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Journal of Food Protection, Vol. 53, No. 8, Pages 674-679 (August 1990)
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Surface Moisture and Osmotic Stress as Factors That Affect the Sanitizing of Beef Tissue Surfaces

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(Received for publication December 13, 1989)

ABSTRACT

The use of osmotic stress or desiccation in combination with 2% acetic acid reduced the numbers of *Salmonella typhimurium* or *Listeria monocytogenes* on beef tissue to a greater degree than the acid alone. Bacteria were osmotically stressed by NaCl, $CaCl_2$, or sucrose solutions. Pre-washing with 20% NaCl followed by sanitizing with acetic acid reduced the numbers of *S. typhimurium* an additional 1.5-to 2-log cycles compared with acetic acid alone. Similar reductions were seen by allowing the tissue surfaces to dry before the application of the acid. Desiccation in combination with acetic acid was also effective in reducing the numbers of *Enterobacteriaceae* on inoculated beef carcasses.

Salmonellae and Listeria monocytogenes are human pathogens that have been associated with fresh meat and meat products *(5,17)*. While salmonellae have historically been a concern with fresh meats, the significance of *L. monocytogenes* has only recently been appreciated. Turkey franks were recently implicated as a vehicle in what is apparently the first documented case of foodborne listeriosis associated with meat products *(3)*.

Pathogenic bacteria can contaminate meat at the carcass level in the abattoir. The source of this contamination is predominantly from parts of the animal itself, with the hide and the adhering material being the primary contributors to this contamination *(4)*. Because of this, virtually all the contamination of the meat is confined to the surfaces of the carcass. Although proper slaughtering and dressing procedures are effective in reducing the initial levels of bacteria (4,8), a certain amount of contamination is unavoidable in the process of converting a large live animal into a food product.

Since most meat contamination is initially confined to the surface of the carcass, many proposed treatments for reducing this contamination involve washing and sanitizing the carcasses with a water-based system. Most of the research has involved either chlorine *(12,14)* or short chain organic acids *(1,18)* as sanitizers, with varying degrees of success. However, a problem that has been encountered in this research is that bacteria that are attached to a tissue surface are significantly more resistant to sanitizers than the same bacteria in liquid media *(9,13)*. The exact mechanism of this protective effect is not known, although it has been speculated that the surface moisture is at least partially involved. The objectives of the research presented in this manuscript were to evaluate the role of surface moisture in sanitizing beef tissue surfaces and to improve the effectiveness of acid sanitizers by altering the surface moisture on the tissue surface.

MATERIALS AND METHODS

Bacterial cultures

Cultures of *Salmonella typhimurium* (ATCC 14028) and *Listeria monocytogenes* strain Scott A (FDA, Division of Microbiology, Cincinnati, OH) were grown and maintained in tryptic soy broth (TSB, Difco, Detroit, MI) at 37 and 23°C, respectively. Cultures were transferred to TSB 18 h before use, then harvested by centrifugation (3000 G x 10 min, 5°C). The pellets were suspended in Butterfield's phosphate buffer *(10)*, and 2 ml of the washed cell were diluted into 18 ml of phosphate buffer in sterile beakers, which produced a final concentration of approximately 10^8 colony forming units (CFU)/ml based on plate counts of the inoculum.

Tissue preparation

Post rigor lean and fat tissue was obtained from boneless boxed beef prepared in the abattoir at the Roman L. Hruska U.S. Meat Animal Research Center (RLHUSMARC). The tissue was sliced into 0.5 cm thick slices and stored frozen until use (maximum storage time 4 weeks). The tissue slices were cut into 1.0 by 1.0 by 0.5 cm pieces (surface area 4 cm^2) and immersed in the diluted cultures for 5 min. Before immersion, the tissue was shown to contain fewer than 25 CFU/cm^2 of surface.

