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Acetic Acid Action on Beef Tissue Surfaces Contaminated with Salmonella typhimurium

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ABSTRACT

Beef tissue surfaces (lean and fat) were artificially contaminated with *Salmonella typhimurium* and then sanitized with 2% acetic acid. The reduction in bacterial population by the acid treatment was consistently proportional to the initial inoculum level for both tissue types. Increasing the amount of organic material in the inoculating menstra reduced the bactericidal effects of acetic acid on *Salmonella typhimurium* on fat tissue, although there was no change in effectiveness on lean tissue. Acid treatment sublethally injured about 65% of the population on both lean and fat tissue, and the residual effects of the acid resulted in a 1 log cycle reduction in bacterial population on fat tissue over 4 hr.

INTRODUCTION

IN THE CONVERSION of live animals to meat for consumption, microbiological contamination occurs as an unavoidable result of processing. Although the extent of contamination is highly variable, most initial contamination occurs on the surfaces of red meat carcasses, and internal muscle tissues remain essentially sterile. Much contamination is contributed by the hide during the removal process, since exposed surfaces of the hide and hair accumulate dust, dirt, and fecal material (Ayres, 1955). Gill and Newton (1978) and McMeekin (1982) have reviewed published reports concerning total bacterial populations of carcasses and retail cuts. They reported that although some bacteria may have come from diseased animals, the major amounts of the microflora on meat are on the surface of the carcass and originated from dirt and fecal material on the hide, dirt on processing equipment, and as a result of the evisceration process. The majority of the microflora transferred to the tissue surfaces from these sources, while aesthetically undesirable and deleterious to shelf life, are nonpathogenic. However, pathogens such as *Salmonella* (Currier et al., 1986), *Campylobacter* (Bracewell et al., 1985), and *Listeria* (Cottin et al., 1985) may infrequently be transferred to carcasses during slaughter and packinghouse operations.

Because of the public health significance of such foodborne pathogens, research has been conducted to determine methods to reduce or eliminate such pathogens from meat carcasses. Physically removing bacteria remaining on carcass surfaces by washing with water and subsequent sanitizing has been a practical and effective means of improving the microbiological quality of fresh meat (Kotula et al., 1974; Anderson et al., 1981). Organic acids, such as acetic or lactic acids, have been used to sanitize carcasses because they have good bactericidal activities (Acuff et al., 1987; Quartey-Papafio et al., 1980) and are generally recognized as safe (GRAS) additives (FDA, 1982). Khan and Katamay (1969) determined that short chain fatty acids had an antagonistic effect on a mixture of salmonella strains in meat. Chung and Goepfert (1970) found acetic and propionic acids were most effective in inhibiting salmonella in laboratory media. Ockerman et al. (1974) used acetic and lactic acid sprays in concentrations from 6% to 24% on lamb carcasses and reported that 18% acetic acid was the most effective in reducing bacterial populations. However, they noted that concentrations in excess of 12% produced surface discoloration. The effectiveness of organic acids, specifically lactic acid, as a meat decontaminant has been reviewed by Smulders et al. (1986).

Washing and sanitizing processes have been automated (Anderson et al., 1987) and the physical parameters involved in the processing have been extensively studied (Crouse et al., 1988). However, most previous reports have evaluated sanitizing in regard to either natural, passive, contamination or a single type of inoculum. Bacteria which attach to a carcass surface may be from a variety of sources and physiological states and such variations in contamination could alter the outcome of a sanitizing process. Therefore, the objectives of this research were to determine the effectiveness of acid sanitization under different simulated contamination conditions. The initial level of contamination on beef tissue and the level of organic material in the inoculum were varied prior to sanitizing with acetic acid. The residual effects of the acid and the sublethal injury to the total bacterial population were also evaluated.

MATERIALS & METHODS

Bacterial culture

Salmonella typhimurium (ATCC 14028) was grown and maintained in tryptic soy broth (TSB, BBL). Cultures were transferred to TSB and incubated at 37°C for 18 hr prior to use.

