SURVIVAL OF *SALMONELLA TYPHIMURIUM, ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* DURING STORAGE ON BEEF SANITIZED WITH ORGANIC ACIDS

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ABSTRACT

Sterile beef tissue was inoculated with either Salmonella typhimurium, Escherichia coli *O157:H7 or* Listeria monocytogenes *Scott A and washed with 23C distilled water, 1% lactic acid or 1% acetic acid. The washed tissue was subjected to simulated dry chilling or spray chilling followed by storage at 5C. The washed tissue was stored at 5C for up to 21 days at 26% relative humidity, and total bacterial populations were determined by plating on nonselective and selective agars. There was no significant difference in the surviving populations of* S. typhimurium, Escherichia coli *O157:H7, or* L. monocytogenes *after storage, irrespective of chilling method. The surviving populations of bacteria were significantly lower on acid washed adipose tissue, when compared to the comparable water washed tissue. These results indicate that although injury and recovery of pathogenic bacteria may occur as a result of organic acid carcass sanitizing treatments, there was no practical significance of this phenomenon after 3 days of storage.*

INTRODUCTION

Bacterial contamination of fresh meats is undesirable but generally unavoidable during normal slaughter and handling procedures. This contamination is highly variable in both composition and location on the carcass, and can

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be minimized by adhering to good hygienic practices during slaughter (Chandran *et al.* 1986; Dixon *et al.* 1991; Gill 1987; Smith *et al.* 1976). Since the bacteria are confined almost exclusively to the carcass surface (Gill and Newton 1978; McMeekin 1982), as compared to the deep muscle tissue, procedures which can effectively reduce or eliminate bacteria from the tissue surfaces are of interest to both the meat industry and regulatory agencies.

Red meat carcasses are commonly washed with water to remove physical contamination, such as hair or bone dust, as part of the usual slaughter procedures. This washing process has been automated to improve efficiency and also to include a sanitizing treatment, usually consisting of chlorine or short chain organic acids (Anderson *et al.* 1987). While the effects of this washing and sanitizing treatment vary, depending on the nature of contamination and the type and concentration of sanitizer used, the overall effect is a reduction in the population of bacteria on the carcass surface (Dickson and Anderson 1992).

In addition to washing and sanitizing, spray chilling with chlorinated water has been used to control surface bacteria on carcasses (Swift and Co. 1973; Heitter 1975). Hamby *et al.* (1987) incorporated 1% acetic or lactic acid into a spray chill process and reported a general reduction in bacterial counts on carcass surfaces. Dickson (1991) also used acetic acid in a model spray chill system, and found that selected foodborne pathogenic bacteria could be reduced by as much as 3 $\log_{10}$ cycles on beef fat tissue. However, in a later study involving pre- and post-evisceration washing, Dickson and Anderson (1991) reported an apparent increase in population of a nalidixic-acid resistant strain of *Salmonella typhimurium* after spray chilling. The authors speculated that this increase could be a result of recovery of acid injured cells during spray chilling.

The concern is that sublethally injured bacteria might not be as readily detected as noninjured cells and could remain undetected in some cases. Therefore, the objectives of these experiments were to determine if detectable bacterial injury occurred after acid sanitizing treatments and the effect of spray chilling on injury and recovery. The ability of the bacteria to survive during refrigerated storage after these treatments was also evaluated.

MATERIALS AND METHODS

Bacterial Cultures

Salmonella typhimurium (ATCC 14028) and *Escherichia coli* O157:H7 (Food Research Institute, Madison, WI) were grown and maintained in tryptic soy broth (TSB, BBL). Cultures were transferred to TSB and incubated at 37C for 18 h prior to use. *Listeria monocytogenes* Scott A was grown and maintained in tryptic soy broth with 0.5% yeast extract (TSB-YE), and cultures

were transferred to TSB-YE and incubated at 25C for 18 h prior to use. The cultures were harvested by centrifugation (3000 × G, 5C, 10 min) and the pellets suspended in Butterfield's phosphate buffer (Pertel and Kazanas 1984).

