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Effects of Acetic Acid, Lactic Acid and Trisodium Phosphate on the Microflora of Refrigerated Beef Carcass Surface Tissue Inoculated with *Escherichia coli* O157:H7, *Listeria innocua*, and *Clostridium sporogenes*†

WARREN J. DORSA,* CATHERINE N. CUTTER, and GREGORY R. SIRAGUSA

United States Department of Agriculture, Agricultural Research Service, Roman L. Hruska U.S. Meat Animal Research Center, P.O. Box 166, Clay Center, Nebraska 68933-0166, USA

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ABSTRACT

The microbial profiles of inoculated beef carcass tissue (BCT) were monitored during prolonged refrigerated vacuum-packaged storage following antimicrobial treatment. An industrial spray wash cabinet was used to deliver water (W), 1.5 and 3.0% lactic (LA) or acetic (AA) acid, or 12% trisodium phosphate (TSP) washes. Fresh unaltered bovine feces spiked with antibiotic-resistant strains of *Escherichia coli* O157:H7, *Listeria innocua*, and *Clostridium sporogenes* were used to inoculate BCT prior to all treatments. The effect of treatments on bacterial populations was tracked by monitoring levels of specific-antibiotic-resistant (marked) bacteria along with mesophilic aerobic bacteria (APC), lactic acid bacteria (LAB), and pseudomonads for up to 21 days of storage at 5°C. Initial APC levels of approximately 5.6 log CFU/cm^2 were reduced by 1.3 to 2.0 log CFU/cm^2 by LA, AA, and TSP treatments. Marked bacteria were reduced to <1.3 log CFU/cm^2, remaining that way throughout the 21-day storage. TSP treatments were not different in effectiveness from acids for controlling growth of *E. coli* O157:H7 and *C. sporogenes*, but were less effective for APC, *L. innocua*, or LAB. The aerobic bacteria, *L. innocua*, and LAB had counts ≥7 log CFU/cm^2 by 7 days in all but one case and by 14 days all had counts >7 log CFU/cm^2 on the untreated controls and water-washed samples. Treatments generally added a degree of safety regarding the foodborne pathogens and pathogen models used for the present study when beef tissue was stored up to 21 days and in no case did the treatments appear to offer any competitive advantage to select microorganisms on BCT.

Key words: Beef, organic acids, trisodium phosphate, *Escherichia coli* O157:H7, *Listeria innocua, Clostridium sporogenes*

The effectiveness of organic acids and trisodium phosphate (TSP) for the inactivation of pathogenic and nonpathogenic bacteria associated with beef has been thoroughly studied and reviewed *(2, 4, 9, 16, 19, 23–25, 27)*. This large body of knowledge comprises conclusions from experiments representing a great diversity of experimental parameters. Few of these studies are representative of the actual beef carcass surface *(15, 17, 18, 21)*, with the bulk of knowledge being derived from cut beef surfaces (i.e., beef cores) dipped into organic acids and other antimicrobial agents. Consequently, the literature offers some conflict regarding the efficacy of organic acid sprays to reduce numbers of pathogenic bacteria such as *E. coli* O157:H7 on beef carcass surfaces. Unfortunately, few efforts have been made to determine the fate of specific bacteria on beef carcass surfaces after organic acid treatments and subsequent refrigerated storage *(18, 21)*.

The U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) has approved the use of some organic acids and of TSP as antimicrobial treatments for beef carcasses prior to entering chill rooms, thus 24 to 48 h before fabrication *(14)*. Therefore, these antimicrobial agents are not presently and will probably not be applied to cut meat surfaces in the beef slaughter industry but rather to whole carcasses. Additionally, it is highly probable that the contamination menstruum for pathogens being targeted by antimicrobial treatments is feces. Normally in the processing industry, whole carcasses are cut into subprimals, vacuum packed, then shipped and stored under refrigeration for an average of 21 days before consumption. While initial reductions in numbers of microorganisms by antimicrobial agents is important, the effect of the antimicrobial treatment becomes increasingly important as the product approaches the point of consumer preparation and consumption.

