# INHIBITION OF *LISTERIA MONOCYTOGENES, SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI* 0157:H7 ON BEEF MUSCLE TISSUE BY LACTIC OR ACETIC ACID CONTAINED IN CALCIUM ALGINATE GELS[1]

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## ABSTRACT

*A method of applying organic acids contained in a calcium alginate gel was tested for inhibiting bacteria contaminating sterile lean beef tissue surfaces. Treated samples were incubated at 5C under controlled moisture conditions for up to 7 days and viable populations of the pathogens determined. For counts of* L. monocytogenes, *recovered on tryptic soy agar, alginate/lactic acid treatment reduced the* $log_{10}$ *counts 1.8 units vs 0.96 for acid treatment without alginate. With acetic acid,* $log_{10}$ *reductions were 1.51 vs 2.33 for the alginate/acetic acid vs acetic acid treatment alone.* S. typhimurium *was reduced 2.11* $log_{10}$ *units vs 1.11 for alginate/lactic acid and acid treatments alone, respectively. The same trend held for* E. coli *0157:H7, although the reduction was considerably less, 0.74* $log_{10}$ *reduction vs 0.5 for alginate/lactic acid application vs acid alone. Both Gram-negative organisms were less inhibited by acetic acid treatments.*

[1]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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## INTRODUCTION

Microbial decontamination of meat animal carcasses by various treatments has been studied for several years (for review see Smulders 1987; Dickson and Anderson 1992). The overall objective of decontamination is to reduce the populations of pathogens and spoilage microflora, thus increasing the microbial food safety and shelf-life of raw meat. The initial microbial load on raw meats determines to a large extent both the shelf-life and the microbial safety of a product (NRC/NAS 1985). Of antimicrobial agents, organic acids and chlorine have receive the most attention (Dickson and Anderson 1992).

Immobilization techniques have been used for many microbial and enzymatic processes (Chibata *et al.* 1986). Generally, the active agent is either immobilized in a gel (i.e., calcium alginate) or diffusible matrix (such as polysaccharide beads) or is adsorbed to a support that is in contact with a substrate.

Calcium alginate has been used to reduce shrink loss in poultry (Mountney and Winter 1961), beef and pork (Berlin 1957), lamb (West *et al.* 1975; Lazarus *et al.* 1976) and other foods (McKormick 1975). Earle (1968) developed a process known as Flavor-Tex$^{R}$, which was used to prevent shrinkage loss in sheep carcasses (Lazarus *et al.* 1976). Organic acids have also been incorporated into alginates for use as additives to prolong salad ingredient shelf-life (Kirsop and Brocklehurst 1989).

We have demonstrated that applying acetic and lactic acids in an alginate gel enhances the antimicrobial effect against *Listeria monocytogenes* on beef tissue (Siragusa and Dickson 1992). The objectives of this study were to evaluate this treatment against *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli* 0157:H7 using a modified treatment, which included moisture control of the tissue samples.

## MATERIALS AND METHODS

### Bacteria

Bacterial strains used in this study were *Listeria monocytogenes* Scott A, *Salmonella typhimurium* ATCC 14028, and *Escherichia coli* 0157:H7. Organisms were stored at −20C in 75% (v/v) glycerol. Cultures were grown in Tryptic Soy broth + 0.5% yeast extract (BBL Microbiology systems, Cockeysville, MD, U.S.A. and Difco Laboratories, Detroit, MI). Inocula were prepared by diluting a 14–16 h, 37C culture in 0.1% peptone water to obtain a suspension of approximately $10^4$ cfu/ml.

## Tissue

Lean beef muscle tissue was obtained from the abattoir at the U.S. Meat Animal Research Center (Clay Center, NE), sliced to 5 cm thickness and frozen at −20C. The tissue was sterilized by gamma irradiation to a dosage of 42 kGy. The tissue was allowed to partially thaw and was aseptically cut into sections measuring 2 × 2 × 0.5 cm (12 $cm^2$ total surface area). Tissue sections were brought to 24C and inoculated by immersing the sections in 50 ml of one of the bacterial suspensions described above for 20 min. Immediately following the inoculation step, the sections were attached to individual alligator clip holders on a stiff wire cross bar and subjected to their respective treatments.