Laboratory experiments

Inoculated tissue samples were dehydrated chemically by transferring to individual 20-ml volumes of the appropriate concentrations of the wash solutions (NaCl, $CaCl_2.2H_2O$) or sucrose; all percentages based on wt/vol) and vortexing on a

[1]*Mention of trade names, proprietary products, or specific equipment does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.*

Vortex Genie 2 (Scientific Industries, Bohemia, NY) set at 75% of the maximum setting for up to 120 sec. The wash solutions were decanted and the tissue samples transferred to 2% (vol/vol) acetic acid (sanitizer) and vortexed for 10 sec. Alternately, the tissue samples were dehydrated by physical means by washing for 10 sec in phosphate buffer (vortexing as described above) and suspending on sterile square-jawed alligator clips (Radio Shack) in sterile beakers at 5°C for 0, 2, 4, and 6 h. The tissue samples were then transferred to phosphate buffer and homogenized in a Stomacher 400 (Takmar Inc., Cincinnati, OH) for 2 min. The samples were serially diluted in phosphate buffer and plated on tryptic soy agar (TSA, Difco) using the pour plate technique *(6)*. The plates were incubated at 37°C for 24 h *(S. typhimurium)* or 23°C for 48 h *(L. monocytogenes)*. The results reported are the average of a minimum of two (usually three) independent replications.

The effect of osmotic shock on the bacteria was evaluated by adding 1 ml of 20% NaCl to the cell pellet and vortexing for 10 sec. After 10 sec, the stressed culture was diluted in 99 ml phosphate buffer, diluting the NaCl to 0.2%. Lean tissue was inoculated in 20 ml of the diluted stressed culture for 5 min and then sanitized by vortexing in 2% acetic acid for 10 sec. Control experiments were carried out using the same protocol and culture but substituting phosphate buffer for the 20% NaCl.

Carcass washing

Market weight steers (Bos taurus) were slaughtered in the abattoir at (RLHUSMARC) according to accepted practices. Briefly, the animals were stunned with a captive bolt stunner, bled, and the hide removed by hand. The carcasses were then eviscerated and split into halves. The carcass halves were experimentally inoculated on the chuck with a 1 to 1 mixture of fresh manure and phosphate buffer over an area approximately 20 x 20 cm. The carcass halves were washed and sanitized in an automated carcass washer described by Anderson et al. *(2)*, with a chain speed of 24 ft/min and a wash and sanitizer pressure of 300 psi. Washing and sanitizing times were approximately 10 and 5 sec, respectively. Four carcass halves were washed and sanitized with 2% (wt/vol) acetic acid in sequence and transferred to the chiller (air temperature ca. -1°C). Another four carcass halves were washed and transferred to the chiller. After 6 h, these carcass halves were removed from the chiller and sanitized with 2% acetic acid and returned to the chiller. Samples (2 x 2 cm) were aseptically excised from the carcass halves before washing, after sanitizing, and after 24 h of chilling. The samples were homogenized and plated as described above on TSA (32°C, 48 h; total aerobic count) and Violet Red Bile Glucose Agar (VRBG, Oxoid, Basingstoke, Hants, England) (32°C, 24 h, *Enterobacteriaceae*). After the 24 h samples were taken, the chuck area was trimmed to remove the entire area of inoculation to the satisfaction of the local inspector.

RESULTS

Chemical dehydration with salt solutions in combination with acid washes reduced the populations of both bacteria to a greater degree than the reductions attributable to acid alone. Twenty percent sodium chloride in combination with 2% acetic acid reduced the numbers of *S. typhimurium* an additional 1.5- (lean tissue) to 2- (fat tissue) log cycles (Fig. 1), compared to the acid alone. The effects were seen within the first 10 sec, with little additional effect after increased washing times. Mixed effects were seen with *L. monocytogenes* (Fig. 2), with no real additional effect from the salt solutions compared with the acid alone with lean tissue. The range of bacterial population means for all treatments was less than 1-log cycle

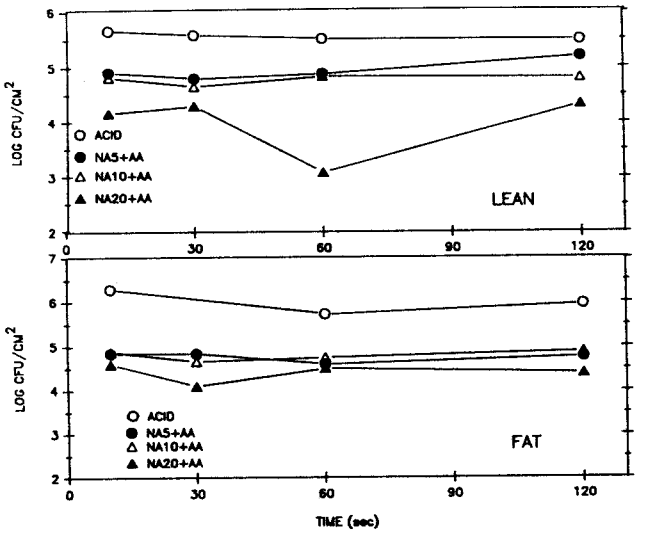

Figure 1. *Populations of viable* S. typhimurium *attached to beef tissue after pre-washing with NaCl solutions. Samples pre-washed for the specified time, followed by sanitizing for 10 sec with 2% acetic acid. Acid = sanitized with 2% acetic acid; NA5+AA = pre-washed with 5% NaCl, followed by 2% acetic acid; NA10+AA and NA20+AA = as with NA5+AA, but using 10% and 20% NaCl, respectively.*

