.57 ± 2.66	66.37 ± 2.23	0.01 ± 1.07	0.36 ± 1.24	-0.65 ± 2.44	5.58 ± 0.96
300	40	25	71.72 ± 3.55	70.42 ± 1.88	0.69 ± 1.65	0.63 ± 0.56	2.41 ± 3.46	6.72 ± 3.73
100	120	25	73.44 ± 2.29	67.35 ± 2.16	0.33 ± 1.04	0.20 ± 1.06	3.26 ± 2.56	8.58 ± 0.82
300	120	25	72.22 ± 3.27	68.08 ± 2.82	-0.22 ± 0.79	0.01 ± 0.71	0.14 ± 2.60	5.84 ± 1.43
200	80	25	69.25 ± 2.24	64.88 ± 2.44	-0.01 ± 1.06	0.04 ± 0.79	-1.46 ± 2.75	5.55 ± 2.67
200	80	25	72.25 ± 1.36	68.21 ± 1.40	-0.08 ± 1.05	-0.21 ± 1.25	0.09 ± 1.48	5.73 ± 2.84
200	80	25	70.90 ± 2.65	64.20 ± 2.58	0.21 ± 1.03	0.49 ± 1.77	2.25 ± 3.25	7.52 ± 3.29
Mean color values[a]			71.20 ± 2.97 A	68.24 ± 3.13 B	0.02 ± 1.09 A	0.23 ± 1.34 A	1.31 ± 2.76 B	6.02 ± 3.74 A
Mean square	Model		445.54		2.39		1128.71	
	Error		9.33		1.49		10.82	
Root mean square error			3.05		1.22		3.29	

[a] Within mean color values for L*a* and b* before and after treatments, means with different capital letters are significantly different ($P < 0.05$)

TABLE 10. *Response surface models for prediction of L*a*b* values of inoculated and treated chicken carcasses as a function of EPL spray concentration (EPL), time interval (TI), ACS spray concentration (ACS)*

Color values	Response surface models
L*	L* = 94.30362 – 0.004045*EPL - 188.15*ACS - 0.067175*TI + 0.000077*EPL*EPL - 0.02675*EPL*ACS - 0.000207*EPL*TI + 379.95*ACS*ACS - 0.100625*ACS*TI + 0.000755*TI*TI
a*	a* = 7.007125 – 0.003354*EPL – 50.5875*ACS - 0.017494*TI + 0.000011*EPL*EPL + 0.019*EPL*ACS - 0.00003*EPL*TI + 90.6*ACS*ACS + 0.026875*ACS*TI + 0.0001*TI*TI
b*	b* = 40.51737 + 0.020655*EPL - 271.5875*ACS - 0.031722*TI - 0.00000395*EPL*EPL - 0.00125*EPL*ACS - 0.000242*EPL*TI + 514.7*ACS*ACS + 0.045*ACS*TI + 0.000442*TI*TI

TABLE 11. *Replications (chicken carcasses) of EPL or LAE and ACS sequential spray applications for reducing* Salmonella *on inoculated chicken carcasses*

Treatments[b]	Time interval (s)	Replications
Control	-	9
Distilled water	40	8
EPL300-ACS30	40	11
EPL100-ACS30	40	9
EPL100-ACS10	40	9
EPL300-ACS10	40	3
LAE200-ACS30	40	9
LAE200-ACS10	40	3
LAE100-ACS10	40	3

[b]EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS30, EPL 100 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS10, EPL 100 mg/liter followed by ACS 10% solution with a 40 s time interval; EPL300-ACS10, EPL 300 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS10, LAE 200 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE100-ACS10, LAE 100 mg/liter followed by ACS 10% solution with a 40 s time interval.