Tissue Preparation

Post-rigor beef tissue was obtained as boneless trim from the abattoir at the Roman L. Hruska U.S. Meat Animal Research Center (RLHUSMARC). The tissue was separated into lean and adipose tissue, sliced into 0.5 cm thick slices, frozen in sealed bags, sterilized with gamma radiation at a minimum dose of 42 kGy, and stored at -20C until use. Prior to use, the slices were cut into 2.0 × 2.0 cm squares (sample size 2.0 × 2.0 × 0.5 cm) and tempered to room temperature. Tissue produced in this manner had previously been determined to be representative of pre-rigor tissue, with respect to numbers of bacteria which would attach and the sensitivity of the attached bacteria to organic acids (Dickson 1992).

Experimental Design

Each bacterium was evaluated separately. The harvested bacteria were diluted 1:100 in phosphate buffer, and tissue samples were inoculated by immersion for 10 min at ca. 23C (9.0 ± 0.25 $\log_{10}$ cfu/ml of inoculum).

Experiment 1: Samples were removed from the inoculum, drained briefly, and processed using the following treatments at approximately 22C: (1) Control; washed in sterile distilled water, (2) washed in sterile distilled water, followed by washing in sterile 1% (v/v) [final concentration 0.11 M] lactic acid, and (3) washed in sterile distilled water, followed by washing in sterile 1% (v/v) [final concentration 0.175 M] acetic acid. A second set of washed samples was prepared as described in (1) to (3) above, and then subjected to a simulated spray chilling treatment. Washing was accomplished by immersing the samples in 250 ml of distilled water and agitating on a gyratory shaker at 200 rpm for 10 s. The samples treated with acid were then transferred to 250 ml of either acetic or lactic acid and agitated as previously described. After treatment, the samples were attached to sterile square jawed alligator clips or hooks and suspended in sterile containers (typically 500 ml beakers) such that the samples did not contact the sides of the containers. The containers were loosely covered to prevent contamination. Spray chilling was simulated by dipping the samples in 5C sterile distilled water for approximately 1 s at 30 min intervals for a 4 h period, similar to the procedure previously described (Dickson 1991). All samples were stored at 5C at a relative humidity (R.H.) of approximately 26% as determined with a dew point hygrometer (Environmental Tectonics Corp., Southampton, PA) for 21 days, with individual samples removed for analysis

approximately every 3 days (day 0 samples analyzed immediately after treatment). This storage condition simulated the conditions which could be found in a holding cooler for beef carcasses.

Experiment 2: A second set of experiments was conducted using lean muscle tissue only and an important change in the method of storage. The treatments were limited to washing in sterile water or washing in 1% lactic acid (equivalent to treatments [2] and [3] in Experiment 1), with and without spray chilling. Samples were hung on clips, placed in containers containing approximately 50 ml of sterile distilled water, and sealed with plastic wrap to maintain a relative humidity (R.H.) of approximately 99%, as determined with a narrow range hygrometer (Newport Scientific, Jessup, MD). These conditions were designed to eliminate the inhibitory effects of the low R.H. used in Experiment 1, specifically by preventing the dehydration of the tissue. Individual samples were removed every day for 3 days for analysis.

Enumeration of Bacteria

Tissue samples were homogenized in 99 ml buffered peptone water (BBL) for 2 min in a Stomacher 400 (Tekmar Inc., Cincinnati, OH). Samples were enumerated using a Spiral Plater Model D (Spiral Systems Instruments, Inc., Bethesda, MD; Messer *et al.* 1984). Injury was determined by a differential plating technique using TSA or TSA-YE as the nonselective agar. The selective agars were Violet Red Bile Glucose agar (BBL; *S. typhimurium* and *E. coli*) and TSA-YE with 5% (wt/vol) NaCl (*L. monocytogenes*; Smith and Marmer 1991; Smith *et al.* 1991). Plates were incubated at 37C for 24 h (*S. typhimurium* and *E. coli*) or 35C for 48 h (*L. monocytogenes*). Both noninjured and injured cells (after repair) form colonies on the nonselective agars, while only noninjured cells would form colonies on the selective agar. The difference between the population estimates on the nonselective and selective agars was considered to represent the sublethally injured portion of the population. The percentage of this sublethally injured population was calculated using the formula:

