APPLICATION OF NOVEL HURDLE TECHNOLOGIES TO MEAT CARCASS TRIMMINGS FOR REDUCTION OF PATHOGENS

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EXECUTIVE SUMMARY

More people are concerned about the safety of the food they consume than at any other time in history. Food-borne diseases are attributed largely to in-home contamination, but food processors bear the greatest responsibility for food safety. They are required by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) or Food and Drug Administration (FDA) to implement strict measures to reduce or eliminate any potential pathogen or hazard that might be introduced to the food during harvest, processing and handling.

One of the largest challenges in the meat industry is keeping the product safe and free from contamination (Keeton and Harris 2004). Small meat plants face a particular challenge due to their limited personnel and financial resources. *Salmonella* Typhimurium (ST), *Escherichia coli O157:H7* (EC) and *Listeria monocytogenes* (LM) are among the pathogens most commonly associated with contamination of meat and poultry products. These organisms pose a serious problem for decontamination of carcasses and/or ready-to-eat (RTE) products due to their ability to survive and grow in extreme and stringent conditions such as low pH or refrigeration temperatures. Contamination can be a problem, especially in small plants, since many small processors deal with both raw (e.g. refrigerated beef trim) and processed product (fully-cooked RTE items) in close proximity, thus increasing the risk of cross-contamination unless proper safety measures are implemented and strict control measures enforced. The use of organic acids in sprays for carcass decontamination and the effective reduction of pathogenic bacteria have been adopted by meat processors industry wide. Treatments designed for use in combination or in sequence, that are cost effective as pathogen reduction interventions for small meat processing plants, have not been validated.

The objectives of this study were to evaluate undeveloped and cost effective pathogen interventions using a multiple hurdle approach suitable for small meat plant operations in an effort to further reduce contamination and growth of pathogens, specifically *Salmonella* spp., *E.coli* O157:H7 and *Listeria monocytogenes.* Pre-rigor, warm beef rounds were surface inoculated with a three-pathogen cocktail of rifampicn-mutant strains of *Salmonella* Typhimurium, *E. coli O157:H7* and *Listeria monocytogenes*. After inoculation, the rounds were left at room temperature for 5 to10 minutes to allow for bacterial attachment, and then sprayed for 15 to 20 sec in a fixed pressure, self-contained spray cabinet. Six decontamination solutions were used: 1:4 acidified calcium sulfate (ACS, Safe2O RTE01®, Mionix Corporation, Rocklin, CA) : water, lactic acid (LA 2.5% L-lactic acid, Purac America, Inc., Lincolnshire, IL), 100 µL (100 ppm) epsilon-polylysine (EPL, Save-ory® PL-25, Chisso, Corporation, Tokyo, Japan), 1:4 ACS RTE01 : water + 100 µL (100 ppm) ε-polylysine, (EPL), 100 µL (100 ppm) EPL, and sterile distilled water (W), and tested for effectiveness. All treatments, once diluted to appropriate concentration, were placed in stainless steel containers and heated to 50-55°C in a water bath. The solutions were then applied using a customized pressurized spray system with nozzles that delivered a specific volume (14 ml/sec) of the treatment solutions. The experiments were replicated three times. All recoveries of inoculated pathogens were done on aseptically sectioned 50 cm^2 portions. A designated portion was removed from each sample and combined separately with 20 ml of sterile PBS in stomacher bags. Counts of rifampicin-resistant E. *coli O157:H7, Salmonella* Typhimurium and *Listeria monocytogenes* were determined by plating appropriate dilutions onto pre-poured plates of a selective-differential medium, LSPR and Modified Oxford Agar base (MOX), respectively.

Our results showed that a sequential application of warm (55°C) ACS followed by EPL at constant pressure for 15 to 20 sec to pre-rigor beef rounds significantly reduced inoculated levels of *Salmonella* Typhimurium, *E. coli* O157:H7 and *Listeria monocytogenes* with an extended effect over seven storage days. This combination was more effective than single treatments of ACS, LA, EPL or W alone. LM appeared to be more sensitive to the initial decontamination treatments on day 0 when compared to ST and EC, but unlike ST and EC did not show further reductions over a 7 day refrigerated storage period. This may have been due in part to LM's increased tolerance to cold temperatures or later becoming resistant to EPL during storage. The separate modes of action of ACS and EPL might contribute to their synergistic effectiveness for inhibiting the growth of pathogens.

From the observations in this study, it appears that a sequential application of ACS + EPL can be a better strategy for pathogen reduction in small meat plants than a single decontamination treatment, and could also provide a more 'fail-safe' pathogen reduction strategy.

Further experiments are currently being performed to investigate the sequence of addition of ACS and EPL, the time interval between the applications (5 to 15 min) on pathogen reduction and the effects of varying the concentrations of each component. It is also worth investigating the antimicrobial effect of LA and EPL when applied in sequence. Additional work is needed to optimize our application techniques for reducing pathogens in small meat and poultry processing operations. When the treatment sequences have been optimized, the procedures will be tested in small meat plants in the College Station area.

ABSTRACT

The use of organic acids as beef carcass decontaminants in the meat and poultry industry is well documented. However, there is limited information on the use of these acids in a multiple hurdle fashion for small meat and poultry plants to reduce contamination and growth of pathogens, specifically *Salmonella* Typhimurium (ST), *E. coli* O157:H7 (EC) and *Listeria monocytogenes* (LM). This study evaluated the effectiveness of the application of warm solutions of 1:4 acidified calcium sulfate (ACS) RTE01:water, 2.5% lactic acid (LA), 1:4 ACS RTE01:water + 100 µL (100 ppm) epsilon-polylysine (EPL), 100 µL (100 ppm) EPL, and sterile distilled water (W) for reducing *Salmonella* Typhimurium, *E. coli O157:H7* and *Listeria monocytogenes* on the surface of fresh, pre-rigor beef rounds secured from a local abattoir. All treatments were applied for 15 to 20 sec at 50-55°C under a constant pressure of 137.9 kPa (20 psi) to deliver 0.2082 L/15 sec (14 ml/sec). ACS followed immediately with EPL significantly reduced levels of inoculated ST, EC and LM with mean log reductions of 2.26, 1.49 and 2.38, respectively, over a 7 day refrigerated storage period. ACS + EPL was more effective than single treatments of ACS, LA, EPL or W alone. Single treatments of ACS, LA and EPL were more effective than water alone for reducing ST and EC, but only LA was effective against LM. Warm water was the least effective for reducing pathogens. LM appeared to be more sensitive to the initial decontamination treatments on day 0 when compared to ST and EC, but unlike ST and EC did not show further reductions over a 7 day refrigerated storage period. Further studies are required to test the effects sequential application on the efficacy of the treatments and to determine if sequence of treatment application, duration of the treatment or frequency of exposure have an effect on the acquisition rates of antimicrobial resistance and virulence of the pathogens.