The present study was designed to determine the effect of water, TSP, acetic acid, or lactic acid washes on both pathogenic and nonpathogenic bacteria associated with beef carcass tissue handled and stored under normal industrial practices.

* Author for correspondence. Tel: (402) 762-4228; Fax: (402) 762-4149; E-mail: dorsa@marcvm.marc.usda.gov.

† Mention of a trade name, proprietary product or specific equipment is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

MATERIALS AND METHODS

Inocula

Escherichia coli O157:H7 CDC B6-914 (*E. coli* MARC1-S), *Listeria innocua* ATCC 33090 (*L. innocua* MARC1-S) and *Clostridium sporogenes* ATCC 11437 (*C. sporogenes* MARC1-N) were made resistant to antibiotics as previously described *(10, 12)*. *E. coli* MARC1-S was enumerated on sorbitol MacConkey agar (SMAC) (Difco Laboratories, Detroit, MI) plus 250 μg of streptomycin per ml as described. Oxoid listeria selective agar (LSA) (Unipath, Ogdensburg, NY) plus 500 μg of streptomycin per ml was used to enumerate *L. innocua* MARC1-S. Clostridium botulinum isolation agar without egg yolk (CBI) *(22)* plus 50 μg of novobiocin per ml was used to enumerate *C. sporogenes* MARC1-N. Using these plating media made it possible to isolate and enumerate each bacterium from fresh feces inoculated with a mixture of all three "marked" bacteria *(10)*.

Prior to the experiment, *E. coli* MARC1-S, *L. innocua* MARC1-S, and *C. sporogenes* MARC1-N were grown separately as described by Dorsa et al. *(10)*. Twenty milliliters of each overnight culture was transferred to separate 50-ml sterile conical centrifuge tubes and centrifuged (Beckman Instruments, Inc., Palo Alto, CA) at 1,690 × *g* for 15 min at 5°C and then resuspended in 20 ml of buffered peptone water (BPW) (BBL, Cockeysville, MD). Appropriate dilutions were made in BPW to yield 6 log CFU/ml of each "marked" bacterium; then 10 ml of each bacterial suspension was mixed and added to 70 ml of BPW. Bovine feces were collected, for each replication, immediately postdefecation from 3 heifers maintained on a hay-silage diet. Thirty-three grams of each fecal sample was combined in a sterile 400-ml beaker along with the 100-ml cell cocktail described above, then hand mixed aseptically by using a sterile spatula for approximately 1 min. This fecal inoculum contained approximately 5 log CFU of each "marked" bacterium per g, yielding a retrievable bacterial population of approximately 4 log CFU/cm^2 from an inoculated 5 by 5 by 1 cm sample, which was excised and stomached in 25 ml of buffered peptone water (BPW) with 0.1% Tween 20 (Fisher Scientific, Pittsburgh, PA) (data not shown).

Inoculation of beef tissue

Beef carcass short plates were taken from cows as described by Dorsa et al. *(11)* at a cow-bull slaughter facility and transported to the lab within 1 h. Beef short plates were used because the predominant surface is lean. Since antimicrobial washes using organic acids and TSP appear to show higher efficacy for bacterial reduction on beef fat surfaces than lean fascia *(7, 8, 19)*, the lean surface of beef short plates was determined to be a worst-case model of a beef-carcass surface. Prior to treatment, seven adjacent areas were marked on the short plates with a sterile 5 by 5 cm template, a sterile cotton swab, and edible ink. The seven marked areas were then inoculated with the fecal inoculum by evenly spreading an adequate volume of the inoculum using a sterile 5.1-cm paint brush so as to cover the entire area. The inoculated plates were allowed to stand undisturbed for 15 min at room temperature (ca. 22°C) before being subjected to the appropriate intervention.

Treatment application

The wash cabinet used for this study was a stainless steel insertable pod of the carcass washer (W.J. Cary Engineering, Inc., Springfield, MO) described by Dorsa et al. *(10)*.