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 fat tissue, sliced into 0.5 cm thick slices, frozen in sterile sealed bags, sterilized with gamma radiation at a minimum dose of 42 kGy, and stored at −20°C until use. Prior to use, the slices were cut into 1.0 × 1.0 cm squares (sample size 1.0 × 1.0 × 0.5 cm) and tempered to room temperature. Alternately, pre-rigor tissue was aseptically excised from the neck area of beef cattle within 5 min of slaughter and flash frozen in liquid nitrogen to facilitate slicing. The pre-rigor tissue was sliced, cut, and tempered to room temperature as described above. Lean muscle and fat tissue were separated to produce two distinct tissue types and used for experimentation within 30 min of excision from the carcass. (Tissue produced in this manner typically had an aerobic plate count of less than 25 colony forming units/cm^2 of surface area and tested negative for *Salmonella* sp.).

Enumeration of bacteria

Tissue samples were homogenized in 99 mL Butterfield's phosphate buffer (Pertel and Kazanas, 1984) for 2 min in a Stomacher 400 (Tekmar Inc., Cincinnati, OH). Samples from the inoculation level experiments were enumerated on tryptic soy agar (TSA, BBL) by the pour plate technique (Busta et al., 1984). Samples from all subsequent experiments were enumerated on TSA using a Spiral Plater Model D (Spiral Systems Instruments, Inc., Bethesda, MD; Messer et al., 1984). Plates were incubated at 37°C for 24 hr.

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Experimental design

Effect of rigor state. Bacterial cultures were serially diluted in phosphate buffer to a cell concentration of about 10^7 colony forming units/mL (CFU/mL). Pre- and post-rigor tissue samples were immersed in 20 mL of the diluted cultures for 5, 10 and 30 min and then transferred to individual 99 mL bottles of phosphate buffer, which were gently inverted 25 times to remove planktonic bacteria which may have been trapped in the moisture layer on the tissue surface. Bacteria remaining on the tissue surfaces were considered to be attached. The tissue samples were removed from the bottles and the attached bacteria were enumerated as previously described. Alternately, pre- and post-rigor tissue samples were immersed in 20 mL of the diluted cultures for 5 min and subjected to an acid treatment, which consisted of vortexing the samples in 20 mL of 2% (vol/vol; 0.33 M) acetic acid on a Vortex Genie 2 (Scientific Industries, Bohemia, NY) at 75% of the maximum setting for 10 sec. The pH of the pre-rigor and post-rigor tissue was determined on representative samples of tissue immediately before and after experimentation by the method of Bendall (1973). Briefly, 2.5 grams of tissue were macerated for 10 sec in 25 mL iodoacetate KCl solution (5 mM sodium iodoacetate in 150mM KCl, pH 7.0) using a Tekmar Tissumizer equipped with an SDT-182 probe (Tekmar Inc., Cincinnati, OH) at 12,000 rpm. The pH of the mixture was determined using a general purpose combination Ag/AgCl electrode and standard pH measurement techniques.

Inoculation level. Bacterial cultures were serially diluted in Butterfield's phosphate buffer (Pertel and Kazanas, 1984) to cell concentrations of about 10^3 to 10^7 colony forming units/mL (CFU/mL). Post-rigor tissue samples were immersed in 20 mL of the diluted cultures for 5 min, transferred to individual 99 mL bottles of phosphate buffer, and gently inverted 25 times as previously described. The tissue samples were removed from the bottles and attached bacteria were enumerated as previously described. These samples were designated control samples. A second set of samples was prepared from each dilution as described above, with the addition of an acid treatment prior to enumeration. The acid treatment consisted of vortexing the samples in 20 mL of 2% (vol/vol; 0.33M) acetic acid on a Vortex Genie 2 (Scientific Industries, Bohemia, NY) at 75% of the maximum setting for 10 sec.