TABLE 12. *Mean* Salmonella *counts after sequential spray application of various concentrations of EPL or LAE and ACS for 20 s on inoculated chicken carcasses*

Treatments[b]	*Salmonella* counts ± SD (log CFU/ml)[a]
Control	6.9 ± 0.2 A
Distilled water	6.6 ± 0.2 A
EPL300-ACS30	4.8 ± 0.5 D
EPL100-ACS30	5.5 ± 0.4 C
EPL100-ACS10	5.8 ± 0.3 BC
EPL300-ACS10	5.7 ± 0.3 C
LAE200-ACS30	4.7 ± 0.5 D
LAE200-ACS10	6.4 ± 0.1 AB
LAE100-ACS10	6.5 ± 0.1 A

[a]Means with different letters are significantly different ($P < 0.05$) within the same column.

[b]EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS30, EPL 100 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS10, EPL 100 mg/liter followed by ACS 10% solution with a 40 s time interval; EPL300-ACS10, EPL 300 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS10, LAE 200 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE100-ACS10, LAE 100 mg/liter followed by ACS 10% solution with a 40 s time interval.

TABLE 13. *Mean L*a*b* color values of chicken carcasses for before inoculation, after inoculation and after treatment sequentially sprayed with various concentration of EPL or LAE and ACS for 20 s*

Treatments[b]		Color space values[a] L* ± SD	a* ± SD	b* ± SD
Distilled water	Before inoculation	70.04 ± 3.39 A	0.07 ± 0.91 A	2.02 ± 2.31 A
	After inoculation	69.74 ± 3.46 A	0.66 ± 2.42 A	1.41 ± 3.28 A
	After treatment	70.52 ± 3.16 A	0.69 ± 1.87 A	0.66 ± 4.76 A
EPL300-ACS30	Before inoculation	68.46 ± 3.55 A	0.77 ± 0.75 A	2.42 ± 2.84 B
	After inoculation	69.58 ± 3.14 A	1.03 ± 0.70 A	2.27 ± 2.23 B
	After treatment	66.67 ± 3.24 A	0.91 ± 1.05 A	6.68 ± 2.52 A
EPL100-ACS30	Before inoculation	71.83 ± 2.28 A	0.31 ± 1.31 A	5.52 ± 3.99 B
	After inoculation	71.95 ± 2.27 A	0.62 ± 1.18 A	3.73 ± 3.51 B
	After treatment	69.01 ± 2.57 B	-0.11 ± 0.90 A	7.95 ± 2.28 A
EPL100-ACS10	Before inoculation	68.48 ± 3.46 A	0.60 ± 1.16 A	2.47 ± 2.04 B
	After inoculation	68.61 ± 3.48 A	0.64 ± 1.02 A	2.01 ± 1.89 B
	After treatment	65.23 ± 3.11 B	0.72 ± 1.45 A	6.75 ± 2.48 A
EPL300-ACS10	Before inoculation	72.51 ± 3.17 A	0.46 ± 1.62 A	2.43 ± 1.89 B
	After inoculation	71.11 ± 2.71 AB	0.11 ± 0.78 A	1.89 ± 1.96 B
	After treatment	68.18 ± 3.48 B	0.86 ± 1.27 A	8.03 ± 2.22 A
LAE200-ACS30	Before inoculation	70.72 ± 2.66 A	0.28 ± 0.91 A	3.21 ± 3.11 B
	After inoculation	69.35 ± 2.70 A	-0.08 ± 0.91 A	2.65 ± 2.73 B
	After treatment	65.42 ± 2.60 B	0.13 ± 1.36 A	6.19 ± 2.72 A
LAE200-ACS10	Before inoculation	71.61 ± 1.35 A	-0.07 ± 0.91 A	4.72 ± 0.89 B
	After inoculation	70.54 ± 1.51 AB	-0.41 ± 0.74 A	3.87 ± 1.59 B
	After treatment	68.56 ± 1.85 B	-0.42 ± 1.14 A	7.62 ± 0.47 A
LAE100-ACS10	Before inoculation	72.04 ± 2.95 A	0.42 ± 0.99 A	4.31 ± 1.67 B
	After inoculation	70.55 ± 1.56 A	-0.02 ± 0.50 A	2.14 ± 1.40 B
	After treatment	68.42 ± 4.40 A	0.07 ± 0.65 A	8.23 ± 3.70 A

[a]Means with different letters are significantly different ($P<0.05$) within the same column for each treatment.