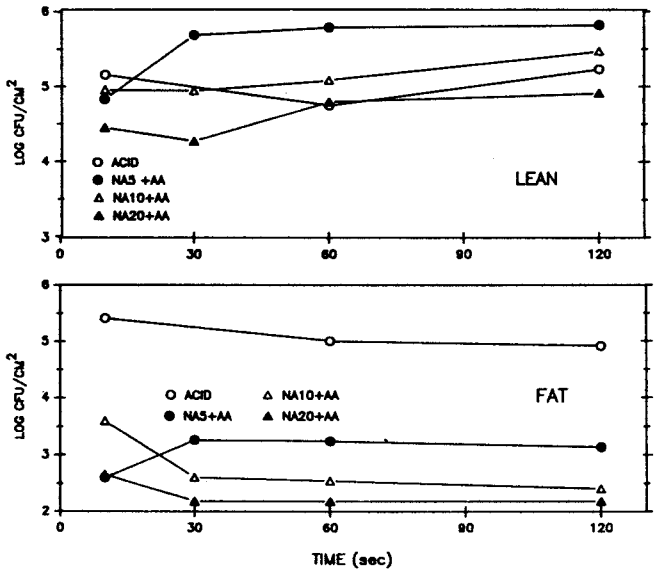

Figure 2. *Populations of viable* L. monocytogenes *attached to beef tissue after pre-washing with NaCl solutions. Samples pre-washed for the specified time, followed by sanitizing for 10 sec with 2% acetic acid. Acid = sanitized with 2% acetic acid; NA5+AA = pre-washed with 5% NaCl, followed by 2% acetic acid; NA10+AA and NA20+AA = as with NA5+AA, but using 10% and 20% NaCl, respectively.*

at 10 and 120 sec. However, an additional 3-log reduction was observed on fat tissue. As with *S. typhimurium*, there was no additional effect seen with increased washing time. When the salt solutions were applied without an acetic acid rinse, there was generally no effect on the population of either bacterium (data not shown).

$CaCl_2$ or sucrose solutions did not appreciably enhance the effect of acetic acid on lean tissue for either *S. typhimurium* (Table 1) or *L. monocytogenes* (Table 2.), although pre-washing for 60 sec with 20% $CaCl_2$ did result in an additional 1-log reduction of *S. typhimurium* on fat tissue, compared to a 60-sec rinse in phosphate buffer. Pre-washing fat tissue contaminated with *L. monocytogenes* with $CaCl_2$ produced an additional 2-to 2.5-log reduction in the bacterial population when followed by a 10-sec rinse with acetic acid. Sucrose had no apparent effect unless used as a 60-sec wash, in which case there were some small (less than 1-log cycle) reductions in the population.

TABLE 1. *Effect of $CaCl_2$ and sucrose pre-wash solutions on* S. typhimurium *on lean and fat beef tissue. Samples pre-washed for the specified time, followed by sanitizing for 10 sec with 2% acetic acid.*

Solution	Concentration (% wt/vol)	Wash time (sec) 10	30	60
LEAN				
$CaCl_2$	0%[1]	5.66[2]	5.58	5.51
	10%	5.20	5.39	5.34
	20%	5.36	5.50	5.38
Sucrose	0%	5.45	5.32	5.56
	10%	5.47	5.32	4.78
	20%	5.43	5.27	5.10
FAT				
$CaCl_2$	0%[1]	6.28	NP[3]	5.72
	10%	5.53	5.69	5.32
	20%	5.38	5.30	4.85
Sucrose	0%	5.22	5.30	5.33
	10%	5.30	5.15	4.99
	20%	5.39	5.47	5.39

[1]Control washed with phosphate buffer.
[2]Log colony forming units /cm^2.
[3]NP = Not performed.