INTRODUCTION

Despite advances in technology and medical sciences, food-borne illnesses continue to be a serious public health problem and of significant concern to the food industry. The Centers for Disease Control and Prevention, one of the 13 operating components of the Department of Health and Human services, has estimated that there are 76 million cases of food-borne illness annually, resulting in 325,000 hospitalizations and 5,000 deaths (Mead and others 1999). Pathogen reduction strategies have been implemented by the regulatory agencies and the food industry to maintain consumer confidence at a time when consumers are increasingly concerned about the safety of what they eat. Agro-terrorism also is of concern due to the hazards that could exist if intervention measures are not put in place to detect, reduce or eliminate pathogens introduced intentionally into the food supply.

Meat and poultry are among the top five items implicated in food-borne illness outbreaks. Pathogen-contaminated meat and poultry are thought to cause at least 2.5 million illnesses and 1,000 deaths every year (Frenzen and others 2000). As a result, several approaches have been developed to decontaminate meat and poultry products during the harvesting process (Mermelstein 2001; Connor 2001; Huffman 2002; White 2002). Some of these methods include cold and hot water rinses; steam pasteurization or steam vacuum treatment; carcass trimming; a variety of chemical rinses including chlorine/chlorine dioxide, ozonated or electrolyzed water, trisodium phosphate, acidified calcium sulfate, and organic acid rinses (such as lactic acid) with or without surfactants. Antimicrobial compounds may also be added to many ready-to-eat (RTE) products including sodium or potassium lactate, sodium diacetate, sodium citrate and a variety of antioxidant compounds that also exhibit antimicrobial properties including various spices or their

extracts (e.g. rosemary extract), fruit preparations (e.g. dried plums) or synthetic antioxidants. Most of these individually will provide a 0.5 to 3 log reduction in pathogens, with water rinses being the least effective. A time lag between treatment of the carcass and fabrication into cuts or trimmings also can allow bacterial attachment to occur (biofilm formation) which decreases the effectiveness of most washing procedures. For these reasons, the immediate application post harvest of a combination of more than one intervention treatment to carcasses often has been found to produce a greater antimicrobial effect than any single treatment, often working in a synergistic manner. The latter has been referred to as hurdle technology. Combined hurdles have been found not only to enhance pathogen reduction, but also serve to improve the quality of meat/poultry resulting in more shelf-stable products.

The United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) issued a final regulation on July 25, 1996 (USDA-FSIS 1996) establishing pathogen reduction requirements applicable to meat establishments. These were designed to reduce the occurrence and numbers of pathogens in or on meat and poultry products, thus reducing the risk of food-borne disease affecting millions of people (Mead and others 1999). The principal source of transmission of microbes such as *E. coli* O157:H7, *Salmonella spp., Campylobacter*, *Staphylococcus aureus* and others are from the hides of animals arriving at processing plants or carcasses that become cross-contaminated with intestinal contents during processing (USDA-FSIS, 2004). For ready-to-eat (RTE) products, cross-contamination or recontamination by pathogens in the processing plant (e.g. human handling, contaminated processing equipment) is generally the major concern (Borch and Arinder, 2002).

Recontamination of cooked products can result in a more serious problem for decontamination than untreated raw products, especially for spore forming microbes like

Clostridium or cold-tolerant, psychrotrophic bacteria such as *Listeria monocytogenes*, because of a lack of competing microflora (e.g. lactic acid bacteria). Listeriosis acquired from the consumption of RTE products represents a serious public health concern because of the high mortality rates associated with the illness. However, contamination of raw materials (e.g. refrigerated beef trimmings) by *Listeria* also can be a problem, especially in small plants. Since many small processors deal with both raw and processed products, often in close proximity, this increases the risks of cross-contamination unless proper safety measures are implemented and strictly controlled.

The regulation entitled "Pathogen Reduction: Hazard Analysis and Critical Control Point (HACCP) systems," has four basic components, one of which is that all establishments develop and implement a system of preventive controls to improve product safety. Federal regulations (CFR 2002; USDA-FSIS 1993) now specify the minimum thermal processing requirements for cooked meat/poultry products as well as establishing 'zero tolerance' for contamination of beef products. In response to demands from consumers (CAST 2004) and government regulations for safer meat products, a wide range of studies testing possible interventions have been conducted, particularly in the last ten years.

The most commonly used chemical decontamination methods are rinses containing chlorine, chlorine dioxide, acidified sodium chlorite, electrolyzed water, ozone, trisodium phosphate (TSP) and cetylpyridinium chloride (CPC). The latter compound has been evaluated in several studies (Ransom and others 2003; Pohlman and others 2002 a, b; Huffman 2002) and was just recently approved for food use by the USDA-FSIS. The 'gaseous' antimicrobials (chlorine, chlorine dioxide, ozone, acidified sodium chlorite which generates an oxy-halogen) are usually applied as an aqueous solution and generally have resulted in a 2 to 4 log reduction of

pathogens depending on concentration, temperature of application and contact time. However, the suppression tends to be transient, providing no extended bactericidal/bacteristatic effects after treatment. The primary reason for this effect is that these compounds are readily reactive with organic compounds, thus quickly removing them from solution and/or negating further action against bacterial cells. TSP on the other hand is an alkaline salt solution that can leave residual reactive hydroxyl radicals in the treated products and suppress further growth. It has been found to improve the color of meat products, but the treatment also generates large amounts of alkaline phosphates, which can be environmentally harsh and create a problem for disposal. The use of organic acids as a carcass washing intervention has been a particularly active area of study, with the most commonly used acids being lactic and acetic acid (Dorsa and others 1998; Castillo and others 1998 a, b, 1999, 2000, 2001 a, b; Mermelstein 2001, Huffman 2002; Pohlman and others 2002 a, b; Ransom and others 2003). Both lactic and acetic acid are generally recognized as safe (GRAS) by the U.S Food and Drug Administration. Lactic acid and acetic acid tend to offer the best residual efficacy for suppressing further pathogen proliferation during long-term refrigerated storage in which the meat is subsequently ground (Pohlman and others 2002 a, b; Castillo 2001 a, b; Dorsa and others 1998). The rinse concentrations used are usually 2 to 5% and both acids are most effective if applied immediately after hot water washes (95°C) or as heated solutions (usually ~55°C) on hot, pre-rigor carcasses. While such applications are both effective, high heat treated products can acquire an undesirable color, loss of ground emulsion stability and increased acidic flavor if the residue is too high.