## Treatment Protocols

Treatments were similar to those previously described (Siragusa and Dickson 1992). A flow chart of the treatment procedure is given in Fig. 1. One percent (w/v) high viscosity sodium alginate (Sigma Chemical Co., St. Louis, MO) was prepared in distilled water, dispensed to a plastic screw cap centrifuge tube (40 ml per tube) and autoclaved for 17 min at 121C. Acids were prepared as follows: acetic acid (Fisher Scientific,Pittsburgh, PA), 2% (v/v; 0.35 M) was prepared

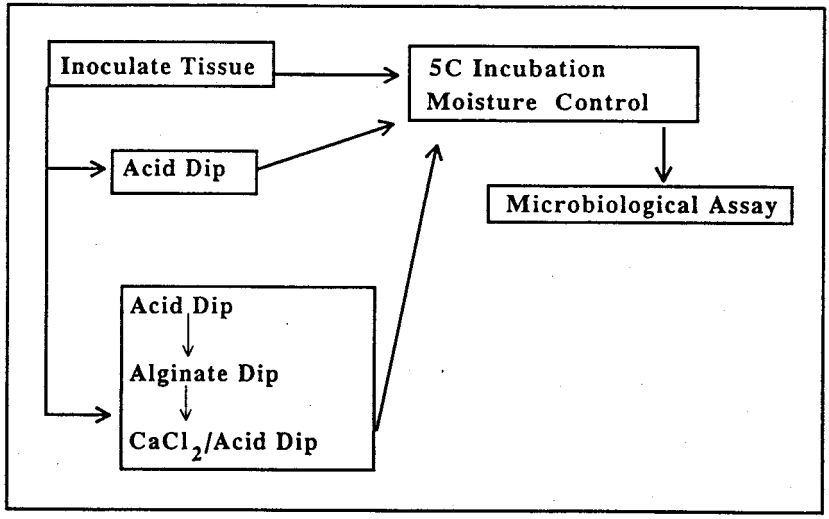


FIG. 1. FLOW CHART AND TREATMENT DIAGRAM OF ORGANIC ACID APPLICATION PROTOCOLS
See Materials and Methods section for details.

in both distilled water and in 90 mM $CaCl_2$; for lactic acid, 1.7% (v/v; 0.19 M) D,L-lactic acid (Sigma Chemical Co., St. Louis, MO) was prepared in distilled water and in 90 mM $CaCl_2$. Solutions were autoclaved at 121C for 17 min. Resulting pH values for the acid solutions were 2.8, 2.3, 2.8, 2.3, for acetic acid + $CaCl_2$, lactic acid + $CaCl_2$, acetic acid and lactic acid solutions, respectively.

All treatments were performed at room temperature. Each dipping step of the flow chart was performed for 1 min (Fig. 1). To control moisture loss, treated samples were placed in a sterile beaker containing 1/10th volume of sterile distilled water and covered with Saran wrap then aluminum foil and bound with a rubber band. Relative humidity was poised at approximately 99% under these conditions when measured with a narrow range hygrometer (Newport Scientific, Jessup, MD).

### Microbiological Assays

Tissue sections were diluted in buffered peptone water (100 ml) and blended in a Stomacher for 2 min. Samples were plated using a Spiral Plater (Spiral System Instrument, Bethesda, MD). Plates were incubated for 36–48 h at 37C before counting. Organisms were enumerated using the following media: *E. coli*, Tryptic Soy Agar (TSA) and Violet Red Bile agar (VRBG); *S. typhimurium*, TSA and Brilliant Green + Sulfadiazine (BGS); *L. monocytogenes*, TSA and Listeria Selective agar, Oxford formulation. All media and diluents were obtained from BBL Microbiology Systems (Cockeysville, MD).

### Statistical Analysis

Three replications of each treatment were performed for each organism. Data were analyzed using SAS (1982) and the general linear model. Inoculum counts were used as a covariant to normalize data from treatment replications.

## RESULTS

The $\log_{10}$ reductions of bacteria per tissue section after 7 days at 5C are shown in Tables 1–3. Under the moisture controlled conditions of this study, alginate immobilization of organic acids did not significantly ($p > 0.05$) enhance the antimicrobial effect of lactic or acetic acids for cells enumerated on TSA (Tables 1–3). However, lactic acid immobilized in alginate caused a numerically greater reduction of all 3 pathogens than did lactic acid applied above.