$$\text{percent injury} = [(NS - S)/ NS] * 100 \quad (1)$$

where NS is the arithmetic population per cm^2 on nonselective agar and S is the arithmetic population per cm^2 on the selective agar. After extensive data analysis at the completion of the project, it was found that the control populations of *S. typhimurium* and *E. coli* O157:H7 were slightly but statistically lower ($P<0.05$) on VRBG than on TSA, indicating that the selective medium was somewhat inhibitory to the noninjured population. To correct for this, a media

adjustment factor was calculated as the ratio of the NS to S populations for each of these two bacteria, using the formula:

$$MAF = NS/S \quad (2)$$

where MAF is the media adjustment factor and NS and S are the populations on nonselective and selective agars, respectively. The percent injury for *S. typhimurium* and *E. coli* O157:H7 was calculated using the modified form of Eq. 1, which was:

$$\text{percent injury} = [((NS - (MAF*S))/ NS] *100 \quad (3)$$

This modified formula corrected for the inhibition of the noninjured portion of the population by adjusting the population estimates on the selective agar by the ratio required to produce a 0% injury of control populations. It should be noted, however, that while this modified formula (Eq. 3) changed the actual numbers of percent injury, it did not change the interpretation or conclusions of the data when compared to calculations using the original formula (Eq. 1).

Statistical Analysis

The estimates of bacterial populations were converted to $\log_{10}$ cfu/cm^2 values. Statistical analysis was conducted using the General Linear Models procedure of the Statistical Analysis System (1985), using models appropriate to the completely randomized design of the experiments. Reported means are the average of three independent replications of each experiment, with the population estimates of the initial inocula used as covariates in the analysis to normalize the results between replications. Unless stated otherwise, significance is expressed at the 0.05% level.

RESULTS

Initial experiments were conducted by storing the treated tissue samples at 5C and 26% relative humidity (R.H.) (Experiment 1). *S. typhimurium* survived on beef tissue for 21 days irrespective of treatment, although there were distinct differences in the numbers of surviving bacteria related to treatment and tissue type (Fig. 1). However, since there was no significant effect of spray chilling on bacterial populations within a given treatment, the data from both sample groups (sprayed and nonsprayed) were pooled. All figures represent the means from the pooled data. There was a decline in the total populations as determined with nonselective agar on lean tissue of

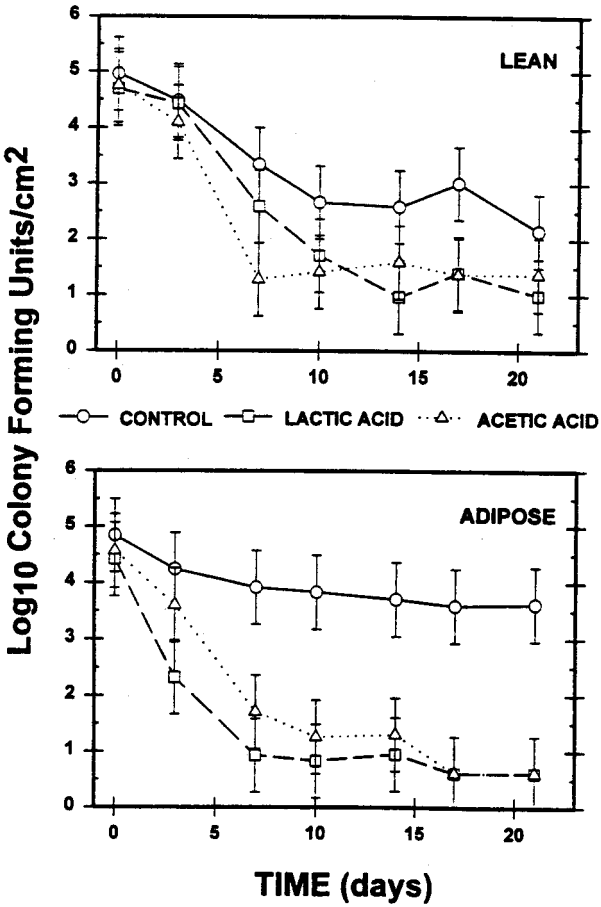

FIG. 1. POPULATIONS OF *SALMONELLA TYPHIMURIUM* ON BEEF TISSUE AFTER WASHING WITH STERILE DISTILLED WATER OR ORGANIC ACIDS, ENUMERATED ON TRYPTIC SOY AGAR

