

Effects of Steam-Vacuuming and Hot Water Spray Wash on the Microflora of Refrigerated Beef Carcass Surface Tissue Inoculated with *Escherichia coli* O157:H7, *Listeria innocua*, and *Clostridium sporogenes*[†]

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ABSTRACT

The fates of several bacterial populations on beef carcass surfaces were examined immediately following hot water washes (W) delivered through a beef carcass wash cabinet or application of steam-vacuum (SV). Additionally, the long-range effectiveness of W and SV on several bacterial populations was also determined during storage up to 21 days at 5°C under vacuum-packaged conditions. Fresh, unaltered bovine feces spiked with antibiotic-resistant strains of *Escherichia coli* O157:H7, *Listeria innocua*, and *Clostridium sporogenes* were used to inoculate beef carcass tissue prior to W or SV treatment. All treatments were equally effective as is indicated by bacterial populations immediately following any of the treatments ($P > 0.05$); however, the combination of SV followed by W consistently produced arithmetically greater bacterial reductions. In general, all treatments produced initial reductions of up to 2.7 log CFU/cm² for APC, lactic acid bacteria, and *L. innocua*, but by 14 days bacterial numbers had increased to levels of at least 7 log CFU/cm². *E. coli* O157:H7 was initially reduced by as much as 3.4 log CFU/cm² and did not grow to original inoculation levels for the duration of the experiment. Vegetative counts of *C. sporogenes* were initially reduced by as much as 3.4 log CFU/cm², and numbers continued to decline for the duration of the study. These results indicate that the use of W and SV effectively reduces bacterial populations from beef carcass tissue immediately after treatment. Additionally, storage of treated tissue up to 21 days at 5°C did not appear to offer any competitive advantage to potentially pathogenic microorganisms.

Key words: Beef, steam-vacuum, hot water washes, *Escherichia coli* O157:H7, *Listeria innocua*, *Clostridium sporogenes*

As part of an effort to reduce the occurrence and

numbers of pathogenic microorganisms on beef products, the U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) is presently proposing substantial changes in beef slaughter facility process requirements (3). One of the effects of these changes is that beef processors are looking for new and innovative antimicrobial treatments to incorporate into their slaughter process prior to chilling of beef carcasses.

Hot water washes (>70°C) have been evaluated and determined superior to ambient water washes for reducing general bacterial populations, *E. coli*, and salmonellae on beef carcasses (2, 4, 5). Dorsa et al. (5) evaluated the effects of steam and a commercially available steam-vacuum sanitizer as well as hot water washes administered through a commercial carcass washer. They concluded that moist heat interventions were effective for reducing aerobic bacterial populations as well as *E. coli* and coliforms on beef and sheep carcasses, regardless of the application method.

The effect of thermal inactivation of specific pathogens within the genera *Escherichia*, *Listeria*, and *Clostridium* found in food and beef products (ground beef), is well documented (7, 13, 14). However, the effects of thermal inactivation followed by storage at refrigerated temperatures has been investigated to a lesser extent (8). To our knowledge, no investigations have considered the long-term effect that using moist heat to decontaminate beef carcasses has on the remaining microbial populations. The objective of the present study was to determine the effect of two types of moist heat interventions, hot water wash and steam-vacuum, on several bacterial populations associated with beef carcass tissue handled and stored under ordinary industrial practices.

MATERIALS AND METHODS

Inoculum

Bacteria were marked using isolation methods for antibiotic-resistant mutants as described by Eisenstadt et al. (6). *E. coli* O157:H7 CDC B6-914 (*E. coli* MARC1-S) and *L. innocua* ATCC 33090 (*L. innocua* MARC1-S) were resistant to streptomycin

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† 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.

(Sigma Chemical Co., St. Louis, MO) at levels of 250 and 500 µg/ml, respectively. *C. sporogenes* ATCC 11437 (*C. sporogenes* MARC1-N) was resistant to novobiocin at 50 µg/ml. All stock cultures were cultured in antibiotic media then suspended in glycerol and stored at -20°C for use during the study.