Sensitivity of single cells compared to cell clusters. Cultures were inoculated and grown simultaneously in 9 mL of TSB (for single cells) and on TSA (for cell clusters) surface plates (Busta et al., 1984) for 18 hr at 37°C. The broth cultures were vortexed on a Vortex Genie 2 (Scientific Industries, Bohemia, NY) at 75% of the maximum setting for 15 sec. Alternately, 5 mL of phosphate buffer was added to the TSA plates and the surface growth gently removed from the entire plate by scraping with a sterile teflon spatula. Two mL of each culture was added to 18 mL phosphate buffer in separate sterile beakers and gently swirled to produce a uniform mixture. Microscopic examination of wet mounts revealed that bacterial cells prepared from broth culture were primarily single cells, while those from the plate were primarily multiple cell clusters. Post-rigor tissue samples were inoculated by immersion in the cultures for 5 min, subjected to the 2% acid treatment, and then enumerated as described previously. Control samples were prepared in an identical manner, with substitution of phosphate buffer for acetic acid for vortexing. Bacterial populations from the control samples were used as a covariate in the statistical analysis to normalize the populations to a common initial inoculum.

Effects of organic material. Inoculating menstra were prepared by diluting 2 mL of an overnight TSB culture into 18 mL phosphate buffer, 18 mL TSB, 18 mL autoclaved rumen fluid, or 18g of an autoclaved 1:1 mixture of manure and phosphate buffer. Rumen fluid was collected from fistulated cows while manure was collected by rectal grab sampling of cattle fed a corn silage diet. Control samples were prepared as previously described with post-rigor tissue, and the populations used as a covariate in the statistical analysis.

Bacterial injury. Post-rigor tissue samples were inoculated in a 1:10 dilution of the culture (2 mL culture, 18 mL phosphate buffer) and then subjected to an acid treatment (previously described). The samples were transferred to sterile square-jawed alligator clips (Radio Shack, Ft. Worth, TX), suspended in sterile beakers, and bacterial populations were enumerated at 0, 15, 30, 60, 120, and 240 min. Injury was determined by a differential plating technique using TSA and Bismuth Sulfite agar (BSA, BBL). Both noninjured and injured cells (after repair) form colonies on TSA, while only noninjured cells would form colonies on the selective agar (BSA) within 24 hr. The difference between the population estimates on TSA and BSA was considered to represent the sublethally injured portion of the population. The percentage of this sublethally injured population was calculated using the arithimetic population estimates and the formula: [(population on TSA − population on BSA)/(population on TSA)] × 100. This experiment evaluated the immediate and residual effects of acetic acid on total population and cell injury due to acetic acid. A second set of tissue samples was prepared in an identical manner and the pH of the tissue surface was determined with a flat surface pH probe and a Corning 140 digital pH meter (Corning Scientific Instruments, Medfield, MA).

Statistical analysis. The numbers of bacteria attached to the tissue surfaces were converted to $\log_{10}$ CFU/cm^2 values. Statistical analysis was conducted using the General Linear Models procedure of the SAS Institute, Inc. (1985), using models appropriate to the completely randomized design of the experiments. Reported means are the average of three independent replications of each experiment. Unless stated otherwise, significance is expressed at $P \leq 0.05$.

RESULTS & DISCUSSION

BACTERIA which become associated with tissue surfaces exist as either free cells in the water film on the tissue surface, or as cells which are physically attached to the surface. These cell types have been described as "loosely" and "strongly" attached cells (Firstenberg-Eden et al, 1978; Farber and Idziak, 1984), or as planktonic and sessile (Costerton and Lappin-Scott, 1989). Most researchers consider "attached" bacteria to refer to strongly attached bacteria, and usually rinse tissue samples after treatment to remove any bacteria which are simply associated in the water film on the surface (i.e., the loosely attached bacteria, Butler et al., 1979; Chung et al., 1989; Farber and Idziak, 1984; Firstenberg-Eden et al., 1978; Lillard, 1986). The experimental design and terminology used in our experiments with pre- and post-rigor tissue was consistent with those of the previously cited reports.