Acidified sodium chloride (ASC) has shown increased antibacterial effects when combined with lactic acids. Castillo and others (1998 b) investigated the effectiveness of ASC solutions activated with phosphoric or lactic acid for reducing pathogens on inoculated beef

carcass surfaces and found a reduction in pathogen numbers though still within countable limits. Acidified calcium sulfate (ACS) (sold by Mionix Corp., Rocklin, CA under the trade name Safe2O®) has been found to be very effective as a beef and poultry carcass washing agent (Huffman 2002; Dickens and others 2004) as well as a rinsing agent for RTE meats with considerable residual listericidal/listeristatic activity (Nuñez de Gonzalez and others 2004). It is a GRAS ingredient for use on food products and consists of a complex blend of sulfuric acid, calcium sulfate, calcium hydroxide, and an organic acid (e.g. lactic acid) adjusted to a final pH of ~1.5. According to Mionix, ACS plus organic acids disable the proton pumps in bacterial membranes and act as a metabolic inhibitor, thus attacking bacteria in a different fashion than organic acids alone (e.g. lactic acid). However, only a few studies have shown its potential residual antimicrobial effects. This promising intervention demands additional study and evaluation, especially as a component of hurdle technology. In addition, epsilon polylysine (EPL) has been found to enhance the antimicrobial effects of ACS especially against lactic acid spoilage bacteria (Hiraki 2002). Epsilon polylysine is also GRAS and is thought to act in a different manner than ACS by causing disruption of the bacterial cell surface (Yoshida and others 2002). EPL concentrations of 0.02% and 0.04% have been shown to have antimicrobial activity against *E.coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes,* with *Salmonella* Typhimurium being the most sensitive of all three pathogens (Geornaras and Sofos 2005). When combined with other antimicrobials EPL has enhanced antimicrobial activity. The potential use of these combined treatments merits further evaluation in food systems and especially because of their ease of application for small plant operators.

Against this background, the present study was aimed at evaluating relatively undeveloped and cost effective pathogen reduction interventions at multiple levels for the small

meat plant as hurdles to reduce contamination and growth of pathogens, specifically *Salmonella* Typhimurium, *E. coli* O157:H7, and *Listeria monocytogenes.* This study used rinses consisting of the application of a 1:4 acidified calcium sulfate (ACS) RTE01:water, 2.5% lactic acid (LA), 1:4 ACS RTE01:water + 100 µL (100 ppm) ε-polylysine (EPL), 100 µL (100 ppm) EPL and sterile distilled water (W) as a control. All treatments were applied hot (50-55^{o}C) using a self-contained spray cabinet, on pre-rigor beef rounds destined for use as ground beef or trimmings to be incorporated into further processed products (e.g. raw and cooked sausages). One or two washes on carcasses/trimmings in succession, in a hurdle fashion, were proposed such that initial bactericidal agents also may provide residual bacteriostatic effects to improve antimicrobial efficacy while minimizing undesirable effects on meat quality. The long term objectives were to develop interventions that will benefit the small processor by enhancing safety, being simple to apply, cost-effective and increasing shelf-life by reducing spoilage bacteria in addition to pathogens. The specific objectives were to**:**

1) Evaluate warm water (control), lactic acid, acidified calcium sulfate and/or epsilon-polylysine as beef carcass/trimmings decontamination agents that are applied as a warm rinse following standard washing procedures. This was done initially in a model system using minimal application procedures to reduce the time and cost of the intervention for small plant. Different concentrations of the treatment were examined to determine optimal reduction of *Salmonella* Typhimurium, *E.coli* O157:H7 and *Listeria monocytogenes*.

2) Determine the residual antimicrobial effects of ACS with and without EPL over time on carcasses/trimmings and selected raw products.

3) Evaluate the efficacy of the best intervention identified above in two to three small local processing plants and to determine its efficacy as part of the HACCP plan.

4) Evaluate the bactericidal/bacteriostatic efficacy of the best treatment on a cooked ready-to-eat (RTE) product contaminated with a *Listeria* cocktail. Due to time limitations, this objective was not covered, but it merits further study.

MATERIALS AND METHODS

Decontamination Procedures

Six decontamination solutions were used: 1:4 acidified calcium sulfate (ACS, Safe2O RTE01®, Mionix Corporation, Rocklin, CA):water, lactic acid (LA 2.5% L-lactic acid, Purac America, Inc., Lincolnshire, IL), 100 µL (100 ppm) epsilon-polylysine (EPL, Save-ory® PL-25, Chisso, Corporation, Tokyo, Japan), 1:4 ACS RTE01:water + 100 µL (100 ppm) ε-polylysine, (EPL), 100 µL (100 ppm) EPL, and sterile distilled water (W), and tested for effectiveness. All treatments, once diluted to appropriate concentration, were placed in stainless steel containers and heated to 50-55°C in a water bath. Treatment solutions were applied using a pressurized spray system with nozzles that delivered a specific volume (14 ml/sec) of the treatment solutions (Figures 1 and 2).

Spray Cabinet

A custom-built isolation spray cabinet designed by CHAD Corporation was used to apply treatment solutions (Figures 1 and 2). Capture and containment of all spray, residue, pathogens and meat particles was accomplished with biohazard autoclave bags. Internal decontamination of the spray cabinet was performed using 20% hypochlorite (Chlorox®) in a removable reservoir attached to a cleaning/decontamination nozzle through the lid. After each treatment, the cabinet was rinsed with hot water which was collected in a biohazard receptacle and sterilized before discarding. This step was necessary to prevent any possible contact of ACS and chlorine. The

cabinet was then disinfected using Chlorox®. External cabinet surfaces were swabbed with a 70% alcohol (ethanol) solution to insure decontamination of the stainless steel cabinet. All run-off treatment solutions from the cabinet were collected in a Biohazard® bag and autoclaved, except for treatments containing ACS that were decontaminated by adding a 1:2 ratio of ACS to the soiled solution. In all procedures, care was taken to avoid contact of ACS and chlorine which are incompatible.