E. coli MARC1-S was plated on sorbitol McConkey agar (SMAC; Difco Laboratories, Detroit, MI) plus 250 µg/ml streptomycin. While it has been shown that SMAC may fail to adequately detect sublethally heat injured cells (1), preliminary trials indicated minimal reductions in recovery of this marked strain occurred when heated in a fecal menstruum (data not presented). Oxoid Listeria selective agar (LSA; Unipath, Ogdensburg, NY) plus 500 µg/ml streptomycin was used to enumerate *L. innocua* MARC1-S, and Clostridium botulinum isolation agar without egg yolk (CBI) (15) plus 50 µg/ml novobiocin was used to enumerate *C. sporogenes* MARC1-N. Incorporating these antibiotics into the appropriate medium made it possible to isolate and enumerate each specific bacterium from fresh feces inoculated with a mix of all three marked bacteria (data not presented). As with SMAC, preliminary trials indicated minimal reductions in recovery of *L. innocua* MARC1-S and *C. sporogenes* MARC1-N on the appropriate selective media occurred when heated in a fecal menstruum (data not presented).

Prior to the experiment, *E. coli* MARC1-S and *L. innocua* MARC1-S were removed from -20°C storage, then grown overnight (ca. 18 h) quiescently at 35°C in tryptic soy broth plus 1.0% yeast extract (TSBYE; Difco Laboratories, Detroit, MI). *C. sporogenes* MARC1-N was removed from -20°C storage, then grown overnight quiescently at 35°C in Schaedler broth plus 0.05% (wt/vol) sodium thioglycollate and 0.1% (wt/vol) resazurin (BBL, Cockeysville, MD). Twenty milliliters of each overnight culture was transferred to separate 50-ml sterile conical centrifuge tubes and centrifuged at 2,800 × g for 15 min at 5°C (model GPKR Beckman Instruments, Inc., Palo Alto, CA) then resuspended in 20 ml buffered peptone water (BPW; BBL). Ten milliliters of each bacterial suspension (*E. coli* O157:H7, *L. innocua*, and *C. sporogenes*) was mixed and added to 70 ml of BPW.

Bovine feces were collected, for each replication, immediately post-defecation from three 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 mixed aseptically by hand for approximately 1 min. This fecal inoculum contained approximately 7 log CFU/g of each marked bacterium yielding a retrievable bacterial population of approximately 6 log CFU/cm² from an inoculated sample (5 by 5 by 1 cm) excised and stomached in 25 ml of BPW with 0.1% Tween 20 (Fisher Scientific, Pittsburgh, PA) (data not shown).

Inoculation of beef tissue

Beef carcass short plates were taken from 24 cow carcasses as described by Dorsa et al. (5) at a cow/bull slaughter facility and transported to Roman L. Hruska U.S. Meat Animal Research Center (Clay Center, NE) within 1 h post-exsanguination. Prior to treatment, seven adjacent areas were marked on each short plate with a sterile template (5 by 5 cm), a sterile cotton swab, and edible ink. The seven marked areas were then inoculated with the fecal inoculum using a sterile 5.1-cm paintbrush. The inoculated short plates were allowed to stand undisturbed for 15 min before being subjected to the appropriate intervention.

Wash cabinet and steam-vacuum

The wash cabinet used for this study was a stainless steel insertable pod of the commercial carcass washer described by Dorsa et al. (5) (W. J. Cary Engineering, Inc., Springfield, MO) and

housed at Roman L. Hruska U.S. Meat Animal Research Center. The external dimensions of the pod are 77 by 61 by 32 cm. This size pod was designed to fit into a biological safety hood (Model S6-400, Baker Corp., Inc., Sanford, ME) for the application of various washes to beef short plates inoculated with pathogens. The single spray bar contained three nozzles set 13 cm apart and 10 cm from the meat surface. The nozzles were of medium-capacity elliptical orifice design (Spraying Systems Co., Wheaton, IL), designed to deliver 1 gallon (3.8 liters) of water per min as a 45° angle flat spray at 40 lb/in² (270 kPa). All other physical parameters were set to parallel those used in previous research involving the commercial carcass washer (5). A thermocouple (type T; Omega Engineering, Inc., Stamford, CT) attached in the spray line, approximately 90 cm upstream of the nozzles, was used to monitor water temperature of 74 ± 2°C for the hot water and 33 ± 1°C for warm water treatments. A bleeder valve was placed downstream of the thermocouple to allow water to continually flow through the lines between treatments. Pretrials with this system indicated 74°C at the nozzle would yield a maximum, consistent temperature of 70°C at the carcass surface (data not presented), and 70°C at the carcass surface is achievable by commercial hot water wash systems.