[b]EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS30, EPL 100 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS10, EPL 100 mg/liter followed by ACS 10% solution with a 40 s time interval; EPL300-ACS10, EPL 300 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS10, LAE 200 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE100-ACS10, LAE 100 mg/liter followed by ACS 10% solution with a 40 s time interval.

TABLE 14. *Mean* Salmonella *counts on inoculated chicken carcasses after individual spray applications of ACS, EPL or LAE for 20 s*

Treatments[b]	*Salmonella* counts ± SD (log CFU/ml)[a]
Control	6.9 ± 0.1 A
Distilled water	6.9 ± 0.1 A
ACS10	6.4 ± 0.1 B
ACS30	4.5 ± 0.3 C
EPL300	6.5 ± 0.1 B
LAE200	6.5 ± 0.1 B

[a]Means with different letters are significantly different ($P < 0.05$) within the same column.

[b]ACS30, ACS 30%; ACS10, ACS 10%; EPL300, EPL 300 mg/liter; LAE200, LAE 200 mg/liter.

TABLE 15. *Comparison of mean* Salmonella *counts with individual and sequential spray applications of EPL, LAE and ACS on inoculated chicken carcasses*

Sequential treatments[b]	*Salmonella* counts ± SD (log CFU/ml)[a]
Control	6.9 ± 0.2 A
Distilled water	6.6 ± 0.2 A
EPL300-ACS30	4.8 ± 0.5 D
EPL100-ACS30	5.5 ± 0.4 C
EPL100-ACS10	5.8 ± 0.3 BC
EPL300-ACS10	5.7 ± 0.3 C
LAE200-ACS30	4.7 ± 0.5 D
LAE200-ACS10	6.4 ± 0.1 AB
LAE100-ACS10	6.5 ± 0.1 A
Individual treatments[c]	
Control	6.9 ± 0.1 A
Distilled water	6.9 ± 0.1 A
ACS10	6.4 ± 0.1 AB
ACS30	4.5 ± 0.3 D
EPL300	6.5 ± 0.1 A
LAE200	6.5 ± 0.1 A

[a]Means with different letters are significantly different ($P < 0.05$) within the same column.

[b]EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS30, EPL 100 mg/liter followed by ACS 30% solution with a 40 s time interval; EPL100-ACS10, EPL 100 mg/liter followed by ACS 10% solution with a 40 s time interval; EPL300-ACS10, EPL 300 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS10, LAE 200 mg/liter followed by ACS 10% solution with a 40 s time interval; LAE100-ACS10, LAE 100 mg/liter followed by ACS 10% solution with a 40 s time interval.

[c] ACS30, ACS 30%; ACS10, ACS 10%; EPL300, EPL 300 mg/liter; LAE200, LAE 200 mg/liter.

TABLE 16. *Mean L*a*b* color values of chicken carcasses for before inoculation, after inoculation and after treatment individually sprayed with various concentration of ACS, EPL and LAE for 20 s*