Calculation of Treatment Volume

Two nozzles were situated inside the cabinet near the top and bottom of the cylindrical spray chamber, through which treatments were delivered (Nozzle specification: H 1/8 VVSS 65015 Nozzle with a spray angle of 65°). At a spraying pressure of 137.9 kPa (20 psi), the flow was 0.4164L/min (0.11 GPM) per nozzle, resulting in a spray angle of 51°/nozzle. Solution delivery was 0.2082 L/15 sec as shown in the following calculations:

1US Gal = 3.785 Liters

1L = 0.2642 Gal

0.11 Gal x 3.785 L/gal = 0.4164 L/min

2 nozzles x 0.4164 L/min ÷ 60 sec/min x 15 sec

0.01388 L/min/60sec/min x 15 sec = 0.2082 L/15 sec or 14 ml/sec

After inoculation, individual beef round samples were sprayed for 15-20 seconds while rotating at constant rate of ~5 revolutions/15 sec in a uniform spray stream. Three, 50 cm^2 portions of each pre-rigor beef round were then excised from the skin side surface using sterile stainless steel forceps and a scapel. Two portions were stored at 4°C in separate sterile Zip-Lock® bags and marked for analysis at 2 and 7 days post harvest, respectively. The third portion was immediately placed in a stomacher bag into which 20 ml of sterile phosphate buffered saline

(PBS) solution was added and the contents pummeled in a stomacher for one minute to dislodge pathogens before examination.

Collection of Beef Rounds

Fresh, pre-rigor beef round samples were secured at a local abattoir by excising a 15 x 25 x 5 cm surface portion of muscle from the dorsal side of a wholesale round immediately after skinning and prior to evisceration of market weight steer/heifer carcasses. Samples were taken randomly and immediately placed in sterile plastic bags, stored in an insulated container and transported to the isolation laboratory at the USDA-ARS Southern Plains Agricultural Research Center. Inoculations were initiated approximately 2.5 hours post sampling and treatments randomized for each replication. Inoculations were applied to a fixed surface area 15 x 10 cm^2 as described below.

Bacterial Strain and Inoculum Preparation

To negate the effect of indigenous microorganisms and/or pathogens that could reside on the beef rounds at harvest, rifampicin-resistance mutants were used for inoculation. Rifampicin-resistance mutants were derived from parent strains of *Listeria monocytogenes* strain Scott A (serotype 4b) obtained from Dr. J. F. Frank, University of Georgia. *E. coli O157:H7* strain ATTC 43895, and *Salmonella* Typhimurium strain NVSL 95-1776 (kindly provided by Dr. R. Anderson of USDA-ARS Food and Food Safety Unit, College Station Texas) were used to inoculate pre-rigor beef rounds in this study. The selected mutants were maintained on tryptic soy agar slants at 4°C.

Rifampicin-resistant strains of *Salmonella* Typhimurium, *E.coli O157:H7* and *Listeria monocytogenes* were resuscitated on three consecutive days using tryptic soy broth. A 12-hour culture of the pathogens was used to prepare a cocktail of inoculum. The procedure was as

follows: two consecutive transfers into tryptic soy broth were carried out and incubated at 35°C for 24 hours. Twelve-hour cultures were diluted 100 fold and plated on TSA to determine initial cell numbers. Equal volumes of the 12-hour culture of each pathogen strain were centrifuged and the cells re-suspended in 5 ml of PBS, combined to form a cocktail and diluted 100 fold using PBS to provide an inoculation medium of 6.5 CFU/ml of PBS for each pathogen.

Selection Media

PBS was used as a recovery medium. A selective differential medium (lactose-sulfite-phenol red-rifampicin agar, LSPR) was prepared as described by Castillo and others (1998 b) with slight modifications. The medium simultaneously enumerates rifampicin-resistant mutants *Salmonella* Typhimurium and *E.coli O157:H7*. The lactose-sulfite-phenol red-rifampicin agar used in this experiment consisted of the following ingredients per liter: tryptic soy agar (TSA, Difco, Detroit, MI) 40 g, yeast extract (Difco) 3 g, beef extract (Difco) 3 g, lactose (EMI Industries, Inc., Gibbstown, NJ) 5 g, sodium sulfite (MCB Reagents, Cincinnati, OH) 2.5 g, ferrous sulfate (MCB Reagents) 0.3 g, phenol red (Fisher Scientific, Fair Lawn, NJ) and 25 mg, rifampicin (Sigma Chemical) 0.1 g. Phenol red was dissolved in 2 ml 0.1N NaOH before adding to the medium. The medium without rifampicin was autoclaved at 121°C for 15 min and cooled to 50°C. Rifampicin was dissolved in 5 ml methanol, filter-sterilized, and added to the sterile medium prior to pouring into Petri plates. The medium did not contain the 0.1 g cycloheximide contained in the original formulation. Rifampicin-resistance *E.coli* O157:H7 produced yellow colonies on the medium, whereas the rifampicin-resistant *Salmonell*a Typhimurium developed colonies with a black center surrounded by a pink halo. TET and RV were used for *Salmonella* Typhimurium enrichment and UVM and Fraser broth were used for *Listeria monocytogenes* enrichment as prescribed by USDA-FSIS (2002).

Rehydrated commercial Modified Oxford Agar base (Oxoid) was used for recovery of *Listeria monocytogenes*. The medium without supplement was autoclaved at 121°C for 15 min, cooled to 50°C and then, one vial of the supplement dissolved in 10 ml of sterile distilled water was added per liter before dispensing into Petri plates. *Listeria monocytogenes* detection is based on the hydrolysis of esculin to 6, 7-dihydroxycoumarin (esculetin) and its reaction with ferric ions in the medium; hence *Listeria's* presence is identified by a dark (blackened) zone due to the esculin hydrolysis usually within 24 hours.

Sample Excision and Inoculum Recovery

A 15 x 10 cm^2 section was outlined on the surface of a freshly harvested beef round using a sterile template. A 2 ml aliquot of the pathogen cocktail (6.8 CFU) was inoculated onto each template area and the inoculum spread uniformly with a sterile swab to yield an initial count of 6.35 logs on a 50 cm^2 area. The inoculated beef round was set aside for 10 min at room temperature to allow for bacterial adhesion to the meat surface. Individual beef round samples were then sprayed in a sealed, stainless steel cabinet (manufactured by CHAD Corporation) for 15-20 sec using heated treatment solutions (50-55°C) as previously described. Data are reported as the mean log_{10} reductions of each respective organism from an initial inoculation level of 6.35 CFU/microorganism, recovered from a surface area of 50 cm^2. Mean log reductions were calculated by subtracting the mean log CFU/50 cm^2 recovered after treatment and storage as described earlier, from the initial log CFU/50 cm^2 inoculum counts before treatment: [Log 6.35 CFU/50 cm^2 of each organism in the cocktail before treatment – mean log CFU/50 cm^2/organism recovered post treatment = Mean log reduction] .