The steam-vacuum and its preset parameters used for this study, trade name Vac-San (Kentmaster Mfg., Monrovia, CA), were described in detail by Dorsa et al. (5).

Experimental design

Inoculated short plates were subjected to 1 of 3 different interventions, or left untreated. The three treatments used were (i) steam-vacuum (SV), (ii) hot water spray wash of 74 ± 2°C sprayed at 20 lb/in² followed by a 30 ± 1°C sprayed at 125 lb/in² (W), and (iii) the combination of the two interventions, SV followed by W (SV + W). Three even passes of the Vac-San nozzle (nozzle opening 10.5 by 1.5 cm), lasting about 1 s per pass, were used for the SV and SV + W treatments while spray washes of 15 s per wash temperature were used for W and SV + W. Each treatment was replicated two times per sampling day on three separate days for a total of six replications.

Tissue sampling

Immediately after treatments, samples were taken by aseptically excising the premarked 5-cm × 5-cm tissue section (5 by 5 cm and approximately 1 mm thick). A premarked 25-cm² area of each inoculated short plate was sampled prior to and immediately after intervention treatments. The treated short plates were then placed on UV-sterilized plastic trays or tubs and covered with a polyurethane bag which was loosely tented around each short plate and tray so as to not touch the marked areas of the tissue. The short plates were then placed into a 5°C incubator for 48 h. After 48 h, the bag was removed and a third 25-cm² area was excised for bacterial enumeration. The remaining four marked areas were excised and individually placed into separate stomacher bags (Sterifil, Spiral Biotech, Bethesda, MD), which were inserted into vacuum bags and vacuum sealed (>26 in mm Hg [>3.5 kPa]; Vacusave, Tilia, Inc., San Francisco, CA) according to the manufacturer's instructions. The oxygen transmission rate of the nylon/olefin/polyethylene bags was less than 2.6 cm³/100 in²/24 h at 25°C and 0% relative humidity; moisture vapor transmission was less than 0.7 g/100 in²/24 h at 100°C and 90% relative humidity. 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 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

Each day of sampling, 25 ml 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). From the filtered stomachate, suitable sample dilutions were made in BPW and spiral plated in duplicate using a Model D spiral plater (Spiral Systems Instruments, Bethesda, MD) on the appropriate medium. Total mesophilic aerobic bacteria (APC) were enumerated on Trypticase soy agar (TSA; BBL), pseudomonads on Pseudomonas isolation agar (PIA; Difco Laboratories, Detroit, MI), and lactic acid bacteria (LAB) on Bacto Lactobacilli agar (MRS; BBL). The marked strains of *E. coli* MARC1-S, *L. innocua* MARC1-S, and *C. sporogenes* MARC1-N were enumerated on media as described above. Typical *E. coli* O157:H7 isolates present on the plates were randomly selected and confirmed serologically (*E. coli* O157 Test kit, Unipath Ltd., Basingstoke, Hampshire, UK). TSA, SMAC, LSA, and PIA plates were incubated for 18 to 24 h aerobically at 37°C. CBI plates were incubated anaerobically for 36 to 48 h in GasPak jars with GasPak Anaerobic Systems (BBL) at 37°C. MRS was incubated in 5% CO₂ in a Cellstar incubator (Lab Research Products, Inc., Lincoln, NE) for 36 to 48 h at 35°C. Number of colony forming units (CFU) per square centimeter was calculated using a digital counter or a CASBA IV computer-assisted colony image analyzer (Spiral Biotech, Inc., Bethesda, MD).

