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Inhibition of *Listeria monocytogenes* on Beef Tissue by Application of Organic Acids Immobilized in a Calcium Alginate Gel

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ABSTRACT

Organic acids added to calcium alginate gels and immobilized on lean beef tissue inoculated with *Listeria monocytogenes* (Lm) reduced the population significantly more than did acid treatment alone. Lactic acid (1.7% v/v) immobilized in alginate reduced counts by 1.3 $\log_{10}$ units vs 0.03 log unit decrease from the acid treatment alone. Acetic acid (2% v/v) reduced counts 1.5 and 0.25 log units, respectively. Over 7 days, Lm proliferated in samples without acid and/or alginate treatment. Differential counts on selective and non-selective agars indicated sublethal cellular injury occurred. Alginate coatings did not enhance acid inhibition on fat tissue. Immobilized agents may have potential for raw meat decontamination.

INTRODUCTION

THE USE OF sanitizing agents to decontaminate meat surfaces has been well studied (Smulders, 1987). Various workers have examined the use of organic acids such as acetic acid (Anderson et al., 1977, 1979), lactic acid (Snijders et al., 1985; Woolthuis and Smulders, 1985), chlorine (Kotula et al., 1974), and other chemicals (Reynolds and Carpenter, 1974; Crouse et al., 1988; Dickson, 1988; Acuff et al., 1987; Hamby et al., 1987) to reduce the microbial load on raw meat as a means to increase shelf life and reduce the potential of foodborne pathogens to cause disease. The major source of microorganisms in meat processing is the animal itself as well as the slaughtering and dressing process (Ayres, 1955). The initial microbial load on raw meat determines to a large extent both the safety of the product and its shelf life (NRC/NAS, 1985). Most microbial inhibitors are applied prior to carcass chilling and refrigeration or further handling. Acuff (1987) studied the effect of first decontaminating then vacuum packing subprimal cuts prior to fabricating steaks, and found no significant effect from decontamination of the vacuum packaged subprimal cut.

Immobilization techniques in microbial processes have been used for many applications, including production of chemicals (Chibata et al., 1986), microbial transformation and detoxification of environmental toxins and pollutants (O'Reilly and Crawford, 1989), and the preservation of milk (Champagne, 1990). That technology exploits the metabolism of whole, viable microbial cells that have been entrapped in a diffusible gel or immobilized in a matrix to carry out biochemical processes.

For food preservation, calcium alginate gels as coatings have been used to reduce shrinkage loss in chicken pieces (Mountney and Winter, 1961), beef and pork (Berlin, 1957), lamb (West et al., 1975; Lazarus et al., 1976), and a variety of other foods (McCormick, 1975) including fish, shrimp, onion rings, and mushrooms. A commerical alginate coating process known at Flavor-Tex® (Earle, 1968) was previously evaluated as a means of preventing shrinkage loss in lamb carcasses (Lazarus et al., 1976). Kirsop and Brocklehurst (1989) developed a system for incorporating organic acids into coatings and additives for cabbage and salad ingredients, respectively, to prolong shelflife. Kearney et al. (1990) used calcium alginate immobilized and lyophilized lactic acid bacteria to perform meat fermentations.

The objective of our study was to test the use of organic acids immobilized on the surface of inoculated beef tissue as a means of inhibiting microbial growth. The foodborne pathogen *L. monocytogenes,* which has been shown to grow and persist on beef tissue surfaces (Dickson, 1990), was chosen as a test organism.

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MATERIALS & METHODS

Bacterium

Listeria monocytogenes (Lm), strain Scott A, a human isolate of serotype 4b, was maintained in 75% glycerol at −20°C. Cultures were propagated in Tryptic Soy Broth (Difco Co., Detroit, MI) + 0.5% (w/v) Yeast extract broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) at 37°C. Five mL of a 16–18 hr culture was added to 495 mL of 0.1% (w/v) peptone water, pH 7.2, to obtain a viable cell density of about 10^7 CFU/mL for use as an inoculum.