Initial experiments were conducted to verify that the irradiated, frozen post-rigor tissue did not alter the response of the bacteria when compared to pre-rigor tissue. There was no difference in the attachment of *S. typhimurium* attributable to rigor state (Table 1). In addition, rigor state did not alter the sensitivity of *S. typhimurium* to acetic acid on lean tissue (Table 2). Although there was a statistically significant difference in response of the bacteria to acid on fat tissue, this difference was 0.14 $\log_{10}$ cycles, and the biological significance of such a difference is questionable. While there were numerous phys-

Table 1—Populations of attached Salmonella typhimurium *cells on pre-rigor tissue and post-rigor (irradiated and frozen) tissue*

Tissue	Rigor state[b]	Time (min)[a] 5	10	30
Lean	Pre	6.95[c]	6.98[c]	7.23[c]
	Post	6.78[c]	6.98[c]	7.16[c]
Fat	Pre	6.87[c]	7.06[c]	7.15[c]
	Post	6.93[c]	7.00[c]	7.10[c]

[a] Inoculation time of tissue samples.
[b] Pre = pre-rigor tissue, flash frozen in liquid nitrogen and thawed, pH 6.4; Post = post rigor tissue, frozen to −20°C and irradiated to a minimum dose of 42 kGy and thawed prior to use, pH 5.5.
[c] Mean $\log_{10}$ colony forming units/cm^2; means within tissue type with different superscripts are significantly different ($P < 0.05$). Inoculum level used as a covariate in data analysis to standardize treatments and replications.

Table 2—Populations of Salmonella typhimurium *on beef tissue after washing with acetic acid; effect of tissue rigor state*

Rigor State[b]	Bacterial population[a] Lean	Fat
Pre	6.35[c]	6.21[d]
Post	6.40[c]	6.42[c]

[a] Mean $\log_{10}$ population; average of three independent replications. Populations of control samples (washed with Butterfield's phosphate buffer) used as a covariate in data analysis to standardize treatments and replications.
[b] Pre = pre-rigor tissue, flash frozen in liquid nitrogen and thawed, pH 6.8; Post = post rigor tissue, frozen to −20°C and irradiated to a minimum dose of 42 kGy and thawed prior to use, pH 5.5.
[c,d] Means with different superscripts are significantly different ($P < 0.05$).

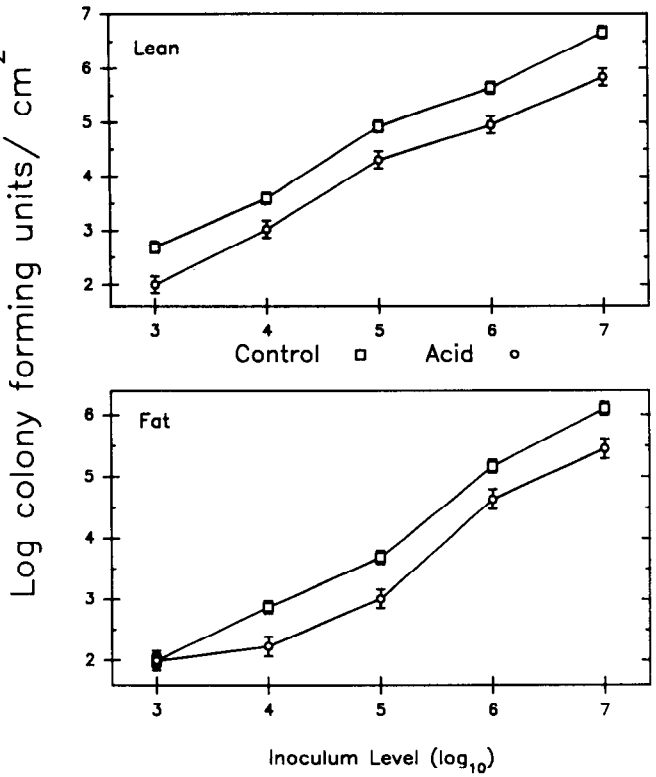

Fig. 1—Reduction in the population of Salmonella typhimurium *on post-rigor beef tissue by acetic acid as affected by initial cell population. Control samples were washed with phosphate buffer, while acid samples were washed with 2% acetic acid.*