Treatments[b]		Color space values[a] L* ± SD	a* ± SD	b* ± SD
Distilled water	Before inoculation	70.95 ± 2.51 A	0.61 ± 0.85 A	2.99 ± 2.18 A
	After inoculation	70.39 ± 2.24 A	0.68 ± 0.65 A	1.43 ± 1.63 A
	After treatment	70.04 ± 2.89 A	0.59 ± 0.86 A	1.66 ± 2.52 A
ACS10	Before inoculation	69.83 ± 2.63 A	1.14 ± 1.38 A	4.24 ± 2.45 B
	After inoculation	70.16 ± 2.14 A	0.99 ± 1.55 A	2.72 ± 2.06 B
	After treatment	68.51 ± 2.65 A	1.16 ± 1.29 A	6.31 ± 2.25 A
EPL300	Before inoculation	70.62 ± 2.29 A	1.26 ± 1.40 A	3.51 ± 2.57 A
	After inoculation	70.64 ± 1.72 A	0.96 ± 0.78 A	2.02 ± 1.72 A
	After treatment	70.82 ± 2.19 A	1.28 ± 1.25 A	2.00 ± 1.82 A
LAE200	Before inoculation	70.39 ± 2.45 A	0.99 ± 1.38 A	3.09 ± 1.69 A
	After inoculation	71.16 ± 1.89 A	0.89 ± 1.01 A	2.04 ± 1.26 A
	After treatment	71.71 ± 1.54 A	0.79 ± 0.94 A	1.97 ± 1.70 A
ACS30	Before inoculation	72.10 ± 1.61 A	2.10 ± 1.54 A	3.22 ± 1.28 B
	After inoculation	71.97 ± 2.01 A	1.91 ± 1.30 A	1.88 ± 1.35 C
	After treatment	69.57 ± 2.23 B	2.16 ± 1.62 A	6.89 ± 2.14 A

[a]Means with different letters are significantly different ($P < 0.05$) within the same column for each treatment.

[b] ACS30, ACS 30%; ACS10, ACS 10%; EPL300, EPL 300 mg/liter; LAE200, LAE 200 mg/liter.

TABLE 17. *Mean counts of* Salmonella o*n chicken carcasses sprayed with distilled water, EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution and stored at 4.4℃ for up to 6 days*

Treatments[b]	*Salmonella* counts ± SD (log CFU/ml)[a]		
	Day 0	Day 3	Day 6
Control	6.2 ± 0.0 A a	6.4 ± 0.1 A a	6.3 ± 0.1 A a
Distilled water	6.0 ± 0.1 A a	6.1 ± 0.2 AB a	5.9 ± 0.0 AB a
EPL300-ACS30	4.7 ± 0.3 B a	5.2 ± 0.6 AB a	5.1 ± 0.3 BC a
LAE200-ACS30	4.4 ± 0.3 B a	5.0 ± 0.4 B a	4.5 ± 0.5 C a

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 18. *Mean L*a*b* color values of chicken carcasses for before inoculation, after inoculation and after treatment sequentially sprayed with distilled water, EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution*

Treatments[b]	Color measurement time	Color space values[a] L* ± SD	a* ± SD	b* ± SD
Distilled water	Before inoculation	69.51 ± 3.08 A	0.77 ± 1.08 A	3.82 ± 2.70 A
	After inoculation	68.54 ± 3.44 A	0.83 ± 0.81 A	3.96 ± 2.59 A
	After treatment	68.64 ± 2.97 A	0.62 ± 0.80 A	4.47 ± 2.29 A
EPL300-ACS30	Before inoculation	68.72 ± 2.94 A	0.96 ± 1.06 A	3.34 ± 2.46 B
	After inoculation	68.89 ± 2.22 A	0.75 ± 0.67 A	2.69 ± 1.36 B
	After treatment	66.57 ± 2.55 B	0.89 ± 1.71 A	5.83 ± 2.10 A
LAE200-ACS30	Before inoculation	70.35 ± 2.21 A	0.82 ± 1.36 A	3.43 ± 3.08 B
	After inoculation	68.68 ± 2.60 B	0.61 ± 0.88 A	3.62 ± 2.24 B
	After treatment	64.93 ± 2.27 C	1.06 ± 1.78 A	7.24 ± 2.55 A

[a]Means with different letters are significantly different ($P < 0.05$) within the same column for each treatment.