Alginate enhanced the effectiveness of lactic acid against *L. monocytogenes* Scott A (2.9 vs 2.03 $\log_{10}$ reduction) but had the opposite effect with acetic acid (2.44 vs 3.22 $\log_{10}$ reduction) (Table 1). The greatest reduction in counts due

TABLE 1.
$LOG_{10}$ REDUCTION IN VIABLE COUNT OF *LISTERIA MONOCYTOGENES* SCOTT A ON LEAN BEEF TISSUE AFTER 7 DAYS AT 5C ENUMERATED ON TSA AND LISTERIA SELECTIVE AGARS FOLLOWING ANTIMICROBIAL TREATMENT.

TSA Agar

| Treatment | Acid + Alginate | Acid Alone | p-Value[3] |
|---|---|---|---|
| Acetic | 2.40[a] | 3.22[a] | 0.35 |
| Lactic | 2.90[a] | 2.03[a] | 0.32 |
| Alginate Control[1] | 0.04[a] | -1.33[b] | .13 |
| Control Tissue[2] | -0.03 | | |

Listeria Selective Agar (Oxford Formulation)

| Treatment | Acid + Alginate | Acid Alone | p-Value[3] |
|---|---|---|---|
| Acetic | 3.36[a] | 3.26[a] | 0.87 |
| Lactic | 3.93[a] | 2.10[b] | 0.009 |
| Alginate Control[1] | -0.66[a] | -0.59[a] | 0.90 |
| Control Tissue[2] | -0.13 | | |

[1] Alginate controls included tissue dipped in alginate and $CaCl_2$ with no acid and tissue dipped in $CaCl_2$ with neither alginate nor acid.

[2] Control tissue was inoculated and received no further treatment.

[3] Probability means in a row are equal. Means with a common superscript are not statistically significantly different.

to the alginate/lactic acid treatment occurred after 7 days of storage (Fig. 2). Microbial counts obtained using Listeria selective agar (LSA) indicates that sublethal acid injury may have occurred for lactic and acetic acids when applied in a calcium alginate gel. LSA counts from the samples that were acid treated but without alginate application indicate no such injury was effected by those treatments. Controls of alginate treatment/no acid, $CaCl_2$ only/no alginate/no acid and inoculated control tissue demonstrate that *L. monocytogenes* populations will proliferate or remain stable on sterile beef tissue over 7 days (Fig. 2).

Lactic acid applied in a calcium alginate gel resulted in a numerically greater antimicrobial effect of lactic acid against *S. typhimurium* ATCC 14028 (Table 2) versus lactic acid treatment alone, although this effect was not statistically significant ($P > 0.05$). Acetic acid at the same pH, 2.8, had a markedly reduced

TABLE 2.
$LOG_{10}$ REDUCTION IN VIABLE COUNT OF *SALMONELLA TYPHIMURIUM* ATCC 14028 ON LEAN BEEF TISSUE AFTER 7 DAYS AT 5C ENUMERATED ON TSA AND BGS AGARS FOLLOWING ANTIMICROBIAL TREATMENTS.[1]

Tryptic Soy Agar

| Treatment | Acid + Alginate | Acid Alone | Pr > F |
|---|---|---|---|
| Acetic | 1.24[a] | 1.28[a] | 0.94 |
| Lactic | 2.59[a] | 1.63[a] | 0.07 |
| Alginate Control[1] | 0.62[a] | 0.47[a] | 0.76 |
| Control Tissue[1] | 0.38 | | |

BGS Agar

| Treatment | Acid + Alginate | Acid Alone | Pr > F |
|---|---|---|---|
| Acetic | 1.42[a] | 1.41[a] | 0.98 |
| Lactic | 2.51[a] | 1.66[a] | 0.08 |
| Alginate Control[1] | 0.56[a] | 0.53[a] | 0.94 |
| Control Tissue[1] | 0.37 | | |

[1] See Table 1 footnotes for explanation.

antimicrobial effect on *S. typhimurium* than did lactic acid in the concentrations used. As in the case with *L. monocytogenes* Scott A, the greatest reduction occurred by 7 days of refrigerated incubation (Fig. 3) of the alginate/lactic acid treated samples. Based on selective versus nonselective agar counts, no evidence of sublethal acid injury was observed with *S. typhimurium*.