Inoculated short plates were subjected to one of six different spray wash interventions, or left untreated. All washes were applied for 15 s at ca. 5.5 bar (80 ± 5 psi) at 32 ± 2°C. The six spray wash treatments used were 12% (wt/vol) trisodium phosphate (TSP) (Av-Gard@, Rhone-Poulenc, Washington, PA), 1.5% (vol/vol) lactic acid (LA) (Sigma Chemical Co., St. Louis, MO), 3% (vol/vol) LA, 1.5% (vol/vol) acetic acid (AA) (Eastman Chemical, Kingsport, TN), 3% (vol/vol) AA, and tap water (W). Each treatment was replicated ten times.

Tissue sampling

Before and immediately after treatments, samples were taken by aseptically excising a randomly chosen premarked 5 by 5 cm tissue section approximately 1 mm deep. The treated short plates were then placed on UV-sterilized plastic trays or tubs and covered with a polyurethane bag as described by Dorsa et al. *(10)*. Short plates on trays were placed into a 5°C incubator for 48 h; then a third 25-cm^2 area was excised for bacterial enumeration. The remaining four marked areas were excised, placed into Stomacher bags (Sterifil, Spiral Biotech, Bethesda, MD) and vacuum sealed as described by Dorsa et al. *(10)*. These samples were stored at 5°C and removed for sampling at 7, 14, and 21 days. One sample from each short plate was used throughout the study to obtain surface pH values by using a flat surface combination probe (Corning model 245, Corning, Inc., Corning, NY). The samples were opened, then vacuum-packed and stored at 5°C each time pH values were obtained.

Bacterial enumeration

At each sampling day, 25 ml of BPW plus 0.1% Tween 20 was added to excised samples in filter stomacher bags and pummeled for 2 min with a Model 400 Stomacher (Tekmar, Inc., Cincinnati, OH). One milliliter of stomachate was used to make suitable sample dilutions in BPW. Samples from the first six replications were spiral plated in duplicate with a Model D spiral plater (Spiral Systems Instruments, Bethesda, MD) on the appropriate medium. In order to increase detection sensitivity for *E. coli* MARC1-S and *L. innocua* MARC1-S from the last four replications, samples used for the detection of these two bacteria were spread plated by using 1 ml of appropriately diluted sample divided evenly over 4 separate plates of the appropriate medium. Total mesophilic aerobic bacteria (APC) were enumerated on Trypticase soy agar (TSA) (BBL), pseudomonads on pseudomonas isolation agar (PIA) (Difco), and lactic acid bacteria (LAB) on Bacto lactobacilli MRS agar (BBL). The marked strains of *E. coli* MARC1-S, *L. innocua* MARC1-S, and *C. sporogenes* MARC1-N were enumerated on appropriate selective plate media and incubated as described previously *(10)*. The number of CFU/cm^2 was calculated using a digital counter or a CASBA IV computer-assisted colony image analyzer (Spiral Biotech, Inc., Bethesda, MD).

Calculations and data analysis

Plate counts were converted to log CFU/cm^2. To facilitate statistical analysis of the counts in logarithms, any 0-count plate was assigned a value of 20 CFU/cm^2 based on the lowest limit of detection for the spiral plate counting method. Least squares means (LSM) and population reduction data were analyzed using the general linear model procedure (GLM) of SAS (SAS Institute, Cary, NC) with a probability level of 0.05 used as the level of significance.

RESULTS

All wash treatments were able to physically remove visible fecal contamination from the inoculated areas of the beef tissue. However, it was observed that on a few occasions the inoculated areas were left slightly stained or with small pieces of vegetable material. The resulting surface pH values of the beef tissues after all of the treatment washes and of the untreated control, including the 21-day refrigerated storage period, are reported in Table 1.

The initial aerobic bacteria inoculation level of ca. 5.6 log CFU/cm^2 was significantly reduced ($P < 0.05$) by 1.3 to 2.0 log CFU/cm^2 by all wash treatments (Figure 1). The aerobic bacteria on beef surfaces treated with water began logarithmic growth within the first 2 days of cold storage and by 7 days had attained a population of ca. 7 log CFU/cm^2 (Figure 1). All acid wash treatments inhibited growth for the first 2 days of cold storage and suppressed growth for the duration of the experiment. By 21 days aerobic bacteria on acid-treated tissue had achieved a population of ca. 5.5 log CFU/cm^2, significantly less ($P < 0.05$) than the untreated controls or W or TSP treatments (Figure 1).