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 19. *Mean aerobic plate counts on chicken carcasses after spray treatment with distilled water, EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution, after chilling and after 10 days of storage at 4.4°C*

Treatments[b]	Aerobic plate counts ± SD (log CFU/ml)[a]		
	After treatment	After chilling	After storage
Control	4.7 ± 0.8 A a	3.7 ± 0.3 A b	5.5 ± 0.7 A a
Distilled water	5.0 ± 0.2 A a	3.9 ± 0.3 A b	5.4 ± 0.6 A a
EPL300-ACS30	3.6 ± 0.6 A a	3.7 ± 0.4 A a	4.6 ± 0.9 A a
LAE200-ACS30	3.8 ± 0.2 A a	4.2 ± 0.9 A a	4.0 ± 0.4 A a

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 20. *Mean counts of* Escherichia coli *on chicken carcasses after spray treatment with distilled water, EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution, after chilling and after 10 days of storage at 4.4°C*

Treatments[b]	*Escherichia coli* ± SD (log CFU/ml)[a]		
	After treatment	After chilling	After storage
Control	4.0 ± 0.5 A a	1.80 ± 0.1 A b	0.5 ± 0.4 A c
Distilled water	4.0 ± 0.4 A a	1.9 ± 0.7 A b	0.3 ± 0.6 A c
EPL300-ACS30	1.4 ± 0.2 B a	1.9 ± 0.4 A a	0.1 ± 0.2 A b
LAE200-ACS30	1.1 ± 0.2 B b	3.4 ± 1.1 A a	0.1 ± 0.2 A b

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 21. *Mean counts of coliforms on chicken carcasses after spray treatment with distilled water, EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution, after chilling and after 10 days of storage at 4.4°C*

Treatments[b]	Coliforms ± SD (log CFU/ml)[a]		
	After treatment	After chilling	After storage
Control	4.1 ± 0.4 A a	1.7 ± 0.2 A b	0.2 ± 0.3 A c
Distilled water	3.8 ± 0.3 A a	1.8 ± 0.5 A b	< 0.0 A c
EPL300-ACS30	1.5 ± 0.1 B a	1.9 ± 0.2 A a	0.2 ± 0.3 A b
LAE200-ACS30	1.2 ± 0.2 B b	3.1 ± 1.1 A a	0.1 ± 0.1 A b

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 22. *Mean counts of psychrotrophs on chicken carcasses after spray treatment with distilled water, EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution, after chilling and after 10 days of storage at 4.4°C*

Treatments[b]	*Psychrotrophs* ± SD (log CFU/ml)[a]		
	After treatment	After chilling	After storage
Control	1.2 ± 0.7 A b	2.0 ± 0.1 A b	> 7.0 A a
Distilled water	1.5 ± 0.7 A b	2.4 ± 0.3 A b	> 7.0 A a
EPL300-ACS30	< 1.0 A b	< 1.0 B b	5.5 ± 0.6 B a
LAE200-ACS30	< 1.0 A b	< 1.0 B b	5.7 ± 0.2 B a

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 23. Salmonella *Typhimurium counts on the surface of beef rounds sprayed with distilled water, EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution at 55℃ and stored at 4.4℃ for up to 6 days*

Treatments[b]	*S.* Typhimurium counts ± SD (log CFU/cm^2)[a]		
	Day 0	Day 3	Day 6
Control	6.4 ± 0.1 A a	5.9 ± 1.0 A a	6.1 ± 0.4 A a
Distilled water	5.4 ± 0.3 AB a	5.2 ± 0.3 A a	4.9 ± 0.9 A a
EPL300-ACS30	4.1 ± 1.4 B a	2.8 ± 1.4 B ab	1.6 ± 1.1 B b
LAE200-ACS30	3.9 ± 1.5 B a	3.0 ± 1.3 B a	2.8 ± 1.5 B a

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 24. Escherichia coli *O157:H7 counts on the surface of beef rounds sprayed with distilled water, EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution at 55℃ and stored at 4.4℃ for up to 6 days*