ical and chemical differences between pre- and post-rigor lean tissue (Lawrie, 1974), the decrease in muscle intracellular pH was the most obvious. However, the change in intracellular pH apparently did not alter the surface characteristics of the tissue cells sufficiently to affect either bacterial attachment or acid sensitivity. The pH of pre-rigor lean tissue was 6.4 and 6.8, for the attachment and acid washing experiments, respectively, (within the expected range for this rigor state). The pH of pre- and post-rigor fat tissue was essentially the same (pH 6.2–6.3). We concluded that the irradiated, frozen, post-rigor tissue was a suitable substrate for evaluation of bacterial response to acetic acid. Previous research had also indicated no difference in growth and survival characteristics of *Listeria monocytogenes* on irradiated tissue, which precluded the presence of inhibitory compounds, such as peroxides, which could have influenced results (Dickson, 1990a). Because the irradiated tissue was sterile and could be prepared and stored in advance to provide a consistent tissue source, irradiated tissue was used for all subsequent experiments.

The reduction in population of *S. typhimurium* attributable to acetic acid was consistent, irrespective of the initial cell population for both tissue types (Fig. 1). Treatment with 2% acetic acid reduced the populations by 0.5 to 0.8 $\log_{10}$ cycles. Anderson and Marshall (1990) reported a similar reduction in the population of *S. typhimurium*, when they used a mixture of acetic, lactic, citric, and ascorbic acids on lean tissue. Statistical analysis of the reduction in population ($\log_{10}$ control - $\log_{10}$ acid treatment) indicated no significant difference ($P > 0.10$) in this reduction related to initial populations on either tissue.

In natural environments, bacterial cells may exist as either individual cells or as cell clusters or microcolonies. If a cluster of cells attached to a tissue surface, the cells in the center of the cluster could be physically protected from the acid by cells on the outer edge of the cluster. However, there was no significant difference ($P > 0.10$) in the populations of either individual or clustered cells for each tissue type, although the populations of the clustered cell inoculum were slightly higher (Table 3). The cells attached to fat tissue were more sensitive to the effects of the acid. Previous reports (Dickson, 1988, 1990b) indicated that *S. typhimurium* cells attached to fat tissue were generally more sensitive to sanitizers than those attached to lean tissue. Fat tissue tends to retain less surface moisture than lean tissue, and the reduced surface moisture may account for the increased sensitivity to acid. With less surface moisture, there would be less water to dilute the acid at the tissue/fluid interface. Alternately, bacterial cells can attach in microcrevices which form in lean tissue surfaces (Lillard, 1988), and such crevices could also trap water and interfere with contact between bacterial cells and acid.

Table 3—Populations of Salmonella typhimurium *on post-rigor beef tissue after washing with acetic acid; effect of inoculation with single cells or cell clusters*

	Bacterial population[a]	
Cell Grouping	Lean	Fat
Individual	6.55[b,c]	6.19[c]
Clusters	6.72[b]	6.41[c]

[a] Mean $\log_{10}$ population; average of three independent replications. Inoculum level used as a covariate in data analysis to standardize treatments and replications.
[b,c] Means with different superscripts are significantly different ($P < 0.05$).

Table 4—Populations of Salmonella typhimurium *on post-rigor beef tissue washed with acetic acid; effect of level of organic material in the inoculating menstra*

	Bacterial population[a]	
Menstra	Lean	Fat
Buffer[b]	6.20[c]	5.31[d]
Tryptic soy broth	6.20[c]	5.13[d]
Rumen fluid	6.22[c]	5.42[d,e]
Manure	6.25[c]	5.70[e]

[a] Mean $\log_{10}$ population; average of three independent replications. Inoculum level used as a covariate in data analysis to standardize treatments and replications.
[b] Butterfield's phosphate buffer.
[c,d,e] Means with different superscripts are significantly different ($P < 0.05$).