Lactic acid analysis

One-milliliter portions of stomachate from each meat sample were filter sterilized using a 0.45- μ m sterile Acrodisc syringe filter (Gelman Corp., Bedford, MA) before analysis. Lactic acid content (mM) was determined by high-performance liquid chromatography (HPLC) analysis as described by Varel and Pond (18).

Calculations and data analysis

The mean of duplicate plate counts were converted to log CFU/cm². To facilitate log analysis any plate with a zero count was assigned a value of 20 based on the lowest limit of detection for the spiral plate counting method. Least squares means (LSMs) and population reduction data were analyzed using the general linear model (GLM) procedure of SAS (SAS Institute, Cary, NC) with a probability level of 0.05 used as the level of significance.

RESULTS AND DISCUSSION

All treatment interventions were effective for reducing both APC and specific target bacteria except in the case of pseudomonads, which started at a low level and remained that way after treatments (Figs. 1–6). Immediate reductions were expected, and the efficacy of using these intervention strategies, in particular SV + W, for the reduction of APC as well as *E. coli* and coliforms has been previously documented by the authors (5).

The initial reductions for APCs of 1.6, 2.1, and 2.6 log CFU/cm² for SV, W, and SV + W, respectively, were slightly lower than APC reductions observed by these authors in a previous study (5). This observation might be explained by variations in microflora types in the fecal inocula and variations in equipment settings. However, in both studies, initial reductions were significant ($P < 0.05$), regardless of

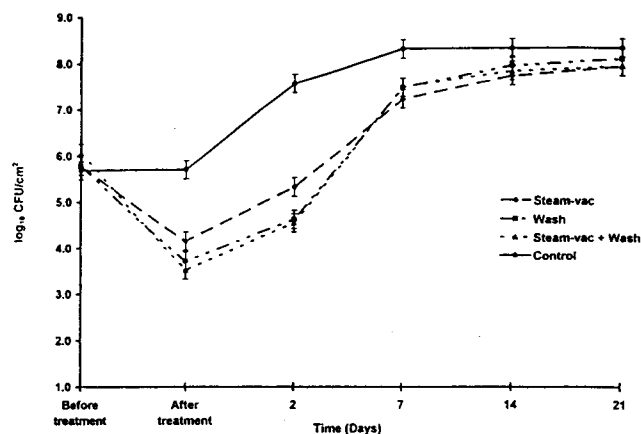


FIGURE 1. Effects of moist heat interventions on the initial levels and subsequent outgrowth of mesophilic aerobic bacterial populations (least squares means, log CFU/cm²; error bars denote standard error of the mean) during a combination of aerobic and vacuum storage at 5°C for 21 days.

the moist heat intervention used. During the present study there was no significant difference between treatment types observed for APC reductions.

Initial reductions of 2.0, 2.5, and 2.6 log CFU/cm² for *L. innocua* MARC1-S and 2.0, 2.2, and 2.7 log CFU/cm² for LAB, were observed after treatments of SV, W, and SV + W, respectively. SV and W were equally as effective when used alone, but SV produced significantly lower initial reductions of *L. innocua* MARC1-S and LAB than those observed for the combination treatment of SV + W.

Population curves for APC, *L. innocua* MARC1-S and LAB are presented in Figures 1 through 3. For all three bacterial groups, growth began within 2 days of all treatments and had reached 7 log CFU/cm² by 7 days. Growth continued for the duration of the study and was equivalent to the untreated control by day 21. As in other studies (2, 4, 5, 16) the reductions in total APC immediately after the moist heat treatments to red meat carcasses indicates an efficacy as