Samples were stored at 5C and 26% relative humidity. Plots are the means of the pooled sprayed and nonsprayed data sets, ± the standard error of the mean.

approximately 3.0 $\log_{10}$ units for the water wash and approximately 4.0 $\log_{10}$ units for the acid sanitizing treatments. Most of the population decline occurred during the first 7 days of storage for the acetic acid sanitizing, while the populations with the water and lactic acid treatments required 10 days to reach the minimum levels. The population trends on adipose tissue for the water

washed samples were quite different from those on lean tissue, with only a 1 $\log_{10}$ reduction after 21 days. The bacterial population trends on the acid sanitized samples were similar to those seen with lean tissue with an overall reduction of approximately 4 $\log_{10}$ units, with most of the reduction occurring within the first 7 days of storage.

The survival of *E. coli* O157:H7 followed a similar pattern to that of *S. typhimurium* (Fig. 2), with some variations. Both water and acid treatments

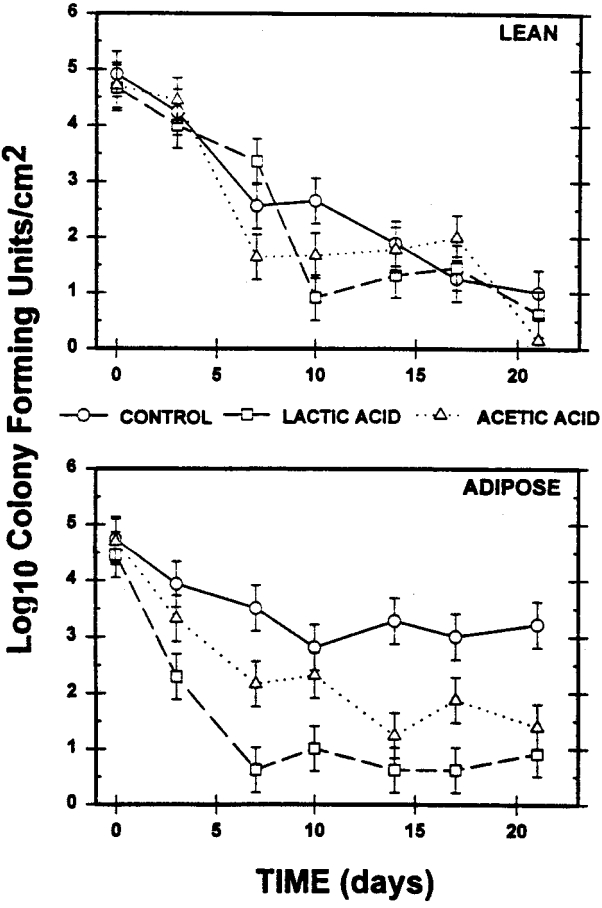

FIG. 2. POPULATIONS OF *ESCHERICHIA COLI* O157:H7 ON BEEF TISSUE AFTER WASHING WITH STERILE DISTILLED WATER OR ORGANIC ACIDS, ENUMERATED ON TRYPTIC SOY AGAR

Samples were stored at 5C and 26% relative humidity. Plots are the means of the pooled sprayed and nonsprayed data sets, ± the standard error of the mean.

demonstrated declines in populations of approximately 4 $\log_{10}$ units on lean tissue, with no significant effect of spray chilling within treatment. As with *S. typhimurium*, the data from both sprayed and nonsprayed sample sets were pooled, and the means of the pooled data set presented in the figures. There was only a 2 $\log_{10}$ reduction of the populations on the water washed adipose tissue samples after 21 days, compared with an approximate 4 $\log_{10}$ reduction in the populations on the acid washed adipose samples. However, while the lactic acid treated population declined rapidly within the first 7 days, the acetic acid treated populations acid declined more gradually over the 21 day period. Both Gram negative populations responded similarly to the acid treatments on adipose tissue. Lactic acid caused a faster population decline for both populations.