The numbers of *E. coli* O157:H7 present in bovine feces were initially reduced by 1.8 log CFU/cm^2 when W treatments were applied (Figure 2). The bacterium began growth within the first 2 days of cold storage, entering a stationary phase about day 7 at 4.6 log CFU/cm^2. The final population density at 21 days, 4.5 log CFU/cm^2, for the W treatment was significantly lower ($P > 0.05$) than the untreated control, 5.8 log CFU/cm^2. For the first six replications, all acid and TSP treatments reduced initial inoculum levels of 4 log CFU/cm^2 to below detectable levels of 1.3 log CFU/cm^2. With a more sensitive plating method used to enumerate the last four replications, LA at both concentrations and TSP reduced *E. coli* O157:H7 to below 1.3 log CFU/cm^2, with several of the replications indicating no *E. coli* O157:H7 present. During the 21-day storage period, *E. coli* O157:H7 remained at or below 2 log CFU/cm^2 after organic acid or TSP treatments and below 0.8 log CFU/cm^2 for both LA treatment concentrations. Three percent LA was the most effective treatment, indicated by the mean *E. coli* O157:H7 population of 0.1 log CFU/cm^2 from the last four replications after 21 days of refrigeration storage.

W treatments were initially able to reduce inoculum levels of *L. innocua* by approximately 2.0 log CFU/cm^2 (Figure 3) immediately after washing. The bacterium began growth within the first two days of cold storage and continued throughout the 21-day storage period. By day 14, the population density of *L. innocua* on the beef tissue was not significantly different ($P > 0.05$) from that of the untreated control.

TABLE 1. *The average pH of beef tissue surfaces under various treatments during 21-day storage at 5°C*

	pH						
	Treatment[a]						
Sample day	None	Water	TSP	1.5% LA	3.0% LA	1.5% AA	3.0% AA
Wash solution[b]	NA[b]	7.34	12.31	2.44	2.27	2.82	2.69
0[c]	6.94	6.87	11.42	3.38	3.13	4.24	3.86
2	6.86	6.18	7.58	4.83	4.29	5.32	5.07
7	6.12	6.13	6.89	5.26	4.87	5.40	5.18
14	5.99	6.09	6.75	5.34	5.09	5.44	5.24
21	5.93	6.12	6.71	5.38	5.04	5.49	5.09

[a] TSP, trisodium phosphate; LA, lactic acid; AA, acetic acid.
[b] pH of wash solutions used for intervention treatments. NA, not applicable.
[c] pH for day 0 taken from beef surfaces immediately after wash treatments.

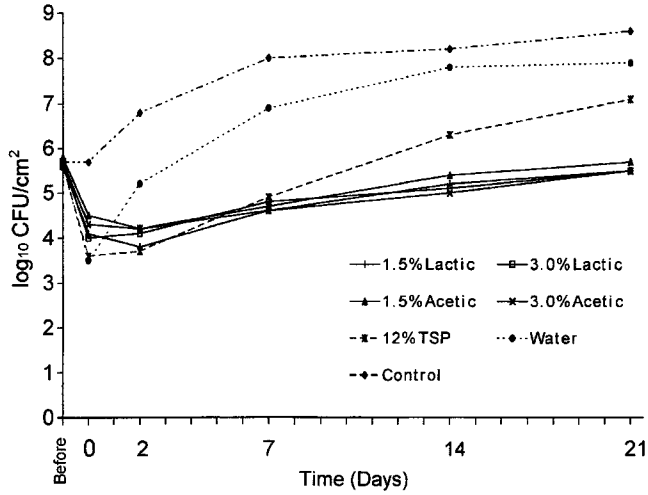

FIGURE 1. *Effects of antimicrobial agents and water wash interventions on beef surface tissue on the initial numbers immediately after treatments (time 0) and subsequent outgrowth of mesophilic aerobic bacterial populations (least squares means log CFU/cm^2:* n = *10) during storage at 5°C aerobically for 2 days; then vacuum packaged and stored for an additional 19 days.*