Preparation and inoculation of beef tissue

Lean beef muscle and fat tissue were obtained from the abattoir at the Roman L. Hruska U.S. Meat Animal Research Center (Clay Center, NE), sliced to 0.5 cm thickness and frozen. The tissue was sterilized by gamma irradiation at 42 kGy. The tissue was allowed to partially thaw and was aseptically cut into pieces measuring 2 × 2 × 0.5 cm (12 cm^2 total surface area). The tissue pieces were brought to room temperature and inoculated by placing the tissue pieces in a vessel containing about 50 mL of the bacterial suspension described above. Immediately following 20 min of room temperature incubation, the tissue pieces were subjected to their respective treatments.

Reagent preparation

One percent (w/v) sodium alginate (high viscosity, Sigma Chemical Co., St. Louis, MO) was prepared in distilled water, dispensed to a plastic screw cap centrifuge tube (40 mL/tube) and autoclaved for 17 min at 121°C. The resulting pH of this solution was 7.22. Two percent (v/v) acetic acid and 1.7% lactic acid solutions, were prepared by adding acid to 90 mM $CaCl_2$ in distilled water. Glacial acetic acid (Fisher Scientific, Pittsburgh, PA) and D,L-lactic acid (Sigma Chemical Co., St. Louis, MO) were used. Resulting pH values were 2.75 for the acetic + $CaCl_2$ and 2.27 for lactic + $CaCl_2$ solutions. The solutions were dispensed in 50 mL capacity centrifuge tubes (40 mL per tube) and autoclaved as above.

Acid immobilization treatments and experimental design

Upon exposure to calcium and certain other divalent cations, alginate or alginic acid solutions of specific concentrations form gels. The cations form ionic bridges between alginate polysaccharide polymers resulting in the formation of a diffusible lattice structure or gel (Grant et al., 1973). To form gels for experimental use, the following treatments were used: (AgA) = alginate + acetic acid, (AgL) = alginate + lactic acid, (Ag) = alginate only, (A) = acetic acid only, (L) = lactic acid only, (CC) = calcium chloride only, (CT) = control, untreated tissue. All treatments were performed at room temperature (25°C). To samples (AgA), (AgL), (Ag) the following procedure was performed: the inoculated tissue was attached to a sterile clamp and immersed in the alginate solution for 1 min., then transferred to the $CaCl_2$ solution containing the respective acid or no acid (alginate con-

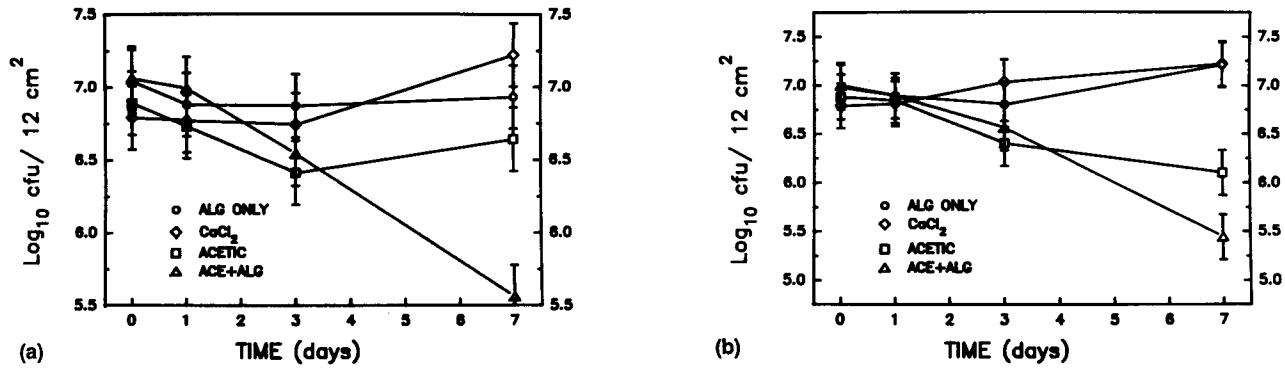

Fig. 1—Growth of L. monocytogenes on lean beef tissue treated with acetic acid both with and without alginate. (1a) enumerated on TSAYE agar; (1b) enumerated on Oxford agar.