TABLE 2. *Effect of $CaCl_2$ and sucrose pre-wash solutions on* L. monocytogenes *on lean and fat beef tissue. Samples pre-washed for the specified time, followed by sanitizing for 10 sec with 2% acetic acid.*

Solution	Concentration (% wt/vol)	Wash time (sec) 10	30	60
LEAN				
$CaCl_2$	0%[1]	5.15[2]	NP[3]	4.74
	10%	4.43	4.62	4.96
	20%	4.17	4.01	4.45
Sucrose	0%	4.76	4.86	5.10
	10%	3.98	4.19	4.63
	20%	4.24	4.44	4.45
FAT				
$CaCl_2$	0%[1]	5.40	NP	5.00
	10%	3.19	2.4	2.65
	20%	2.60	2.60	2.40
Sucrose	0%	3.78	3.45	3.64
	10%	3.78	3.18	2.96
	20%	3.83	3.02	2.93

[1]Control washed with phosphate buffer.
[2]Log colony forming units /cm^2.
[3]NP = Not performed.

In an attempt to separate the dehydration effects of NaCl from the effect of osmotic shock of the concentrated salt solutions, the bacteria were placed in a 20% NaCl solution for 10 sec and then diluted 100-fold (residual salt concentration 0.2%). These stressed cells were inoculated onto lean and fat tissue for 5 min, and then the tissues were sanitized in 2% acetic acid for 10 sec. The osmotically stressed cells were generally more sensitive to the acid than the nonstressed cells (Fig. 3), with approximately a 1-log cycle difference between the two populations.

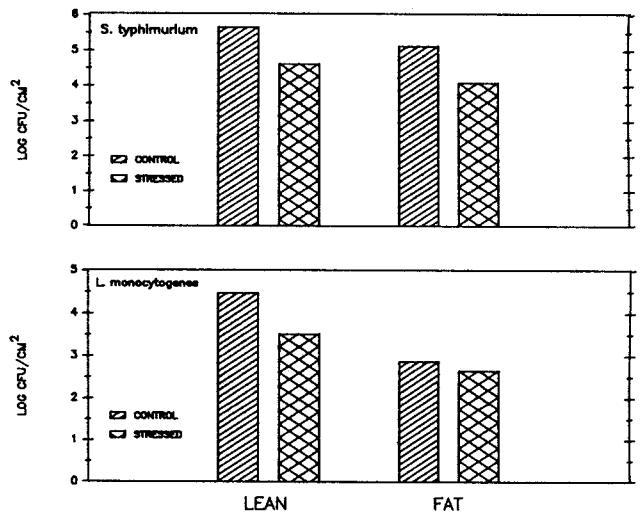

Figure 3. *Effect of pre-stressing with 20% NaCl on the sensitivity of* S. typhimurium *and* L. monocytogenes *to 2% acetic acid.*

When the tissue samples were allowed to dehydrate by physical means, the acid reduced the numbers of *S. typhimurium* by an additional 1-(lean) to 3.5-(fat) log cycles after 4 h (Fig. 4). The control was an unwashed, inoculated treatment and the phosphate treatment consisted of washing the tissue in phosphate buffer with no acid sanitizer. The intent of the control was to monitor the population during storage at 5°C, and the intent of the phosphate treatment was to determine if the bacteria simply became easier to remove by washing during storage. As seen previously, there were mixed effects with *L. monocytogenes* (Fig. 5). There was an initial reduction of approximately 1.5-log cycles attributable to the acid treatment, although there was no additional effect of dehydration on lean tissue over 6 h. However, there was an initial reduction of 1.5-log cycles attributable to the acid when

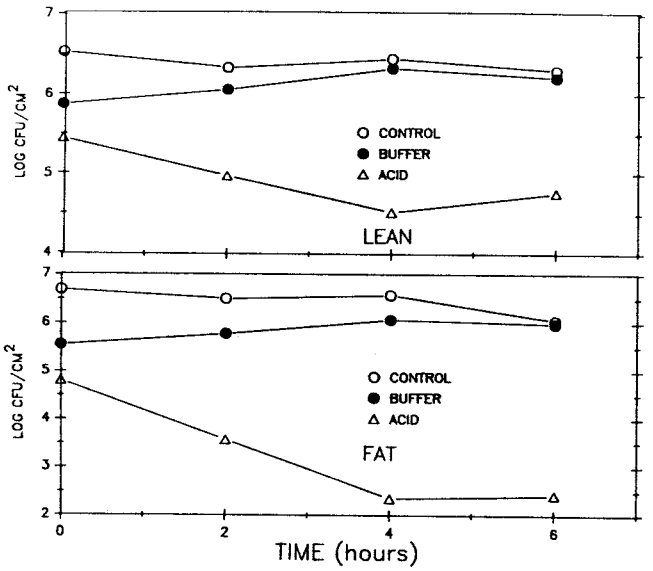

Figure 4. *Populations of viable* S. typhimurium *attached to beef tissue. Samples allowed to dehydrate at 5°C for the specified time interval, and then washed with phosphate buffer or 2% acetic acid.*