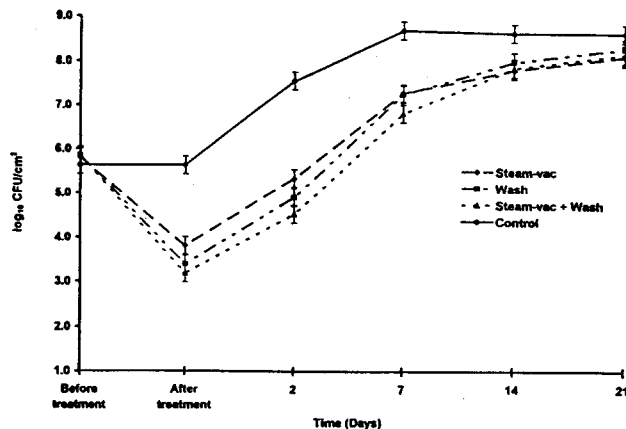


FIGURE 2. Effects of moist heat interventions on the initial levels and subsequent outgrowth of *Listeria innocua* (least squares means, log CFU/cm²; error bars denote standard error of the mean) during a combination of aerobic and vacuum storage at 5°C for 21 days.

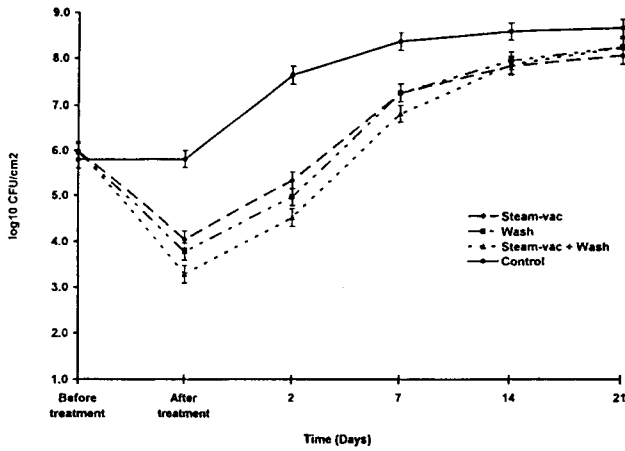


FIGURE 3. Effects of moist heat interventions on the initial levels and subsequent outgrowth of lactic acid bacteria (least squares means, log CFU/cm²; error bars denote standard error of the mean) during a combination of aerobic and vacuum storage at 5°C for 21 days.

an effective antimicrobial treatment. However, when the carcass tissue is allowed to chill at 5°C for 48 h then cut and vacuum packaged for 21 days storage at 5°C, as would occur to a beef carcass in a slaughter/fabrication facility, the use of APC to indicate effectiveness of these treatments indicated that hot water as a comprehensive antimicrobial treatment would be of little value when compared to untreated beef tissue. The growth rate of *L. innocua* (Fig. 2) observed in the present study might also suggest that the use of hot water sprays to eliminate other *Listeria* spp. such as *L. monocytogenes*, if initially present in high numbers, would not be adequate as a single intervention when regarding food safety. However, *L. monocytogenes* has been shown to typically be present at much lower levels on beef carcasses (9) than the worst-case inoculation scenario used during the present study. While lower initial inoculation levels might result in total elimination of the bacterium by these moist

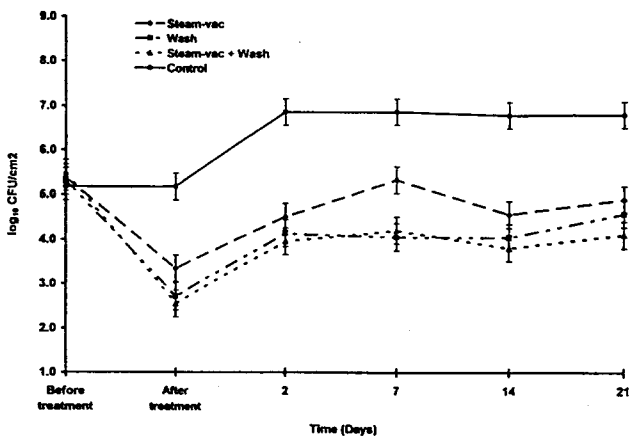


FIGURE 4. Effects of moist heat interventions on the initial levels and subsequent outgrowth/survival of *Escherichia coli* O157:H7 (least squares means, log CFU/cm²; error bars denote standard error of the mean) during a combination of aerobic and vacuum storage at 5°C for 21 days.