In contrast to *S. typhimurium* and *E. coli* O157:H7, the populations of *L. monocytogenes* declined only slightly on water washed lean tissue (Fig. 3). Organic acid washing of lean tissue reduced the populations by approximately 3 $\log_{10}$ cycles after 21 days, with most of the reduction occurring during the first 10 to 14 days. As with the previous bacterial species, spray chilling did not affect the bacterial populations within treatments, and the data from sprayed and nonsprayed samples were pooled. The bacterial population trends on adipose tissue were very similar to those on lean tissue, with essentially no decline in population after 21 days with the water washing and a 3 $\log_{10}$ (acetic acid) or 4 $\log_{10}$ (lactic acid) reduction with the organic acid washing.

Changing the relative humidity of the storage conditions (Experiment 2) slightly altered the basic patterns of survival for the bacteria during the first three days (Fig. 4). The populations of *S. typhimurium* on water washed tissue without spray chilling declined slightly when stored at 26% R.H., but remained constant when stored at 99% R.H. At 99% R.H., the populations of *S. typhimurium* on the nonspray chilled, water washed samples were significantly ($P < 0.05$) higher than those on the spray chilled or organic acid washed samples. The populations of *E. coli* were essentially unchanged after 3 days storage, irrespective of treatment. For *L. monocytogenes*, spray chilling did not alter the populations within treatment, although the populations of the water washed samples were significantly higher than those of the acid washed samples.

Sublethal injury of all bacterial species evaluated in this experiment was evident with all treatments. Although there were individual differences between sprayed and nonsprayed samples at specific times, overall there were no significant differences in injury attributable to spray chilling. There was considerable variation in the data both within and between treatments and because of this, the results will only be discussed in terms of trends in the data. The populations of the Gram negative bacteria demonstrated a general increase in injury with time with the water wash treatments, while the lactic and acetic acids resulted in an initial increase in injury, followed by a slight decline in the

percent of the injured population. The percentage of sublethal injury was generally much lower for *L. monocytogenes* than with the Gram negative bacteria. Water washing of lean tissue, with or without spray chilling, typically resulted in sublethal injury of less than 10% of the total population. The percentages were higher with lactic acid washing, but still averaged 15% without spray chilling and 19% with spray chilling, compared to pooled averages of 38% (*E. coli*) and 50% (*S. typhimurium*).

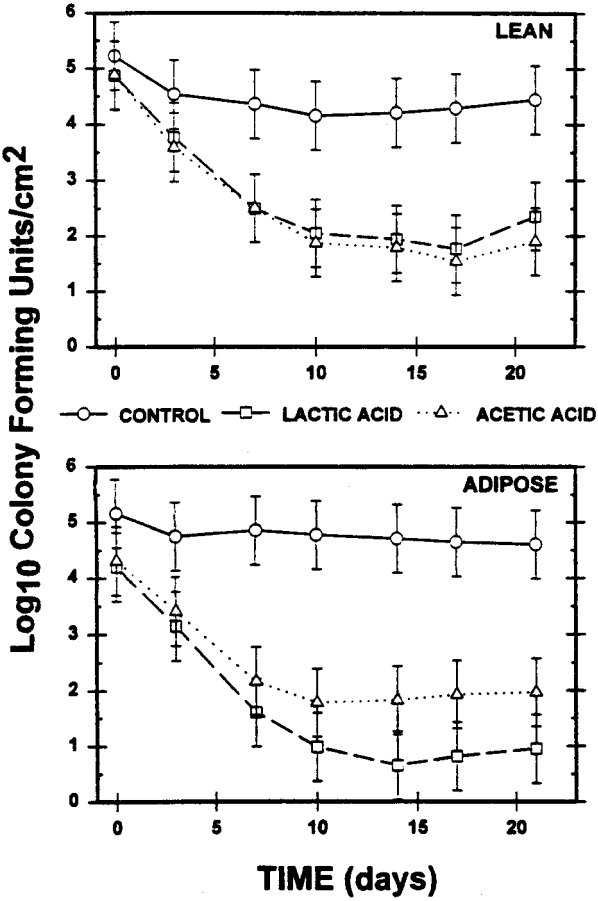

FIG. 3. POPULATIONS OF *LISTERIA MONOCYTOGENES* ON BEEF TISSUE AFTER WASHING WITH STERILE DISTILLED WATER OR ORGANIC ACIDS, ENUMERATED ON TSB-YE AGAR

Samples were stored at 5C and 26% relative humidity. Plots are the means of the pooled sprayed and nonsprayed data sets, ± the standard error of the mean.