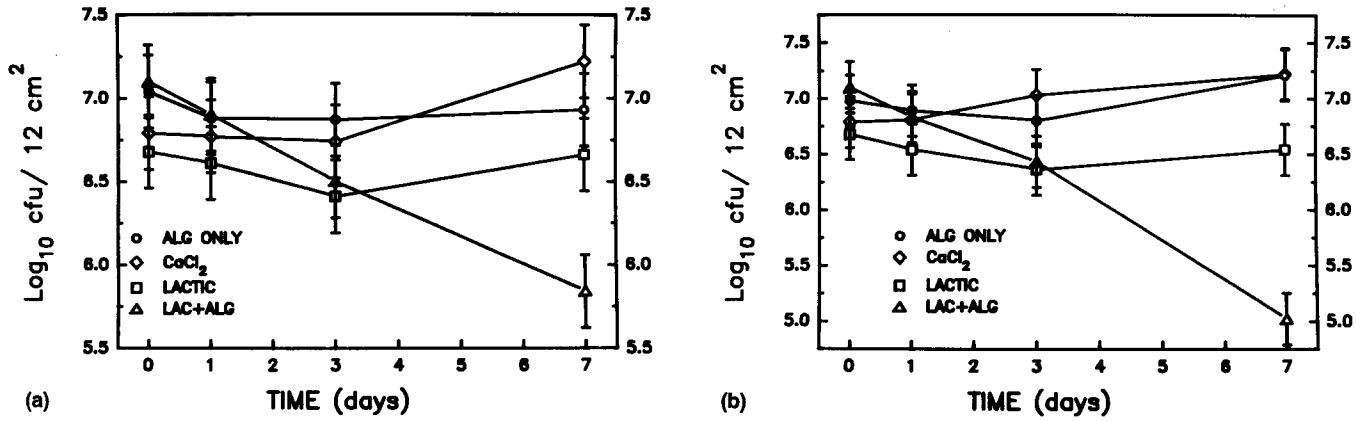

Fig. 2—Growth of L. monocytogenes on lean beef tissue treated with lactic acid both with and without alginate. (2a) enumerated on TSAYE agar; (2b) enumerated on Oxford agar.

Table 1—Log reduction[a] in viable counts of L. monocytogenes on lean beef tissue after 7 days at 5°C

Treatment	Alginate		No alginate
	TSAYE Agar		
Acetic[b]	1.5		0.25
Lactic[b]	1.26		0.02
Alginate control[c]	0.01		−0.44
Control tissue[d]		−0.61	
	Oxford Agar		
Acetic[b]	1.56		0.78
Lactic[b]	2.08		0.14
Alginate control[c]	−0.23		−0.44
Control tissue[d]		−1.16	

[a] Difference in counts within each treatment between day 0 and day 7.
[b] See Materials & Methods section for explanation of treatments.
[c] Alginate control, Alginate = no acid applied in alginate dip. Alginate control, no Alginate = $CaCl_2$ dip only, no acid or alginate applied.
[d] Control tissue = inoculated, untreated lean tissue.

trol) for 1 min. The treated sample was transferred to a holding container and stored at 5°C until assayed.

Treatments (A), (L), and (CC) included all steps except immersion in the alginate solution. Sample (CT) was clamped and placed in a holding container. Four pieces of inoculated meat were subjected to each of the 7 treatments. Following treatment, the tissue pieces were placed in sterile foil covered beakers and held at 5°C until sampled.

Microbiological assay

Following 0, 1, 3, and 7 days of 5°C incubation, the tissue sample was placed in a stomacher bag with 99 mL of 0.1M phosphate buffer (pH 7.2) and stomached for 2 min in a Stomacher 400 (Tekmar, Inc., Cincinnati, OH). Samples were then plated on Tryptic Soy Agar (BBL) or Oxford Listeria Selective agar (Oxoid, USA) by means of a Spiral Plating apparatus (Spiral Systems Instruments, Bethesda, MD). Plates were counted after incubation for 36 hr at 35°C (Messer et al., 1984).