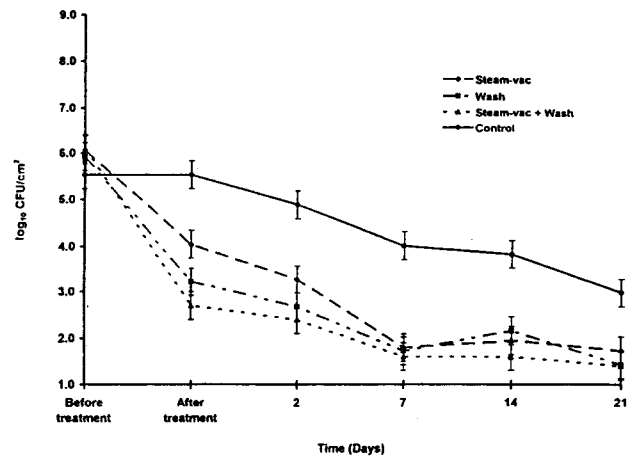


FIGURE 5. Effects of moist heat interventions on the initial levels and subsequent survival of *Clostridium sporogenes* (least squares means, log CFU/cm²; error bars denote standard error of the mean) during a combination of aerobic and vacuum storage at 5°C for 21 days.

heat treatments, the present study did not attempt to address this issue.

Significant initial reductions of 2.1, 2.6, and 3.0 log CFU/cm² for *E. coli* MARC1-S were initially obtained after application of the SV, W, and SV + W, respectively. SV alone produced significantly lower initial reductions of *E. coli* MARC1-S than those observed for the W and SV + W. These reductions are comparable to the log reductions for *E. coli* observed by Smith and Graham (16) when they applied 80°C water for 10 s on sheep carcasses. Hot water treatments of beef carcass tissue previously reported (4, 5) also reduced *E. coli* to levels similar to those in the present study, namely to levels of 3.0 and 3.4 log CFU/cm².

The population curves observed for *E. coli* O157:H7 were very different than for other bacterial groups observed in the present study (Fig. 4). The growth increases of *E. coli* MARC1-S treated with SV, W, and SV + W were 1.2, 1.4,

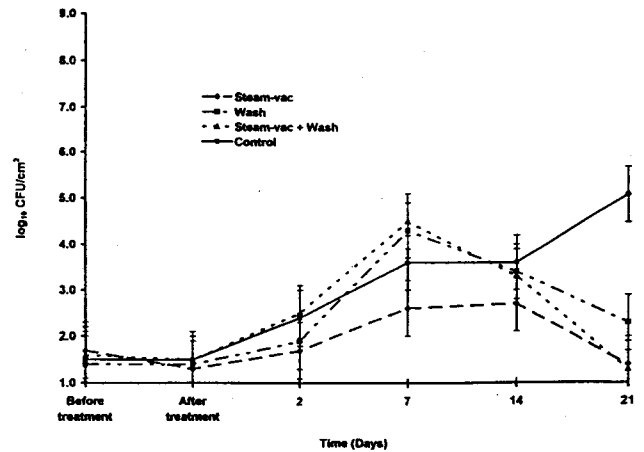


FIGURE 6. Effects of moist heat interventions on the initial levels and subsequent outgrowth/survival of pseudomonads (least squares means, log CFU/cm²; error bars denote standard error of the mean) during a combination of aerobic and vacuum storage at 5°C for 21 days.

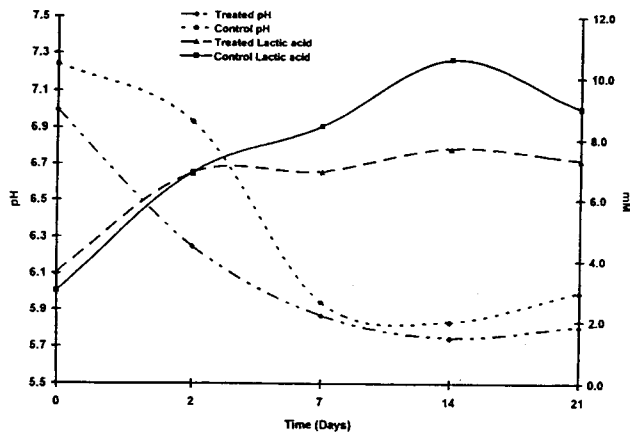


FIGURE 7. Average surface pH ($n = 18$) and lactic acid content (mM; $n = 18$) of sample stomachate from beef tissue of all moist heat interventions immediately after treatment and over time.