Experimental Design and Statistical Analysis

The experiment was a 5 X 3 factorial design where five decontamination treatments (ACS, LA, ACS+EPL, EPL and W) and three storage days (0, 2 and 7) were used as main factors. The effects of decontamination treatments and storage days and their interaction were tested by the General Linear Procedure (GLM) using SPSS (Statistical Package for Social Science) for Windows release 13.0. The least significant difference (LSD) pair-wise multiple comparison test was used to compare the mean differences when the effects of the treatments were significant ($p \leq 0.05$).

RESULTS AND DISCUSSION

Preliminary Trials

Preliminary trials were conducted with the antimicrobial treatments to establish inoculation and recovery procedures, determine the most appropriate method of treatment application (i.e., combine LA with EPL in the same solution or apply separately and sequentially), establish spray cabinet pressure conditions to deliver a specified volume of liquid, and to develop decontamination procedures for the spray cabinet to ensure complete inactivation and containment of pathogens. Preliminary mean log reductions for ST, EC and LM by storage day are presented in Tables 1-3, respectively. Reductions shown are somewhat indicative of treatment effects on the respective pathogens, but not definitive since adjustments were being made in the experimental procedures. Although these means cannot be analyzed statistically, they are presented for information purposes and were used to assist in the selection of subsequent treatment combinations. In the preliminary trials, EPL was added directly to a 2.5% LA solution,

but additional information provided by the distributor of EPL indicated that direct contact with the acid might decrease its antimicrobial efficacy, which was consistent with our observations.

Numeric values shown in Tables 1-3 are the mean log reductions from the initial counts of each pathogen (log 6.35 CFU/50cm^2) that was inoculated onto pre-rigor beef rounds as a cocktail, and then treated with a hot (50-55^{o}C) antimicrobial spray.

***Salmonella* Typhimurium (ST)**

Table 1 shows the mean log reductions of ST due to treatment solutions. On storage day 0, an initial application of ACS and LA showed a mean log reduction of 1.6 and 1.1 logs, respectively. LA combined with EPL (0.7) and EPL (0.7) alone had numerically lower reduction values than ACS and LA alone, but were comparable to water (0.6) for reducing ST counts. Comparable reductions of 2.9 and 2.7 logs on storage days 2 and 7 were noted for ACS and LA, respectively. The hot water treatment had comparable reductions of 2.1 and 2.6. LA + EPL reductions were 1.2 and 1.1 logs on days 2 and 7 while, EPL reductions were 1.1 and 1.4, respectively. These preliminary results for ST were not consistent with the replicated data obtained later. Even in the preliminary trials, it is interesting to note that larger reductions in ST seemed to occur after 2 and 7 days storage at 4^{o}C.

***Escherichia coli O157:H7* (EC)**

In Table 2, an initial application of ACS reduced EC by 1.8 log while LA showed a mean reduction of 1.1 log followed by W (0.9) and LA+ EPL (0.7). EPL alone had a mean log reduction of 0.2. On storage day 2, ACS had the highest mean log reduction (3.1) followed by LA + EPL (2.4). Likewise, LA, EPL and W had greater reductions in EC on days 2 when compared to day 0. The same trends were noted on day 7 as compared to day 2, with slightly lower reductions for LA + EPL and EPL and higher reductions for W. All treatments, to varying

degrees, appeared to inhibit the growth of *E.coli* O157: H7 over a 7 day refrigerated storage period.

***Listeria monocytogenes* (LM)**

Table 3 shows that the largest mean log reductions in LM tended to occur with the initial ACS treatment (3.5) followed by the application of LA + EPL (2.0). All treatments, except ACS, appeared to offer greater suppression of growth on days 2 and/ or 7 than at day 0. It is interesting to note that LA had the greatest numeric reduction (4.1) on day 7 while ACS had less reduction than on days 0 and 2 for most treatments.

Overall, mean log counts in Tables 1-3 seemed to show increased antimicrobial effectiveness over 7 days of refrigerated storage. However, these were only preliminary results. Because of the adjustments being made in testing conditions during the preliminary trials, the changes in procedures may have influenced the log counts and these data may not truly reflect treatment effects. It was determined at this stage, however, that the sequence of application for EPL in combination with ACS or LA could be important. Because of the larger initial bacterial reductions with ACS, it was decided to apply ACS and EPL sprays to pre-rigor beef rounds in a sequential manner (ACS + EPL) in three replicated experiments that followed the preliminary trials. Application of ACS, followed by EPL at different time intervals (data not shown), have also shown reductions in *Salmonella* Typhimurium, *E.coli O157:H7* and *Listeria monocytogenes.*

Results of Decontamination Treatments

***Salmonella* Typhimurium (ST)**

The analysis of variance of the log reduction data for *Salmonella* Typhimurium showed that the interaction between decontamination treatments and storage days was significant ($p \leq 0.05$). Due to the significant interaction between the main factors, a plot of the treatment means and LSD procedure were used to compare all treatment means instead of testing and comparing the individual levels of each factor (Table 4). As shown in Figure 3 and Table 4, the effectiveness of treatments varied depending on the storage day. Initial mean log reductions of ST were not different among the spray treatments initially on day 0, but with slight reductions from the initial inoculum level. By storage day 2, the ACS + EPL treatment had reduced ST by 2.0 log from the initial inoculum level, while ACS, LA and EPL tended to show reductions, but these were not significantly different from the W treatment. W was used as a control to demonstrate the effect of warm water alone and to give a basis of comparison to the other treatments. On storage day 7, ACS + EPL had reduced ST by 4.38 log while the other treatments, except W, reduced ST by $\geq$ 1 log. W tended to have the same level of ST on day 7 as at the time of initial inoculation. Treatment with LA alone was consistent with other studies in which 2.5% lactic acid has been shown to be effective in reducing pathogens on cold beef carcass surfaces and in ground beef produced from the carcass trimmings (Castillo and others 2001). In general, all decontamination treatments, except W, caused a reduction in ST counts after 7 days of refrigerated storage with the ACS + EPL combination being the most effective antimicrobial treatment against ST. EPL concentrations of 0.02% and 0.04% have been shown to have antimicrobial activity against *E.coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes*, with *Salmonella* Typhimurium being the most sensitive of all three pathogens (Geornaras and Sofos 2005). Based on these results and those of Geornaras and Sofos (2005), EPL appears to have enhanced antimicrobial activity in combination with acidic antimicrobials.