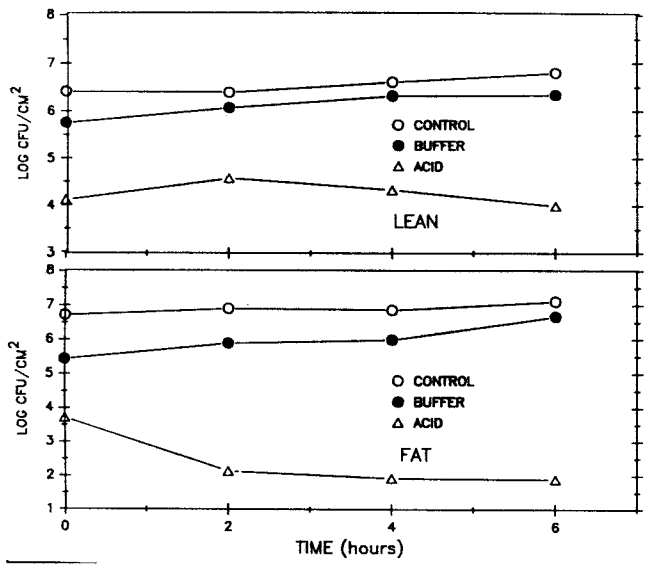

Figure 5. *Populations of viable* L. monocytogenes *attached to beef tissue. Samples allowed to dehydrate at 5°C for the specified time interval and then washed with phosphate buffer or 2% acetic acid.*

compared to the buffer wash, with an additional 1.5-log reduction on fat tissue after 2 to 4 h of dehydration.

When the process of dehydration was used with carcasses, the population of *Enterobacteriaceae* was reduced by 1-log cycle on carcass surfaces that were allowed to dry out in the chiller for 6 h before sanitizing with acetic acid when compared with those that were sanitized immediately after washing (Fig. 6). However, after 24 h, there was no difference between the two treatments, probably because of the residual effect of the acid. There were no real differences in the total aerobic flora between the two treatment groups.

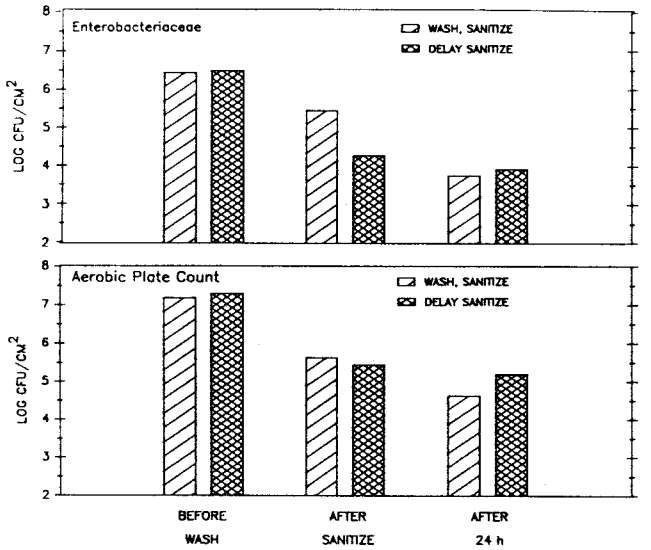

Figure 6. *Effect of delayed application of 2% acetic acid on the populations of* Enterobacteriaceae *and aerobic mesophiles on artificially contaminated beef carcasses. Carcasses were washed and sanitized either immediately (wash, sanitize) or after 6 h of chilling (wash, delay sanitize).*

DISCUSSION

Moisture is essential for the growth and survival of bacteria. Moisture reduction by either desiccation or osmotic pressure deprives the cell of moisture necessary for metabolism and results in plasmolysis *(21)*. Bacteria which have been stressed by moisture reduction are, in many cases, more susceptible to other forms of stress. The combined effects of stress have been variously described as "superimposed limiting factors" *(16)* or the "hurdles concept" *(20)*. The change in resistance varies with the strain of bacteria involved and the type of secondary stress employed with the process.