Statistical analysis

Three replications of the experiment were carried out. Data were analyzed using SAS® (SAS Institute, Inc., 1982) and the general linear model. Inoculum counts were used as a covariate to normalize data from treatment replications.

RESULTS & DISCUSSION

IMMOBILIZED organic acids resulted in a significantly greater ($p<0.05$) reduction in viable count of Lm attached to lean beef surfaces at day 7 when compared to acid treatments without alginate (see Table 1 and Fig. 1 and 2). The effect was not observed on samples tested from days 0, 1, and 3. Untreated inoculated meat samples supported the growth of Lm through day 7, at which point the populations increased more than one $\log_{10}$ cycle (data not shown).

After day 0, Lm counts on the inoculated control treatments Ag and CC began to increase or remained constant through day 7. At day 7, the TSAYE counts of lean tissue from treatment CC were about 0.16 $\log_{10}$ units higher than treatment Ag (Fig. 1a, 2a). Slight decreases in surface counts on meats treated with alginate have previously been reported (West et al., 1975; Lazarus et al., 1976). There were no significant differences between treatments AC and CC counts whether obtained from TSAYE or Oxford agar at day 7 (Table 1,2).

The largest reduction in viable numbers (1.5 $\log_{10}$ units) occurred with immobilized acetic acid (Table 1) and immobilized lactic acid (1.26 $\log_{10}$ units) which were not significantly different when assayed using TSAYE agar. Counts from TSAYE agar represent the remaining viable population of Lm including both injured and uninjured cells. Counts obtained from Oxford agar (Table 1) showed that the immobilized lactic acid treatment reduced the viable cell counts 0.52 $\log_{10}$ units

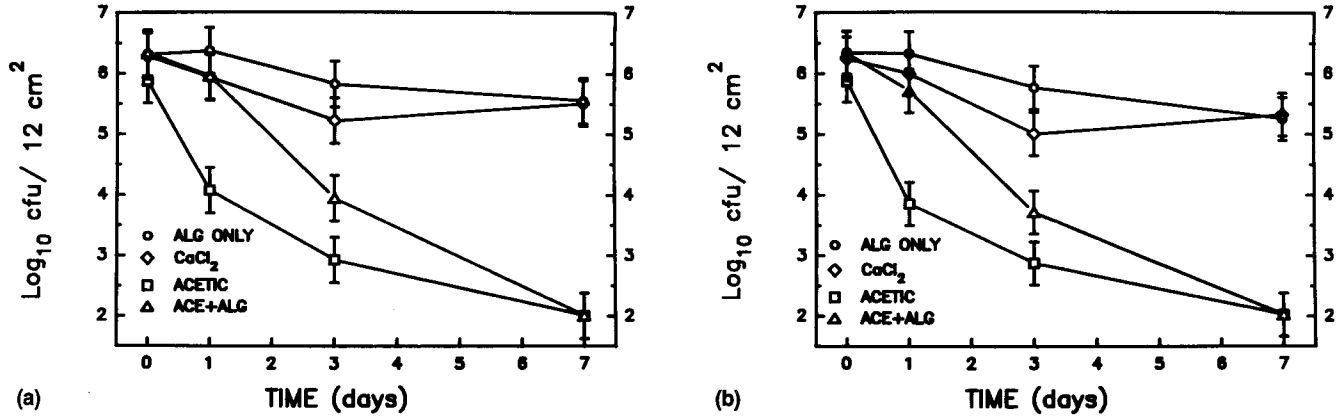

Fig. 3—Growth of L. monocytogenes on fat beef tissue treated with acetic acid both with and without alginate. (1a) enumerated on TSAYE agar; (1b) enumerated on Oxford agar.