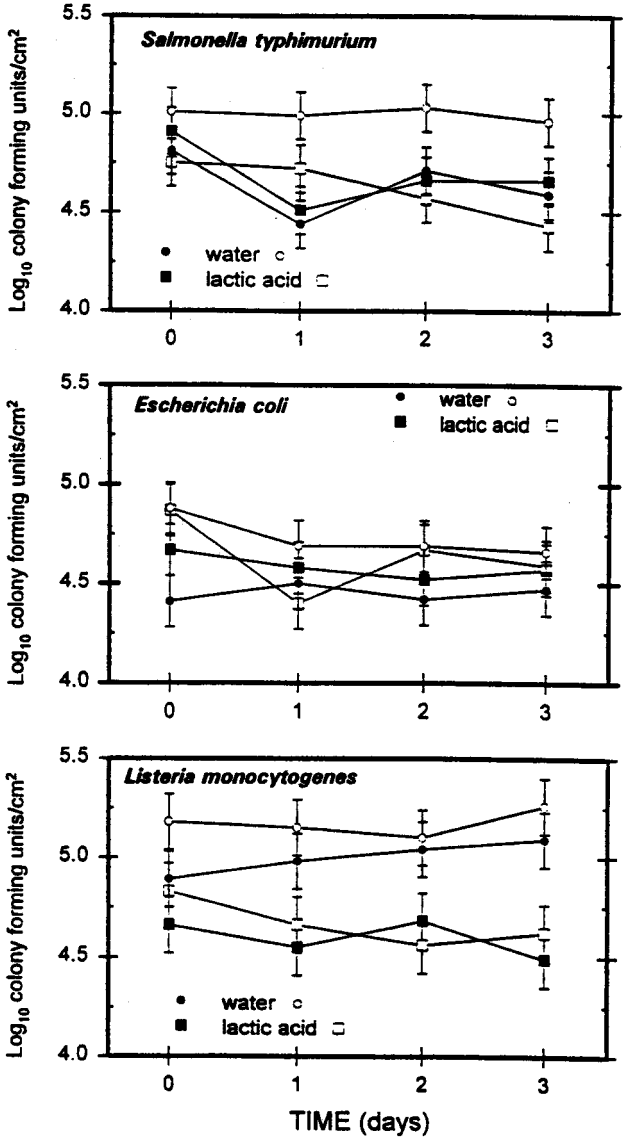

FIG. 4. BACTERIAL POPULATIONS ON BEEF TISSUE AFTER WASHING WITH STERILE DISTILLED WATER OR LACTIC ACIDS, ENUMERATED ON NONSELECTIVE AGAR

Open symbols indicate washed samples; closed symbols indicate samples which were washed and then subjected to simulated spray chilling. Samples were stored at 5C and 99% relative humidity. Plots are the means of the pooled sprayed and nonsprayed data sets, ± the standard error of the mean.

The high R.H. of the storage conditions in Experiment 2 greatly reduced the sublethal injury for *S. typhimurium* on water washed lean tissue. Sublethal injury after 3 days at 99% R.H. was approximately 5% for the nonsprayed and sprayed treatments, respectively, compared to 25% at 26% R.H. Little difference was seen in sublethal injury with the lactic acid washed samples without spray chilling, with injury levels of approximately 43% at R.H. of both 99% and 26%. Spray chilling resulted in injury levels of 17% and 35% at R.H. of 99% and 26%, respectively. The percentages of the population of *E. coli* which were sublethally injured at 99% R.H. were approximately half of those seen with the samples stored at 26% R.H. In contrast, the percent injury of the *L. monocytogenes* populations stored at 99% R.H. was much higher than the percentages at 26% R.H. storage. The percent injury for the water washed samples at 99% R.H. was 33.9% (nonsprayed) and 38.9% (sprayed), compared to 9.1% and 7.1% for the same conditions at the lower R.H.