The amount of organic material present in the inoculating menstra had no effect ($P > 0.10$) on reduction of the population of *S. typhimurium* on lean tissue (Table 4). However, there was less reduction of populations on fat tissue after acid treatment as the relative level of organic material increased. Fewer bacteria attached to lean and fat beef tissue surfaces when manure was used as the inoculating menstra (Dickson and MacNeil, 1990), but the cells which attached were more resistant to the effects of acetic acid. Apparently, there was an interaction between the fat tissue surface and the manure. The manure was the most viscous inoculum tested, and this may have provided physical protection for the bacterial cells. Alternately, the manure may have neutralized the acid in the microenvironment around the cells. Since much of the contamination of beef is in the form of dirt, manure, or rumen contents (Ayres, 1955), laboratory studies regarding contamination and sanitization of beef should consider the nature of inoculating menstra in the experimental design.

Acetic acid, when used as a sanitizing agent on beef tissue, had an immediate lethal effect on part of the bacterial population, as well as sublethally injuring more of the initial population (Fig. 2). There was no significant difference ($P > 0.10$) in population estimates of uninjured (control) bacteria plated simultaneously on TSA and BSA. This indicated that any subsequent difference in estimates after treatment was attributable to failure of the acid injured bacteria to grow on the selective media. There was no difference ($P > 0.10$) in the average percentage of the population which was sublethally injured (71.6%, lean; 62.9%, fat), although there was some variation over time. Since acid remained on the tissue surface after treatment, it had a residual inhibitory effect on the remaining population, although this residual effect was only obvious on fat

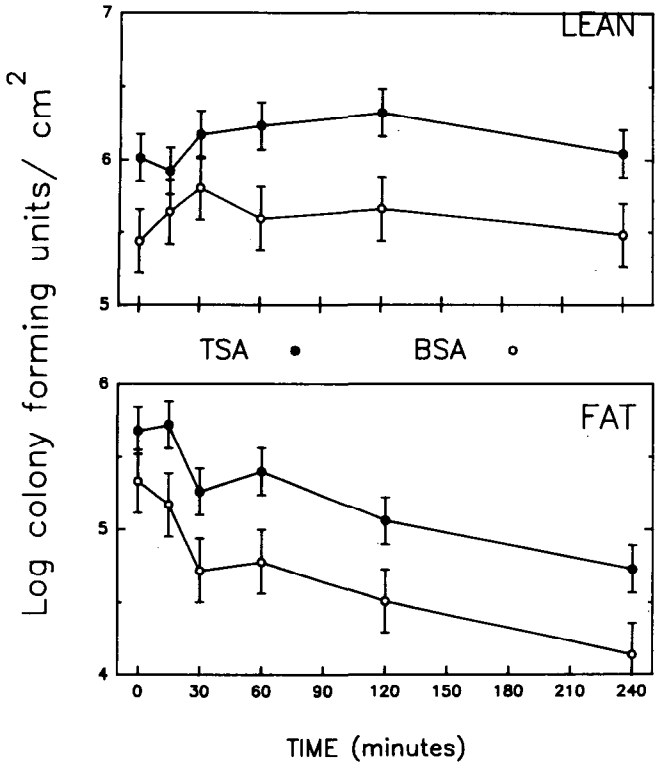

Fig. 2—Residual effect of acetic acid on the population of Salmonella typhimurium *on post-rigor beef tissue. BSA population estimates indicate the number of uninjured cells, while the TSA population estimates indicate the total population of uninjured and sublethally injured cells.*