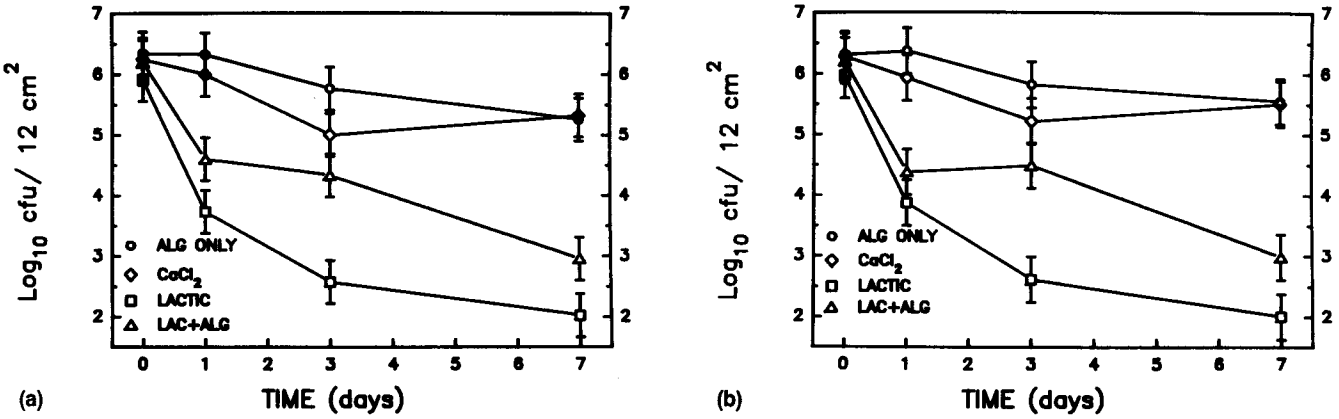

Fig. 4—Growth of L. monocytogenes on fat beef tissue treated with lactic acid both with and without alginate. (2a) enumerated on TSAYE agar; (2b) enumerated on Oxford agar.

more than the immobilized acetic acid treatment. Differences in the degrees of inhibition as determined by nonselective TSAYE agar and selective Oxford agar ($\log_{10}$ 1.26 vs 2.08, respectively) indicated that sublethal cellular injury probably occurred in the case of the lactic acid treatments. This same effect was not observed in the case of A + Ag treatments (AgA, 1.50 vs 1.56 log reduction as calculated from TSAYE and Oxford agars) and to a lesser extent in the case of the acetic control treatments (A, 0.25 vs 0.78 log reduction as calculated from TSAYE and Oxford agars, respectively). Comparing log reductions in the control samples (Ag and CC, Table 1) calculated from TSAYE agar counts and Oxford agar, there was no evidence of sublethal cellular injury. Initial inoculum counts determined on TSAYE and Oxford agars were not significantly different ($p<0.05$, data not shown).

Within acid treatments of inoculated fat tissue, there was no significant difference ($p>0.05$) between Lm counts and reductions by day 7 with or without alginate coatings (Table 2, Fig. 2 and 3). Lm did not proliferate on the control fat tissue as they did with lean tissue but declined in numbers over 7 days by almost 1 log cycle (Table 2). A similar trend has been reported by Dickson (1990a, b) who reported that after 14–21 days at 5°C, viable counts of Lm decreased about 2 log cycles but remained constant for 42 days on beef tissue.

Acetic acid treatments reduced the numbers of viable Lm more than the lactic acid treatments. Although not statistically significant, the treatment of fat tissue with acetic acid + alginate coating reduced the population by 0.45 $\log_{10}$ units more than without alginate (Table 2). Inhibition of Lm by acetic acid alone occurred rapidly between days 0 and 1 and continued to day 7. In the case of lactic acid treated fat tissue, acid

Table 2—Log reduction[a] in viable counts of L. monocytogenes on fat beef tissue after 7 days at 5°C

Treatment	Alginate		No alginate
	TSAYE Agar		
Acetic[b]	4.33		3.88
Lactic[b]	3.23		3.97
Alginate control[c]	0.76		0.78
Control tissue[d]		0.89	
	Oxford Agar		
Acetic[b]	4.31		3.86
Lactic[b]	3.24		3.89
Alginate control[c]	1.09		0.92
Control tissue[d]		1.12	

[a] Difference in counts within each treatment between day 0 and day 7.
[b] See Materials & Methods section for explanation of treatments.
[c] Alginate control, Alginate = no acid applied in alginate dip. Alginate control, no Alginate = $CaCl_2$ dip only, no acid or alginate applied.
[d] Control tissue = inoculated, untreated lean tissue.

treated samples without alginate had a 0.67 $\log_{10}$ unit greater reduction in counts than the alginate + acid treated samples (Table 2). Within the treated fat tissue samples, there was no evidence of sublethal cellular injury from selective and nonselective count data (Table 2) as occurred with Lm populations on lean tissue.