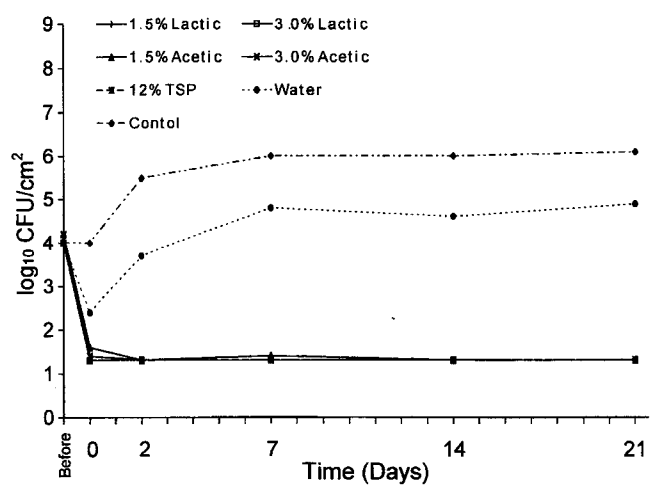

FIGURE 2. *Effects of antimicrobial agents and water wash interventions on beef surface tissue for the first six replications, on the initial numbers immediately after treatments (time 0) and subsequent outgrowth and/or survival of* Escherichia coli *O157:H7 (least squares means log CFU/cm^2;* n *=6) during storage at 5°C aerobically for 2 days; then vacuum packaged and stored for an additional 19 days.*

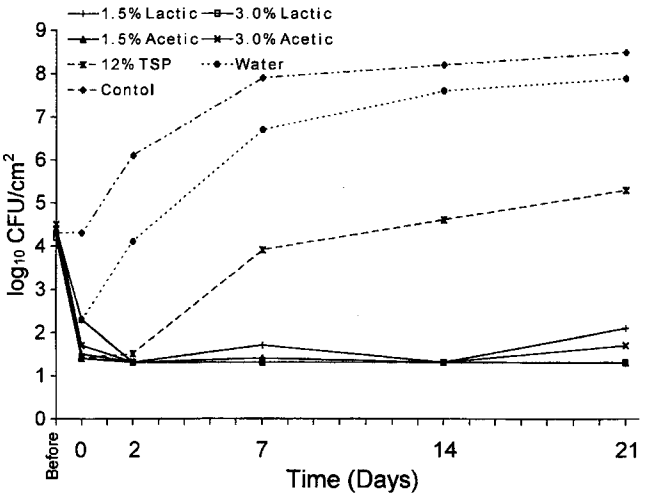

FIGURE 3. *Effects of antimicrobial agents and water wash interventions on beef surface tissue for the first six replications, on the initial numbers immediately after treatments (time 0) and subsequent outgrowth of* Listeria innocua *(least squares means log CFU/cm²; n = 6) during storage at 5°C aerobically for 2 days; then vacuum packaged and stored for an additional 19 days.*