Treatments[b]	*E. coli* O157:H7 counts ± SD (log CFU/cm^2)[a]		
	Day 0	Day 3	Day 6
Control	6.5 ± 0.2 A a	6.0 ± 0.9 A a	6.2 ± 0.5 A a
Distilled water	5.5 ± 0.3 AB a	5.4 ± 0.4 A a	5.1 ± 1.1 A a
EPL300-ACS30	4.2 ± 1.5 B a	2.9 ± 1.2 B ab	1.9 ± 0.8 B b
LAE200-ACS30	4.2 ± 1.3 B a	3.2 ± 0.9 B a	2.6 ± 1.6 B a

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 25. *L*a*b* color values of beef rounds before and after treatment with distilled water, EPL 300 mg/liter followed by ACS 30 % solution or LAE 200 mg/liter followed by ACS 30 % solution at 55℃ for 20 s*

Treatments[b]		Color space values[a] L* ± SD	a* ± SD	b* ± SD
Control	Before treatment	43.30 ± 8.21	12.01 ± 3.97	3.64 ± 1.72
Distilled water	Before treatment	45.40 ± 6.62 A	10.23 ± 4.43 A	2.29 ± 3.34 A
	After treatment	44.07 ± 3.22 A	11.44 ± 2.27 A	4.18 ± 1.74 A
EPL300-ACS30	Before treatment	61.26 ± 7.55 A	2.63 ± 2.08 A	-0.97 ± 3.16 A
	After treatment	48.80 ± 8.88 B	3.98 ± 5.75 A	-0.11 ± 2.95 A
LAE200-ACS30	Before treatment	40.28 ± 2.39 A	13.21 ± 1.46 A	3.92 ± 0.80 A
	After treatment	39.63 ± 1.01 A	9.14 ± 0.69 B	2.87 ± 0.85 B

[a] Means in the same column with different capital letters within before and after measurements of L* a* b* values are significantly different ($P < 0.05$) for each treatment.

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 26. Salmonella *Typhimurium counts of ground beef manufactured from beef rounds treated with EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution at 55℃ and stored at 4.4℃ for 0, 2 and 4 days*

Treatments[b]	*S.* Typhimurium counts ± SD (log CFU/g)[a]		
	Day 0	Day 2	Day 4
Control	5.7 ± 0.2 A a	5.9 ± 0.3 A a	5.7 ± 0.1 A a
EPL300-ACS30	4.1 ± 0.4 B a	4.0 ± 0.4 B a	3.7 ± 0.4 B a
LAE200-ACS30	3.8 ± 0.4 B a	3.5 ± 0.8 B a	3.7 ± 1.1 B a

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

TABLE 27. Escherichia coli *O157:H7 counts of ground beef manufactured from beef rounds treated with EPL 300 mg/liter followed by ACS 30% solution or LAE 200 mg/liter followed by ACS 30 % solution at 55℃ and stored at 4.4℃ for 0, 2 and 4 days*

Treatments[b]	*E. coli* O157:H7 counts ± SD (log CFU/g)[a]		
	Day 0	Day 2	Day 4
Control	6.7 ± 0.3 A a	6.5 ± 0.4 A a	6.5 ± 0.3 A a
EPL300-ACS30	5.1 ± 0.4 B a	4.9 ± 0.4 B a	5.0 ± 0.7 B a
LAE200-ACS30	4.7 ± 0.7 B a	4.5 ± 0.9 B a	4.6 ± 0.9 B a

[a] Means in the same column with different capital letters are significantly different ($P < 0.05$). Means in the same row with different lowercase letters are significantly different ($P < 0.05$).

[b] EPL300-ACS30, EPL 300 mg/liter followed by ACS 30% solution with a 40 s time interval; LAE200-ACS30, LAE 200 mg/liter followed by ACS 30% solution with a 40 s time interval.

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