Overall, the bactericidal effect of acid sanitizers on Lm was much more pronounced on fat than lean tissue. This was reported by Dickson (1988) in a study of the reduction of bacteria attached to fat tissue. Possibly the reduced surface population of Lm on fat tissue was due to the inability of non-detergent diluents to rinse off the organisms attached to fatty material. It has been demonstrated that addition of Tween 80 to rinse

diluents enhanced recovery of organisms bound to fat (Dickson, 1990b) when compared to lean beef tissue.

The reductions in populations were similar to those reported by many others, which ranged from $0-3$ $\log_{10}$ units. Snijder et al. (1985) reported a 2.4 log reduction in aerobic plate count 3 days post-spraying of beef carcasses with 1% lactic acid. The same group treated pig bellies with 5% lactic acid and reported as much as a 3.4 log reduction in aerobic plate count 2 days post-treatment. The delay in the lethal effect on lean tissue was not unusual considering the short time of initial exposure to the acid treatments. Smulders and Woolthius (1985) reported a delayed inhibitory effect on lactic acid bacteria applied to meat surfaces. Anderson et al. (1977, 1979) reported a 3 log reduction in aerobic plate count on beef carcasses treated with a 3% acetic acid spray. The bactericidal effect of our immobilized acid treatments was dependent on the diffusion of the acid through the gel to contact the meat surface. Lethality or inhibition caused by direct application of the acid was not dependent on diffusion time of the agent to reach the target organism.

We hypothesized the enhanced bactericidal effect on lean tissue by the alginate immobilization was due to the alginate coating increasing the contact time that the inhibiting acid maintained with the meat surface before evaporation or neutralization. Additionally, the rate of moisture loss was slowed by alginate coatings (West et al., 1975; Lazarus et al., 1975) which could have maintained a moist surface environment for the acid to act on the tissue surface. Alternatively, with no coating, the lean tissue surfaces would dehydrate rapidly and the acid would have less time in a hydrated environment to inhibit attached bacteria. Since these experiments were performed using sterile tissues, we did not speculate regarding the effects of competing microflora on the inhibition enhancement from alginate coatings.

This same concept could be tested using other immobilizing agents or coatings such as agar, xanthan, corn starch or calcium pectate gels. The thickness and the structural integrity of the calcium alginate gel is dependent on specific ratios of calcium ion to sodium alginate, the mannuronic to guluronic acid ratio of the alginate, and the reaction time between alginate and the calcium ion source (Grant et al., 1973; Morris, 1986).

Using calcium alginate offers many advantages and variations for immobilizing antimicrobial agents. First, the gel system fluidity is not temperature dependent, as are agar coatings. Although sodium alginate will gel at pH extremes, we were able to circumvent this problem by including the acid inhibitor in the cross linking agent ($CaCl_2$). Secondly, alginate can immobilize whatever is suspended or immobilized within the sodium alginate solution when cross-linked with calcium. Other types of inhibitors could be included directly in the alginate solution. Thirdly, living cells can be immobilized in alginate gels (Kearney et al., 1990) which includes the prospect of using bacteriocin producing microorganisms to inhibit the growth of specific pathogens on food surfaces. Lastly, alginate can be sprayed on the surface of carcasses (West et al., 1975; Lazarus et al., 1975) and has GRAS status so it would fit into currently used meat processing operations.

CONCLUSIONS

LACTIC AND ACETIC acids applied in immobilized calcium alginate gels were more effective at inhibiting the growth of *Listeria monocytogenes* than acids applied alone on lean beef tissue. No treatments were effective until after 3 days of incubation at 5°C. Alginate immobilization of lactic and acetic acids did not enhance the bactericidal effects on fat tissue. Organic acids incorporated into alginate gels showed potential for use in sanitizing and preserving raw meat.

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