Neither lactic nor acetic acids affected large reductions in the numbers of viable *E. coli* 0157:H7 at the concentrations used (Table 3). Lactic acid applied in calcium alginate caused a numerically larger reduction than without alginate, although this was not a statistically significant interaction ($p > 0.5$). Figure 4 indicates the recalcitrance of this isolate to lactic acid and alginate/lactic acid treatment. Population reductions determined using selective VRBG agar were greater than those determined with nonselective TSA, thereby indicating sublethal acid injury may have occurred.

Under the conditions of this study, neither of the Gram-negative bacteria tested proliferated on the lean beef muscle tissue.

TABLE 3.
$LOG_{10}$ REDUCTION IN VIABLE COUNT OF *ESCHERICHIA COLI* 0157:H7 ON LEAN BEEF TISSUE AFTER 7 DAYS AT 5C ENUMERATED ON TSA AND VRBG AGARS FOLLOWING ANTIMICROBIAL TREATMENTS.

Tryptic Soy Agar

| Treatment | Acid + Alginate | Acid Alone | Pr > F |
|---|---|---|---|
| Acetic | 0.44[a] | 0.64[a] | 0.32 |
| Lactic | 1.03[a] | 0.91[a] | 0.54 |
| Alginate Control[1] | 0.94[a] | 0.78[a] | 0.41 |
| Control Tissue | 0.37 | | |

VRBG Agar

| Treatment | Acid + Alginate | Acid Alone | Pr > F |
|---|---|---|---|
| Acetic | 1.00[a] | 1.24[a] | 0.42 |
| Lactic | 2.25[a] | 1.70[a] | 0.09 |
| Alginate Control[1] | 1.41[a] | 1.55[a] | 0.64 |
| Control Tissue | 1.22 | | |

[1] See Table 1 footnotes for explanation.

## DISCUSSION

Previous work was performed using a similar procedure (Siragusa and Dickson 1992) without moisture control. In that study, alginate application was proven to significantly enhance the antimicrobial effect of lactic acid against *L. monocytogenes* on lean beef. Tissue samples in that study, which were not alginate coated, showed signs of dehydration by 7 days of refrigeration. Alginate treated samples showed no signs of dehydration. Numerical reductions in bacterial populations from alginate/acid treatment reported in the present study were comparable to results obtained previously for *L. monocytogenes* Scott A (Siragusa and Dickson 1992). However, the addition of moisture controlled environmental conditions affected a greater $log_{10}$ reduction by the acid treatment alone (i.e., without alginate). Other techniques using calcium alginate have shown that alginate coatings are an effective means of reducing moisture loss (West *et al.* 1975; Lazarus *et al.* 1976). In the current study, noncoated samples under moisture

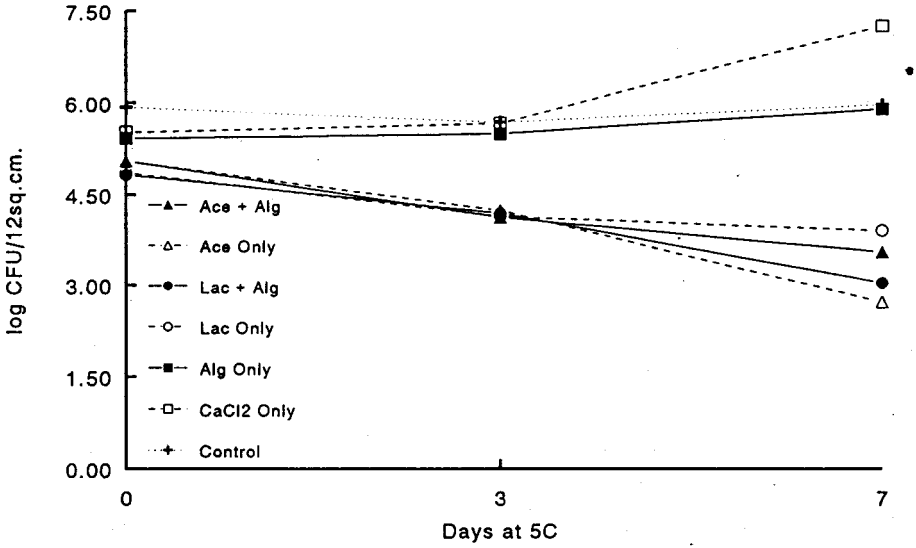


FIG. 2. SURVIVAL OF *L. MONOCYTOGENES* STRAIN SCOTT A ON STERILE LEAN BEEF TISSUE TREATED WITH LACTIC AND ACETIC ACIDS APPLIED ALONE OR IN A CALCIUM ALGINATE GEL