***Escherichia coli* O157:H7 (EC)**

The analysis of variance of the log reduction data for *Escherichia coli* O157:H7 demonstrated that the interaction between decontamination treatments and storage days was not significant. As a result, the data were pooled across treatments and across storage days and reported as main effect means in Tables 7 and 8. Mean log reductions in *E.coli* O157:H7 segregated by treatment and storage are presented in Table 5 for information only. Table 7 shows the independent effect ($p<0.05$) of decontamination treatments and Table 8 the effect of storage days on mean log reductions of EC. Least significant difference (LSD) comparisons of decontamination treatments over all storage days indicated that ACS + EPL was the most effective treatment with an overall reduction in EC of 1.49 log. ACS, LA and EPL reductions were approximately half (0.89, 0.88 and 0.65 log, respectively) the ACS + EPL treatment, but significantly more effective than W alone (0.32 log). The effects of ACS, LA and EPL were not significantly different from one another in reducing EC, though all treatments were effective to varying degrees for reducing EC below the initial inoculation level of 6.35 CFU/50 cm^2.

Reductions in EC after spraying with warm (50-55^oC) decontamination solutions for 15 to 20 seconds on storage day 0 (0.49 log) were ($p\leq0.05$) less than the reductions observed for days 2 and 7 (0.92 and 1.14 log, respectively). Overall, mean log reductions for all treatments were greater after the second day. The ACS + EPL combination was the most effective antimicrobial treatment against EC while warm water showed the least reduction.

***Listeria monocytogenes* (LM)**

The analysis of variance of the mean log reductions for *Listeria monocytogenes* demonstrated that interaction between decontamination treatments and storage days was not significant. As a result, the data were pooled across treatments and across storage days and

reported as main effect means in Tables 7 and 8. Mean log reductions in *Listeria monocytogenes* after spraying with warm (50-55°C) decontamination treatment solutions for 15 to 20 seconds are presented in Table 6 for information only. The effects of decontamination treatments over storage (Table 7) were significant while storage alone (Table 8) did not affect mean log reductions of LM ($p \geq 0.05$). Least significant difference (LSD) comparisons of decontamination treatments in Table 7 showed ACS + EPL to cause the greatest reduction (2.38 log) in LM. A similar result when ACS was used in combination with EPL has been shown by Geornaras and Sofos (2005). LA (1.54 log) was more effective at reducing LM than W alone (0.78 log), but ACS (1.48 log) and EPL (1.36) were not different from the W control. LA treatment was not different from ACS or EPL treatments. Reductions in LM were not different across storage (Table 8) and ranged from 1.39 to 1.61 log.

Previous studies in our laboratory have demonstrated the antimicrobial effectiveness of ACS (bactericidal effect) and LA (bacteriostatic effect) on LM inoculated onto the surface of frankfurters and stored at 4.5^{o}C (Nuñez de Gonzalez and others 2004). In this study, the greatest mean log reductions in ST, EC and LM on pre-rigor beef rounds were obtained when ACS was applied followed immediately by EPL. ST showed the greatest susceptibility to the ACS + EPL treatment over storage followed by EC. LM appeared to be more sensitive to the initial decontamination treatments on day 0 when compared to ST and EC, but unlike ST and EC did not show further reductions over a 7 day refrigerated storage period. This may have been due in part to LM's increased tolerance to cold temperatures. In addition, Delihas and others (1995) have shown some microorganisms to be highly susceptible to EPL *in vitro* on the first day of treatment, but later becoming resistant to EPL during storage. Other studies have shown that

some microorganisms possess EPL-degrading enzymes, which make them resistant to EPL or compounds containing EPL (Kito and others 2002).

The separate modes of action of ACS and EPL might contribute to their synergistic effectiveness for inhibiting the growth of pathogens. ACS, which contains lactic acid, are believed to disable the proton pumps in bacterial membranes and act as a metabolic inhibitors, thus attacking bacteria in a different fashion than organic acids alone (e.g. lactic acid). EPL is thought to act in a different manner than ACS by causing disruption of the bacterial cell surface (Hiraki 2002). The differences in sensitivity of gram-positive and gram-negative organisms to EPL is not fully understood, but it is speculated that the types and/or number of cell surface receptors or proteases secreted by an organism contribute to its sensitivity as well as differences in their respective cell envelope make up (Delihas and others 1995).

The enhanced antimicrobial activity of EPL against *Salmonella* Typhimurium and *E. coli* O157:H7 when used in combination with sodium acetate or acetic acid has been demonstrated by Geornaras and Sofos (2005). In this study, ST, EC and LM were more sensitive to ACS + EPL than EPL alone. Although Geornaras and Sofos (2005) used different treatment combinations of EPL, they reported ST grown *in vitro* to have more resistance to EPL, than EC or LM. Preliminary results in our laboratory showed the efficacy of EPL to be reduced when combined with LA and used as a decontamination spray for pre-rigor beef rounds.

Castillo and others (2001) have shown 4% LA at 55^{o}C and sprayed for 30 sec or 4% LA at 65^{o}C and sprayed for 15 sec or 30 sec to consistently result in undectable levels of *E. coli* O157: H7 on chilled beef carcasses. In the present study, 2.5% LA showed reductions of ST, EC and LM, but to a lesser extent than the higher concentrations of LA. Castillo and others (1998) also reported mean reductions in *E. coli* O157:H7 and *Salmonella* Typhimurium of 3.7 and 3.8

log, respectively, when a hot water spray (95^{o}C) was used for beef carcass decontamination. However, warm (55°C) water application to beef rounds in this study showed only minimal reductions in pathogens. While high level (5%) applications of LA and hot water (95°C) are both effective, treated products can acquire an undesirable color, loss of ground emulsion stability and increased acidic flavor if the residue is too high.