Calhoun and Frazier *(7)* reported that *Escherichia coli* survived at 60°C to a greater degree in phosphate buffer adjusted to a_w 0.980 with either NaCl or glucose than in phosphate buffer at a_w 0.994. However, *Staphylococcus aureus* was more heat sensitive in buffer which had been adjusted to a_w 0.95 with glucose than in standard buffer or in buffer adjusted to a_w 0.95 with NaCl. McEldowney and Fletcher *(15)* reported that bacterial survival on steel or glass surfaces was influenced by both temperature and relative humidity, with a survival time for *S. aureus* ranging from 148 d at 4°C and 34% relative humidity to 8 d at 25°C and 0% relative humidity. When an additional stress is added in the form of acetic acid, the combined effects should be greater than the individual effects.

The greatest reductions in bacterial counts were seen when acetic acid was used in combination with either NaCl solutions or with desiccation, with the $CaCl_2$ and sucrose solutions having lesser effects. The salt and sucrose solutions, when used without the acid sanitizing step, generally had no effect on the bacterial populations. Although

these solutions would be expected to stress the bacterial cells, the use of nonselective agar (TSA) allowed the stressed cells to recover and to be enumerated.

The differences between NaCl and the other two compounds may be partially attributable to differences in osmolality of the standard solutions. A 10% solution of NaCl has an osmolality of 3.529, compared to 2.106 for 10% $CaCl_2$ (hydrated) and 0.336 for 10% sucrose *(22)*. However, if osmotic pressure were the only factor involved, solutions with equivalent osmolality should have similar effects and the 10% $CaCl_2$ (hydrated) solution (osmolality 2.106) should produce similar effects to a 5% NaCl solution (osmolality 1.638). If the response of *S. typhimurium* to 5% NaCl (Fig. 1) and 10% $CaCl_2$ (Table 1) is compared, there are some similarities, although the 5% NaCl solution generally produced greater reductions, particularly with fat tissue. However, the net reductions with *L. monocytogenes* (Fig. 2 and Table 2) are virtually identical for the two solutions. The NaCl solutions appear to have a biological effect on *S. typhimurium* in addition to the physical effects attributable to osmotic pressure.

Sato et al. *(19)* attributed the detrimental effects of NaCl solutions on *E. coli* to the loss of Mg^{2+} from the cells. NaCl injury was overcome by allowing the injured cells to revive in a medium containing supplemental Mg. The data presented here with *S. typhimurium* and *L. monocytogenes* suggest that this type of injury may be seen primarily with Gram-negative cells, although there apparently is no data in the literature to prove or disprove this.

The effects of desiccation injury were demonstrated both with laboratory cultures (Fig. 4 and 5) and the mixed flora commonly encountered in meat processing (Fig. 6). Acetic acid had a large residual effect after 24 h on the microflora beef carcasses (Fig. 6), and there would appear to be no advantage to delaying the application of the sanitizer. However, most of the major beef processors in the United States currently use spray chilling to improve the cooling rates of the carcasses and reduce the moisture loss (shrinkage) in the chiller *(11)*. The process of spray chilling involves applying chilled water in timed cycles to the carcasses during the initial cooling after slaughter. The use of spray chilling would eliminate the residual effect of the acid simply by washing it off the carcass. The present slaughter and chilling operations could be modified to incorporate a delayed application of sanitizer prior to spray chilling to take advantage of the stress induced by desiccation.

CONCLUSIONS

The combination of osmotic stress and dehydration with acetic acid was more effective in reducing the populations of *S. typhimurium* and *L. monocytogenes* than either form of stress individually. The use of NaCl solutions produced the greatest reductions in bacterial numbers, although in practice physical dehydration may be the more acceptable process. Surface moisture also has a role in the sanitization of beef tissue surfaces, either through dilution of the sanitizer or by maintaining the bacterial cells in a hydrated (nonstressed) state.

ACKNOWLEDGMENTS

The author gratefully acknowledges the assistance of Ms. Tammy Stuehm, Ms. Terri Alberts, Ms. Jane Long, and Ms. Carole Smith in the laboratory. The author also wishes to thank Mr. David Kohmetscher and the staff of the RLHUSMARC abattoir for their assistance with the carcass washing. The author would also like to thank Ms. Carol Grummert for her assistance with the preparation of this manuscript.

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