Samples were enumerated using Tryptic Soy Agar. Closed symbols = alginate application, open symbols = acid applied alone. Triangles = acetic acid; circles = lactic acid; squares = no acid application; + = inoculated control tissue.

control conditions showed no obvious signs of dehydration. Preventing dehydration allowed a comparison to be made between both the untreated acid dipped samples and the alginate/acid treated samples. Comparing results of *L. monocytogenes* inhibition between this experiment and that reported previously (Siragusa and Dickson 1992) indicate moisture control may have enhanced the antimicrobial effects of both acetic acid and lactic acid when applied alone. We previously reported $\log_{10}$ reductions of *L. monocytogenes* Scott A, by acid treatment alone, of 0.25 and 0.02 vs 2.33 and 0.96 in studies without and with moisture control, respectively.

In our initial study, microbial reductions from acid treatments alone were only approximately 50% as effective as the alginate/acid treated samples under conditions lacking moisture control (Siragusa and Dickson 1992). Since no $a_w$ measurements were made in the current study, it is difficult to speculate on the relationship between organic acid inhibition and water content of the meat surface. Under actual carcass processing, the aging process generally occurs after a spray chilling process and offers no means of moisture control. Alginate application of acids in place of spray chilling could enhance the antimicrobial effectiveness of the acid and assist in preventing shrink loss (Lazarus *et al.* 1976; West *et al.* 1975) and reducing water usage.

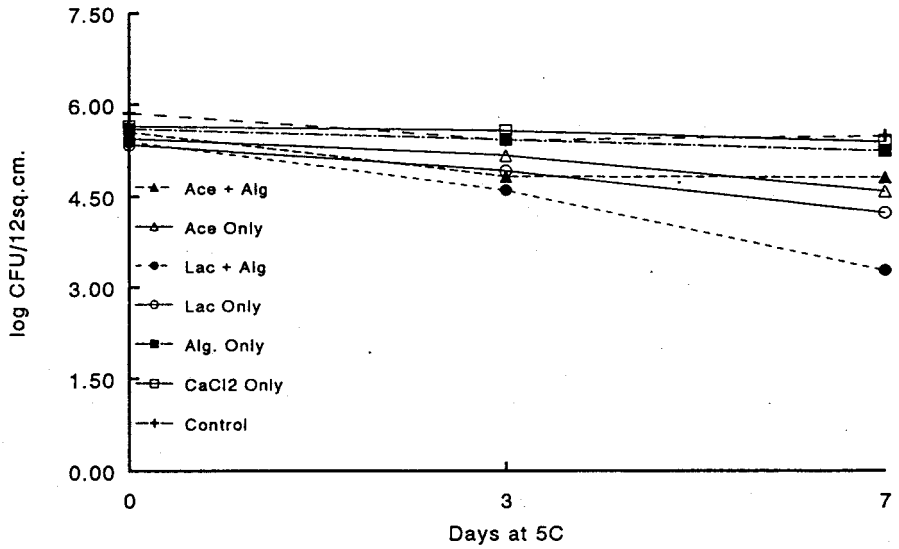


FIG. 3. SURVIVAL OF *S. TYPHIMURIUM* ATCC 14028 ON STERILE LEAN BEEF TISSUE TREATED WITH LACTIC AND ACETIC ACIDS APPLIED ALONE OR IN A CALCIUM ALGINATE GEL

Samples were enumerated using Tryptic Soy Agar. Closed symbols = alginate application, open symbols = acid applied alone. Triangles = acetic acid; circles = lactic acid; squares = no acid application, + = inoculated control tissue.