CONCLUSIONS

A sequential warm (55°C) spray application of ACS followed by EPL at constant pressure for 15 to 20 sec reduced ST, EC and LM inoculated on the skin-side surface of pre-rigor beef rounds more effectively than a single treatment of ACS, LA, EPL or W alone. This confirms the fact that multiple interventions can be a better strategy for pathogen reduction in the small processing plants than single treatments and could also provide a more 'fail-safe' pathogen reduction strategy for small meat and poultry processing plants. Based on observations in this study, it appears that the combination of antimicrobial agents that express different modes of action for suppressing pathogen growth, and the sequential application of different decontamination sprays (e.g. ACS + EPL) are significant factors for obtaining greater reductions in pathogen numbers on beef carcasses at slaughter. Further experiments are currently being performed to investigate the sequence of addition of ACS and EPL, the time interval between the applications (5 to 15 min) on pathogen reduction and the effects of varying the concentrations of each component. It is also worth investigating the antimicrobial effect of LA and EPL when applied in sequence. Additional work is needed to optimize our application techniques for reducing pathogens in small meat and poultry processing operations. When the treatment

sequences have been optimized, the procedures will be tested in small meat plants in the College Station area.

Figure 1.

A custom built isolation spray cabinet designed by CHAD Corporation, Showing containment chamber, three stainless steel reservoirs for application of treatment, White plastic receptacle for application of cleaning solutions, and pressure gauges.

Figure 2.

A custom built isolation spray cabinet designed by CHAD Corporation, showing rotating stainless steel hooks for suspension of beef rounds

Table 1. *Mean log reductions of Salmonella Typhimurium after spraying with warm (50-55°C) decontamination treatment solutions for 15 to 20 seconds.*

	Mean Log Reductions[b]		
	Storage Day at 4°C		
Treatment[a]	**0**	**2**	**7**
ACS	1.6	2.9	2.7
LA	1.1	2.9	2.7
LA+EPL	0.7	1.2	1.1
EPL	0.7	1.1	1.4
W	0.6	2.1	2.6

[a]1:4 acidified calcium sulfate (ACS) RTE01:water ; 2.5% lactic acid (LA), 2.5% lactic acid (LA)+ 100 µL (100 ppm) ε-polylysine, (EPL) , 100 µL (100 ppm) EPL and sterile distilled water (W).
[b] Mean Log reductions = (Log of CFU/50 cm^2 of initial inoculum counts before treatment) – (Log CFU/50cm^2 after treatment). Log CFU/50 cm^2 of each organism in the cocktail before inoculation was 6.35.

Table 2. *Mean log reductions of E. coli O157:H7 after spraying with warm (50-55°C) decontamination treatment solutions for 15 to 20 seconds.*

	Mean Log Reductions[b]		
	Storage Day		
Treatment[a]	**0**	**2**	**3**
ACS	1.8	3.1	2.7
LA	1.1	1.9	2.0
LA+EPL	0.7	2.4	1.8
EPL	0.2	2.1	1.3
W	0.9	1.9	2.5

[a]1:4 acidified calcium sulfate (ACS) RTE01:water ; 2.5% lactic acid (LA), 2.5% lactic acid (LA)+ 100 µL (100 ppm) ε-polylysine, (EPL) , 100 µL (100 ppm) EPL and sterile distilled water (W).
[b] Mean Log reductions = (Log of CFU/50 cm^2 of initial inoculum counts before treatment) – (Log CFU/50cm^2 after treatment). Log CFU/50 cm^2 of each organism in the cocktail before inoculation was 6.35.

Table 3. *Mean log reductions of Listeria monocytogenes after spraying with warm (50-55°C) decontamination treatment solutions for 15 to 20 seconds.*

	Mean Log Reductions[b]		
	Storage Day		
Treatment[a]	**0**	**2**	**7**
ACS	3.5	3.2	2.4
LA	1.6	2.4	4.1
LA+EPL	2.0	2.4	2.7
EPL	1.3	2.4	2.1
W	1.6	1.5	2.1

[a]1:4 acidified calcium sulfate (ACS) RTE01:water ; 2.5% lactic acid (LA), 2.5% lactic acid (LA)+ 100 µL (100 ppm) ε-polylysine, (EPL) , 100 µL (100 ppm) EPL and sterile distilled water (W).

[b] Mean Log reductions = (Log of CFU/50 cm^2 of initial inoculum counts before treatment) – (Log CFU/50cm^2 after treatment). Log CFU/50 cm^2 of each organism in the cocktail before inoculation was 6.35.

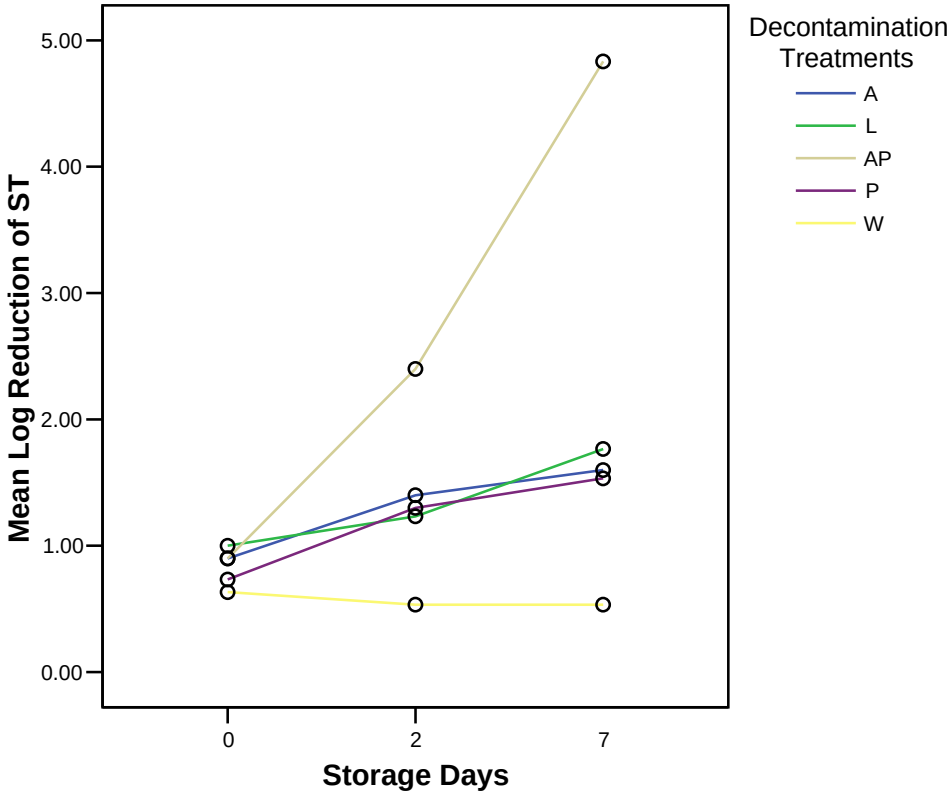

Fig 3. Mean log cell reduction of *Salmonella* Typhimurium after spraying with warm (50 to 55 ºC) decontamination treatment solutions for 15 to 20 seconds.