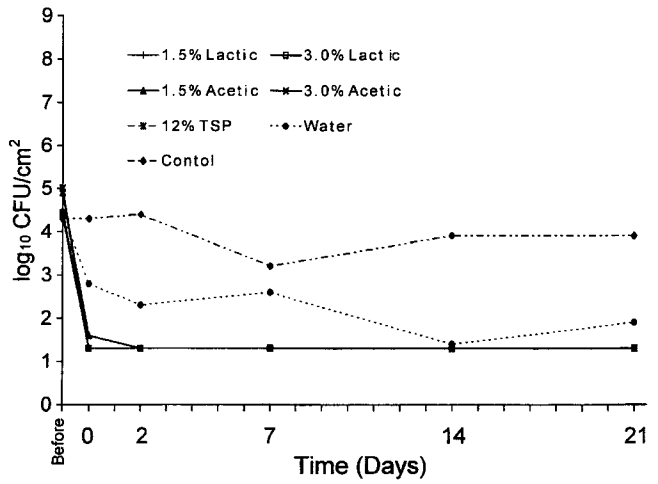

FIGURE 4. *Effects of antimicrobial agents and water wash interventions on beef surface tissue on the initial numbers immediately after treatments (time 0) and subsequent survival of* Clostridium sporogenes *(least squares means log CFU/cm²; n = 6) during storage at 5°C aerobically for 2 days; then vacuum packaged and stored for an additional 19 days.*

The first six replications indicated that LA treatments, regardless of concentration, initially reduced the level of *L. innocua* by 2.8 log CFU/cm² (Figure 3). The final four replications, in which a more sensitive plating method was used, demonstrated that the initial reduction was actually as high as 4 log CFU/cm². Additionally, the bacterium was not detectable in several replications of the 3.0% LA treatment. While AA treatments appeared to be somewhat less effective, there was no significant reduction difference ($P > 0.05$) in the numbers of *L. innocua* for the first six replications. By day two of cold storage the bacterium was not detectable (<1.3 log CFU/cm²) on beef carcass surfaces treated with any of the acid washes in the first six replications. The last four replications demonstrated that indeed the bacterium had been reduced in numbers of viable CFU/cm² to <0.3 log CFU/cm² for LA and to undetectable levels for both concentrations of AA. By day 21 of cold storage, *L. innocua* was recoverable during the first six replications from beef surfaces treated with 1.5% LA; however, at a significantly lower level ($P < 0.0001$) than that of the untreated control (Figure 3). However, in the last four replications the bacterium was present only at levels ≤0.2 log CFU/cm² and was not detectable from the 3.0% LA treated samples.

Treatment with TSP reduced initial inoculum levels of *L. innocua* equally as well as LA and AA (Figure 3). However, *L. innocua* was never reduced in numbers to below 1.3 log CFU/cm² as was the case with both acids. By day 7 of cold storage, the bacterium exhibited growth that continued throughout the 21-day study period (Figure 3). The eventual population of *L. innocua* on beef tissue treated with TSP was significantly less ($P < 0.0001$) than that of either the untreated control or the W treated samples (Figure 3) for the first six replications. However by 14 days during the last four replications there was no significant difference in numbers of *L. innocua* between the untreated controls and TSP or W treatments.

AA, LA, and TSP treatments all reduced vegetative cells of *C. sporogenes* inoculated on beef carcass tissue to 1.6 log CFU/cm² or below detectable levels of 1.3 log CFU/cm² (Figure 4). The bacterium remained undetectable for the 21-day study period. The W treatment demonstrated the efficacy of a water wash to physically remove 1.6 log CFU/cm² of the bacterium, but did not reduce this bacterium to less than detectable levels.

While pseudomonads were initially present at very low levels (1.5 log CFU/cm²), substantial growth occurred on the untreated controls and the W treated samples by 21 days (Figure 5). However, all acid and TSP treatments suppressed counts to below detectable limits (1.3 log CFU/cm²) for the 21-day study period. For lactic acid bacteria, the various

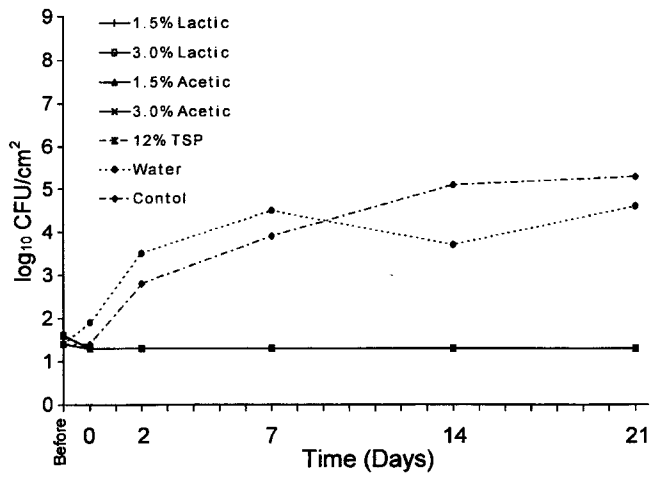

FIGURE 5. *Effects of antimicrobial agents and water wash interventions on beef surface tissue on the initial numbers immediately after treatments (time 0) and subsequent outgrowth and/or survival of pseudomonads (least squares means log CFU/cm²; n = 6) during storage at 5°C aerobically for 2 days; then vacuum packaged and stored for an additional 19 days.*