At a fixed pH of 2.8, the concentrations used for this study, lactic acid at 1.7% v/v was more inhibitory against the Gram-negative species than was acetic acid at 2.0% v/v. In the case of *L. monocytogenes*, acetic and lactic acid did not produce significantly different reductions ($p > 0.05$). Anderson *et al.* (1992) reported that acetic acid was less effective against *E. coli* and *S. typhimurium* when tested at three different temperatures and two different concentrations than was lactic acid or two test acid mixtures containing various proportions of ascorbic, lactic, acetic, and citric acids. Because of the mode of application in the current study, it is difficult to compare the acid concentrations used in this study with those in other experiments.

Hamby *et al.* (1987) reported acetic and lactic acid treatments significantly reduced the mean aerobic plate counts (APC) of vacuum packaged subprimal beef cuts only if the acid treatment was repeatedly applied over a 12 h period during spray chilling of the carcass. On the other hand, these workers found significant APC reductions in only some vacuum packaged beef cuts when the source carcasses were sprayed a single time. The authors speculated that the antimicrobial activity may have been affected by diluting the applied acid with the spray chill water.

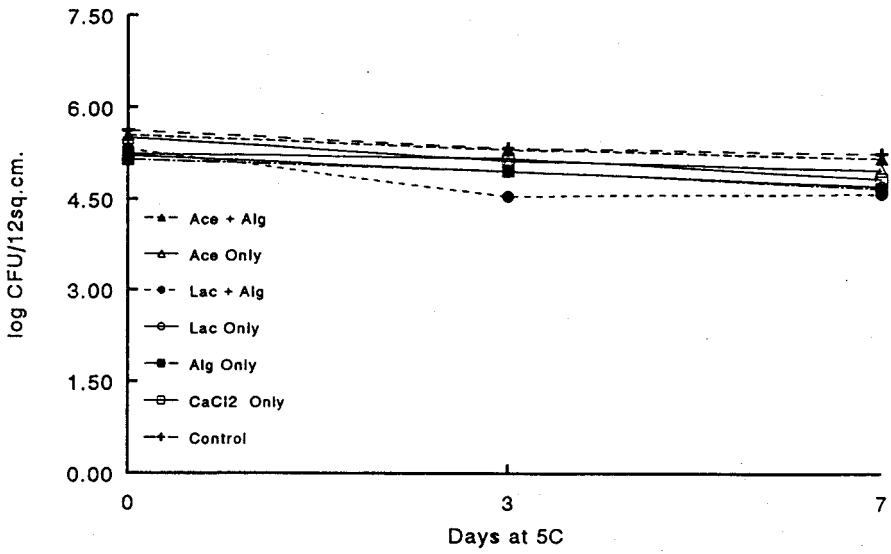


FIG. 4. SURVIVAL OF *E. COLI* 0157:H7 ON STERILE LEAN BEEF TISSUE TREATED WITH LACTIC AND ACETIC ACIDS APPLIED ALONE OR IN A CALCIUM ALGINATE GEL
Samples were enumerated using Tryptic Soy Agar. Closed symbols = alginate application, open symbols = acid applied alone. Triangles = acetic acid; circles = lactic acid; squares = no acid application, + = inoculated control tissue.

The mechanism responsible for the enhanced antimicrobial effect of acids immobilized in alginate is not known. It is possible that the acid remained in contact with the contaminating microflora on the tissue for a longer period of time in a moist microenvironment. This condition may result in an enhanced antimicrobial effect. Using the sodium alginate-$CaCl_2$ system, many different antimicrobial agents may be immobilized in the diffusible gel matrix simply by incorporating the agent in the alginate solution and/or the $CaCl_2$ cross-linking agent. This system has the added advantage of not requiring elevated temperatures for application. The structure and diffusion properties of the alginate gel matrix can be adjusted by varying the gelling or cross-linking agent concentrations. Incorporating acids in $CaCl_2$ solutions for the spray chilling medium would prevent the alginate coating from being removed from the tissue surface and may be a reasonable approach to incorporating this technique into carcass processing. Alginate(s) and $CaCl_2$ are both GRAS (Generally Recognized as Safe) substances. Immobilizing the antimicrobial agent in a calcium alginate gel may offer potential advances in decontaminating raw meat when applied to carcasses in an environment that is not moisture controlled. Experiments to test such methods are

currently underway. Additionally, using higher concentrations of organic acids in the alginate procedure will be examined using an actual whole carcass system.

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