DISCUSSION

The general reduction in the populations of *S. typhimurium* and *E. coli* on lean tissue stored at 26% R.H. are primarily attributable to surface drying of the tissue. Several published reports indicate a decrease in total bacterial counts on carcasses chilled for 24 h, with at least part of this reduction attributed to reduced water activity (a_w) and surface desiccation (Nortje and Naude 1981; Nortje *et al.* 1989). Previous studies had indicated that the a_w of lean beef tissue stored under comparable humidity conditions was less than 0.85 after 7 days at 5C (Dickson 1990), and the samples were visibly dehydrated. Juven *et al.* (1984) reported comparable reductions of salmonellae in meat and bone meal samples at a_w values ranging from 0.43 to 0.75. Organic acid washing increased the rate of these reductions, but there was no significant difference between the water and acid washed populations after 21 days. The increased rate of these reductions is a combination effect of the acid and inhibitory storage conditions. The levels of sublethal injury seen with the populations on the water washed samples are a reflection of the low R.H. of the storage conditions, and are not specifically related to the water wash treatment or spray chilling.

In contrast, the bacterial populations found on the water washed adipose tissue showed relatively little decline. The adipose tissue did not exhibit visible dehydration to the same extent as the lean tissue, and apparently provided a more favorable environment for the survival of the Gram negative bacteria. Population reductions on acid washed adipose tissue were therefore attributable primarily to the acid treatment itself, rather than the adverse storage conditions. Since much of the outer surface of an animal carcass is covered with adipose

tissue, the survival patterns for water and acid washing on this tissue type are significant.

The populations of *L. monocytogenes* showed relatively little decline on water washed lean tissue, in contrast to the 2 to 3 $\log_{10}$ reductions seen with the Gram negative bacteria. Doyle *et al.* (1985) reported that the population of *L. monocytogenes* declined less than 1 $\log_{10}$ in nonfat dry milk (moisture content 4%) after storage at 25C for 28 days. Later, Dickson (1990) reported that *L. monocytogenes* was resistant to desiccation on lean muscle tissue when stored at 5C and approximately 26% R.H., showing only a 1.5 $\log_{10}$ decrease after 42 days storage. A relatively small portion of the surviving population was sublethally injured, averaging 8 to 10% over the 21 day storage period. The decrease in populations on the acid washed tissue was primarily attributable to the effects of the organic acid, since the populations on the water washed tissue remained essentially unchanged, indicating no direct inhibitory effect of dehydration. The portion of the population which was sublethally injured by the acid treatment, while slightly higher than that of the water washed samples, was much less than that seen with the Gram negative bacteria.

Increasing the R.H. to 99% eliminated the inhibition of the storage conditions (Experiment 2). The bacterial populations within treatment remained relatively constant during storage, allowing the differentiation of subtle differences between treatments. The populations of *S. typhimurium* on water washed, nonsprayed tissue were higher than those of the other treatments, indicating that spray chilling had a slight inhibitory effect on the population which had previously been masked by the adverse storage conditions. As expected, the percent of the populations which was sublethally injured for both Gram negative bacteria was less when stored at 99% R.H. than at 26% R.H. for both water and acid washed tissue.

L. monocytogenes had a much higher level of sublethal injury when stored at 99% R.H. for a given treatment, when compared to the more inhibitory storage condition. This increased level of injury was evident after initial treatment (day 0), which may indicate a slight difference in the physiological state of the inoculum. The decreased level of injury seen with storage at 26% R.H. may also be a result of bacterial adaptation, and not specifically related to treatment. The selective agent in the selective media used to enumerate uninjured *L. monocytogenes* was 5% NaCl. This compound lowers the a_w of the medium and increases the osmotic stress on the bacteria. These selective conditions were already present on the tissue itself, such that surviving populations of bacteria on the tissue were already adapted to the restrictive conditions of the selective medium. As such, most of these adapted bacteria would be expected to grow on the selective media, which could account for the apparently lower percentage of sublethally injured bacteria.

CONCLUSIONS

In summary, although sublethal injury of bacterial populations did occur, there was no specific effect attributable to spray chilling. With virtually every treatment and bacterium, there were no differences between either total populations or the percentage of sublethally injured bacteria between sprayed or nonsprayed samples. The Gram negative bacteria were sensitive to surface dehydration on lean tissue as a result of the low relative humidity, and decreases in populations comparable to the acid washed samples were observed after 21 days. In contrast, *L. monocytogenes* was extremely resistant to this dehydration, requiring acid washing to reduce the populations.

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