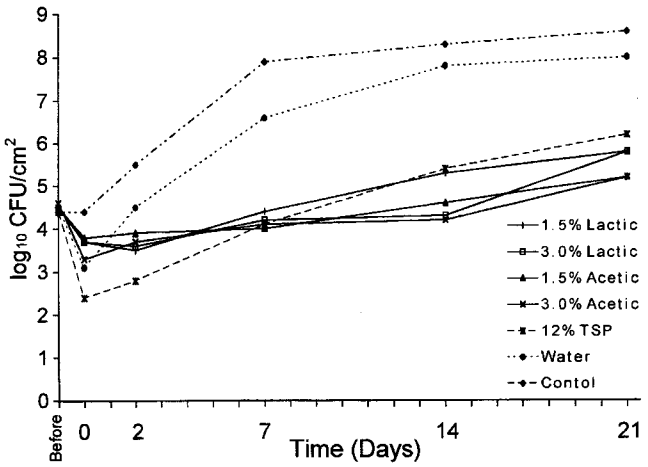

FIGURE 6. *Effects of antimicrobial agents and water wash interventions on beef surface tissue on the initial numbers immediately after treatments (time 0) and subsequent outgrowth of lactic acid bacteria (least squares means log CFU/cm²; n = 6) during storage at 5°C aerobically for 2 days; then vacuum packaged and stored for an additional 19 days.*

wash treatments all demonstrated significant ($P < 0.05$) initial reductions (Figure 6); however, regardless of treatment, this group of bacteria grew throughout the 21-day refrigeration storage period. LA, AA, and TSP treatments on beef carcass surfaces significantly suppressed ($P > 0.05$) lactic acid bacterial growth when compared to the control and W treatments (Figure 6).

DISCUSSION

None of the antimicrobial agents incorporated into wash treatments during the present study was able to produce better initial reductions of aerobic bacteria than water. This observation was not unexpected since the initial effectiveness of water and organic acids as washes for removal of various bacteria from beef carcasses has been shown to be similar in other studies *(7, 17)*. However, growth suppression of aerobic bacteria for the 21-day refrigerated storage period was significantly greater ($P < 0.05$) on beef carcass tissue when any of the antimicrobial agents were part of the wash treatment. Prasai et al. *(21)* and Kenney et al. *(18)* observed that the mean APCs of acid-treated carcasses were significantly ($P < 0.05$) lower than those of the control carcasses for 72 h. TSP, to a lesser extent than any of the organic acid treatments, inhibited but did not eliminate the growth of aerobic mesophilic bacteria.

TSP reduction and suppression of *E. coli* O157:H7 during the present study support earlier findings of Kim and Slavik *(19)*, who noted that TSP is an effective reducer of the bacterium from beef surfaces.

The initial reductions of *E. coli* O157:H7 observed in the present study by LA and AA washes reflect those reported by Hardin et al. *(17)* who also used industrial spray wash parameters to reduce *E. coli* O157:H7 in an unaltered bovine fecal inoculum from prerigor beef carcass surface tissue. In that study, initial inoculation levels of 5 log CFU/cm² were reduced to below minimum detectable levels of 0.5 log CFU/cm². Contrary to these observations, Brackett et al. *(5)*, using acetic, citric, and lactic acid sprays at various temperatures on cut beef surfaces, found no effectiveness of these acids for the reduction of *E. coli* O157:H7. Since it has been well documented that *E. coli* O157:H7 exhibits an acid tolerance *(4, 6)*, the former observation might not be unexpected. However, that study *(5)* was conducted on cut beef surfaces inoculated with pure *E. coli* O157:H7 in a liquid medium, and acid sprays were applied as straight down light mists. The beef industry presently applies acid sprays to a vertically hanging carcass at 1.4 bar (20 psi) after subjecting the carcass to a high-pressure water spray of 8.6 to 27.6 bar (125 to 400 psi).