and 1.5 log CFU/cm², respectively, after 2 days of aerobic 5°C storage. While no growth of the bacterium is expected at 5°C, it is known that *E. coli* held at a minimal growth temperature of 10°C when shifted to the nongrowth temperature of 7°C will continue to grow for a period of one day during which time the growth rate gradually declines (10). As expected, after the initial 2-day period no significant growth was observed between sampling days 2 and 21. The 21-day levels of 4.9, 4.6, and 4.1 log CFU/cm² for SV, W, and SV + W, respectively, were as much as 1.4 log CFU/cm² lower than the original inoculated level, 5.4 ± 0.1 log CFU/cm². The difference between the untreated populations and populations following SV, W, and SV + W at 21 days were 1.4, 2.2, and 2.7 log CFU/cm², respectively. These results indicate that any of these treatments would offer an additional long-term degree of safety to beef that might be contaminated with *E. coli* O157:H7 via feces during carcass processing.

Vegetative cells of *C. sporogenes* MARC1-N were initially reduced on beef carcass tissue by 2.1, 2.7, and 3.4 log CFU/cm² when treated with SV, W, and SV + W, respectively. *C. sporogenes* populations continued to be reduced over time and were 4.4, 4.5, and 4.7 log CFU/cm² lower after 21 days than the original level of ca. 6.0 log CFU/cm² (Fig. 5). Line et al. (13) observed a similar behavior over a 14-day period for *C. sporogenes* spores in heat-treated deboned turkey meat stored at 4°C.

Initial reductions were a result of heat and physical removal. The remaining *C. sporogenes* population would be comprised of spores and undamaged and sublethally damaged vegetative cells. The initial 2-day storage period was aerobic, thus not conducive to the outgrowth of any sublethally damaged vegetative cells. During the initial 2-day storage period the beef surface allowed exponential growth of lactic acid producing bacteria (Fig. 3), concomitant with a drop in surface pH and an increase in lactic acid (Fig. 7). Lower pH and higher lactic acid concentrations of samples after vacuum packaging probably affected the survival of remaining *C. sporogenes* vegetative cells (17) and by day 7 surviving cell populations remained constant for the duration of the study.

CONCLUSION

As has been shown by previous investigators, hot water washes and steam-vacuum treatments are effective for reducing populations of aerobic bacteria and *E. coli* on beef carcass surfaces. The present study demonstrated that these treatments are also effective for significant initial reductions of *L. innocua*, *C. sporogenes*, and LAB.

It has been suggested that the beef industry is producing such a microbially clean carcass that, due to the lack of competitive inhibition, pathogens are posing greater safety or health risks (11). While *L. innocua* did reach the level of the untreated tissue by day 21 in the present study, it at no time exceeded the level of the untreated tissue. The data presented suggest that the microbial progression was similar for APC and LAB and that no competitive advantage was afforded *E. coli* O157:H7 or *C. sporogenes* when compared to the untreated controls. *E. coli* O157:H7 or *C. sporogenes* were never able to recover to initial inoculation levels. These observations indicate that regardless of the application method the use of moist heat interventions on beef carcass tissue surfaces during processing does not increase the outgrowth potential of selected pathogens in the presence of lower numbers of nonpathogenic bacteria. However, post-processing contamination of moist-heat-treated carcasses will need to be determined.

While moist heat interventions would appear to have no long range benefit for beef products if they are initially contaminated with high levels of *Listeria* spp., these interventions do not create an environment that would pose any additional threat. However, moist heat interventions do appear to add a degree of safety to beef products when *Escherichia* spp. and vegetative *Clostridium* spp. are initially present, even at high levels, on the carcass surface.

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