Table 4. *Mean log reductions of Salmonella* Typhimurium *after spraying with warm (50-55°C) decontamination treatment solutions for 15 to 20 seconds.*

	Mean Log Reductions[b] ± SD		
	Storage Day		
Treatment[a]	**0**	**2**	**7**
ACS	$0.45^{ABC} \pm 0.17$	$0.95^{ABC} \pm 0.17$	$1.1^{BCD} \pm 0.44$
LA	$0.55^{ABC} \pm 0.26$	$0.78^{ABC} \pm 0.64$	$1.32^{CD} \pm 0.32$
ACS+EPL	$0.45^{ABC} \pm 0.00$	$2.00^{D} \pm 0.53$	$4.38^{E} \pm 1.9$
EPL	$0.28^{AB} \pm 0.55$	$0.85^{ABC} \pm 0.17$	$1.0^{BCD} \pm 0.11$
W	$0.18^{AB} \pm 0.29$	$0.08^{A} \pm 0.40$	$0.08^{A} \pm 0.23$

[a] 1:4 acidified calcium sulfate (ACS) RTE01: water, 2.5% lactic acid (LA), 1:4 ACS RTE01: water +100 µL (100 ppm) ε - polylysine, (EPL), 100 µL (100 ppm) EPL, and sterile distilled water (W).

[b] Mean Log reductions = (Log CFU/50cm^2 of initial inoculum counts before treatment) – (Log CFU/50cm^2 after treatment). Log CFU/50cm^2 of each organism in the cocktail before inoculation was 6.35

^{ABCDE} Different superscript letters indicate that means are different ($p<0.05$) (LSD).

Table 5. *Mean log reductions of E.coli O157:H7 after spraying with warm (50-55°C) decontamination treatment solutions for 15 to 20 seconds.*

	Mean Log Reductions[b] ± SD		
	Storage Day		
Treatment[a]	**0**	**2**	**7**
ACS	0.65 ± 0.10	0.95 ± 0.17	1.11 ± 0.42
LA	0.68 ± 0.25	0.65 ± 0.40	1.32 ± 0.30
ACS+EPL	0.88 ± 0.35	2.00 ± 0.47	1.62 ± 0.35
EPL	0.05 ± 0.52	0.85 ± 0.17	1.10 ± 0.1
W	0.18 ± 0.06	0.15 ± 0.36	0.62 ± 0.37

[a]1:4 acidified calcium sulfate (ACS) RTE01; 2.5% lactic acid (LA), 1:4 ACS RTE01 + 100 µL (100 ppm) ε-polylysine (EPL), 100 µL (100 ppm) EPL and sterile distilled water (W).

[b] Mean Log reductions = (Log of CFU/50cm^2 of initial inoculum counts before treatment) – (log CFU/50cm^2 after treatment). Log CFU/50cm^2 of each organism in the cocktail before treatment was 6.35.

Table 6. *Mean log reductions of Listeria monocytogenes after spraying with warm (50-55°C) decontamination treatment solutions for 15 to 20 seconds.*

	Mean Log Reductions[b] ± SD		
	Storage Day		
Treatment[a]	**0**	**2**	**7**
ACS	1.25 ± 0.53	1.72 ± 0.49	1.48 ± 0.55
LA	1.55 ± 0.44	1.15 ± 0.26	1.92 ± 0.31
ACS+EPL	2.28 ± 0.59	2.58 ± 2.10	2.28 ± 1.37
EPL	0.98 ± 0.68	1.65 ± 0.36	1.45 ± 0.35
W	0.88 ± 0.49	0.55 ± 0.20	0.92 ± 0.58

[a]1:4 acidified calcium sulfate (ACS) RTE01; 2.5% lactic acid (LA), 1:4 ACS RTE01 + 100 µL (100 ppm) ε-polylysine (EPL), 100 µL (100 ppm) EPL and sterile distilled water (W).

[b] Mean Log reductions = (Log of CFU/50cm^2 of initial inoculum counts before treatment) – (log CFU/50cm^2 after treatment). Log CFU/50cm^2 of each organism in the cocktail before treatment was 6.35.

Table 7. *Analysis of Variance results for log cell reductions[b] of, E.coli O157:H7and Listeria monocytogenes recovered from beef after spraying with warm (50-55°C) decontamination treatment solutions for 15 to 20 seconds.*

	Antimicrobial Treatment[a]					
	ACS	**LA**	**ACS+EPL**	**EPL**	**W**	**STD Error**
***E.coli* O157:H7**	0.89^{B}	0.88^{B}	1.49^{C}	0.65^{B}	0.32^{A}	0.11
Listeria monocytogenes	1.48^{AB}	1.54^{CB}	2.38^{D}	1.36^{AB}	0.78^{A}	0.26

[a] 1:4 acidified calcium sulfate (ACS) RTE01: water, 2.5% lactic acid (LA), 1:4 ACS RTE01: water + 100 µL (100 ppm) ε - polylysine, (EPL), 100 µL (100 ppm) EPL, and sterile distilled water (W).

[b] Mean Log reductions = (Log CFU/50cm^2 of initial inoculum counts before treatment) – (Log CFU/50cm^2 after treatment). Log CFU/ 50cm^2 of each organism in the cocktail before inoculation was 6.35.

^{ABCD}Means in same row with different superscript letters are significant ($p \leq 0.05$) (LSD).

Table 8. *Analysis of variance for log cell reductions[a] (log CFU/cm^2) of E. coli O157:H7, and Listeria monocytogenes recovered from beef rounds after spraying with warm (50-55°C) decontamination treatments for 15 to 20 seconds at storage days 0 (immediately after treatment) 2 and 7 at 4^{o}C .*

	Storage days at 4^{o}C[b]			
	0	**2**	**7**	**STD Error**
***E.coli* O157:H7**	0.49 ^{A}	0.92 ^{B}	1.14^B	0.08
Listeria monocytogenes	1.39^A	1.53 ^{A}	1.61^A	0.20

[a] Mean Log reduction = (Log CFU/50cm^2 of initial inoculum counts before treatment) – (log CFU/50cm^2 after treatment). Log CFU/50cm^2 of each organism in the cocktail before treatment was 6.35.
[AB]Means in same row with different superscript letters are significant ($p \leq 0.05$) (LSD).

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