Also a pH below 5.05 was not observed on the cut meat surfaces using the misting application method employed during the Bracket et al. *(5)* study. The pH observed from beef carcass surfaces during the present study was consistently lower than those observed by Bracket et al. *(5)*, with a low of pH 3.13 for lactic acid and pH 3.86 for acetic acid (Table 1), being only slightly higher than those observed by Hardin et al. *(17)*. The resulting low pH of the beef surface and larger volumes of acid presented to the bacterium during the present study and elsewhere *(17)* probably played an important role in the different results obtained. It is known that internal pH homeostasis plays a vital role in the survival of any bacterium *(20)*. Consequently, the inability of *E. coli* O157:H7 to sustain the removal of protons from the cytoplasm contributed by copious volumes of acid, would lead to acidification of cytoplasmic components resulting in growth inhibition and death *(4)*.

The initial reductions of *L. innocua* by LA and AA observed during the present study have been noted previously *(27)*. Van Netten et al. *(27)* found LA effective for the reduction of *L. monocytogenes* from a cut meat surface, and that the acid exhibits an increase in effectiveness as it is applied with a decrease in pH and increase of temperature. Ahamad and Marth *(1)* reported that both LA and AA in concentrations as low as 0.1%, when incorporated into tryptose broth, would inhibit the growth of *L. monocytogenes* and that the degree of inhibition increased as the temperature of incubation decreased. This effect is also supported by El-Khateib et al. *(13)* who found 2% LA on meat surfaces had an immediate and also a delayed listericidal action during 48 h of refrigerated storage. The present study demonstrated some bactericidal effect of LA and AA and an ability to inhibit growth for at least 21 days on beef carcass surface tissue held under refrigerated storage.

The present study demonstrated the growth of *L. innocua* on beef carcass tissue that was stored at 5°C for 21 days after being treated with a TSP wash. While the combined means of all replications indicated that the growth of *L. innocua* was suppressed with the use of TSP when compared to the control and W treatment, this was not always the case among individual replications. Other *Listeria* spp., like *L. monocytogenes*, have been shown to be more resistant to the effects of TSP than *E. coli* O157:H7, *Salmonella typhimurium*, and *Campylobacter jejuni (25)*.

The ability of LA and AA washes to physically remove the bacterium, coupled with the rapid reduction in pH (Table

1), are probably major contributing factors for reduction of *C. sporogenes* by these treatments. Thylin et al. *(26)* showed, using a similar clostridial strain common in silage, that the two most important factors contributing to the suppression of clostridial growth were the rapid introduction of lactic acid and the resulting rapid decrease of pH.

Anderson and Marshall *(3)* found that increasing the application temperature of AA will increase the effectiveness of the acid's ability to reduce *E. coli* from meat. It appears that the combination of inoculation menstruum, acid temperature, volume, and application method along with beef tissue surface type play important roles in the effectiveness of organic acid spray washes. The large differences among the results of the many laboratory studies and the actual industrial practices of applying organic acids and/or antimicrobial agents indicate the inappropriateness of extrapolating. Numbers of viable, recoverable, *E. coli* O157:H7, *L. innocua,* and *C. sporogenes* were reduced to <1.3 log CFU/cm^2 and, on some occasions, to below detectable levels when any of the antimicrobial agents were applied using commercially feasible parameters. Numbers remained at this low level for the entire 21-day cold storage period with the exception of *L. innocua* after TSP treatments. The incorporation of any of these antimicrobial agents into a commercial wash would result in improved product safety, but until the effects of post-treatment cross contamination are studied, the efficacy of antimicrobial spray washes on beef carcasses will not be fully understood.

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