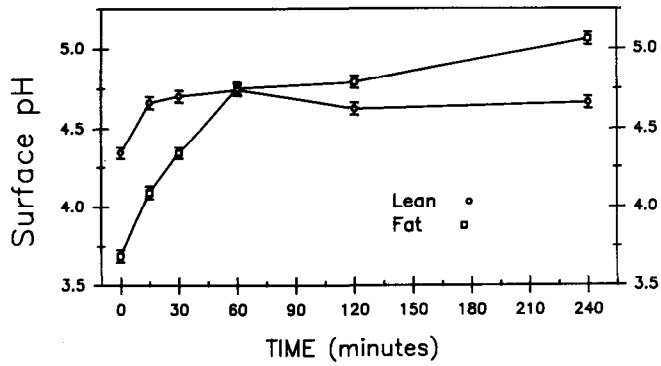

Fig. 3—Surface pH of post-rigor beef tissue after exposure to acetic acid. Initial surface pH's prior to acid treatment were 5.75 (lean) and 6.35 (fat).

tissue. There was no change ($P > 0.10$) in the population estimates (TSA or BSA) on lean tissue over 240 min. In contrast, the population estimates on fat tissue declined about 1 $\log_{10}$ cycle over the same time interval. This decline occurred in both the injured and noninjured populations, and the proportion of injured cells in the total population remained relatively constant over the 240 min time interval.

The surface pH values indicated an immediate lowering of surface pH immediately after acid treatment, followed by a relatively rapid rise over the next 60 min (Fig. 3). The change in surface pH was greater on fat tissue (initial pH 6.35) than on lean (initial pH 5.75) after acid treatment, with immediate drops of about 2.6 pH units and 1.4 pH units, respectively, after treatment with the acid. Anderson and Marshall (1990) reported initial reduction in pH of 1.2 units on lean tissue dipped in 2% mixed acid, followed by an increase of 0.6 units after 3 hr. Snijders et al., (1985) reported that the surface pH of veal carcasses sprayed with 1.25% lactic acid dropped from pH 7.0 to 3.7 (a decline of 3.3 pH units), although they did not specify the surface (i.e., fat or lean tissue) of the veal carcasses. The greater initial reductions in bacterial populations on fat tissue were primarily the result of a greater initial decline in surface pH on that tissue surface.

Lean muscle and fat tissue have apparent buffering effects on organic acids. While the exact nature of this buffering capacity is unknown, lean muscle tissue has a moisture content of about 75% (Lawrie, 1974), compared to 20% (Banks et al., 1976) for fat tissue. It is palusible that moisture in the lean tissue may have either diluted the acid or solubulized components of the tissue cells which buffered the acid on the surface. Alternately, the acid could be absorbed by the tissue cells at a more rapid rate than by the bacterial cells, which would account for the increased observed resistance of the bacteria to acetic acid on lean tissue.The buffering mechanism which prevented further lethal effects of the acid on lean tissue was not sufficient to allow for the recovery of sublethally injured cells. There would likely have been some reduction in bacterial population with longer holding times, since the surface pH remained relatively low.

The decrease in bacterial population estimates on fat tissue was greatest during the first 30 min after acid treatment (Fig. 2). While surface pH increased rapidly after acid treatment, it had only reached pH 4.3 after 30 min, which probably accounts for the population decline. Subsequent decreases in the population are attributable to combination effects of low pH and osmotic stress (dehydration). As previously stated, fat tissue has a lower moisture content than lean muscle tissue, and tissue surface dries out over time. The fat tissue surfaces were noticeably drier than lean tissue after 60 min.

CONCLUSIONS

THE EFFECTIVENESS of acetic acid sanitizing of beef tissue was independent of the level of initial contamination, with a consistent reduction in population, irrespective of cell numbers. The level of organic matter in the contaminating menstra affected sensitivity of the bacterial population to acetic acid, with more bacteria surviving on fat tissue when manure was the contaminating menstrum. Most bacterial contamination of beef carcasses results from contact with rumen fluid, dirt, or manure, and a high proportion of the exposed carcass surface is covered with fat. Sanitizing under commercial processing conditions may not be as effective as some laboratory experiments have shown. Sanitizing sublethally injured about 60% to 70% of the bacterial population, and more research is required to determine the fate of such injured bacteria as the carcass undergoes further processing.

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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.

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