Antimicrobial Effects of Corn Zein Films Impregnated with Nisin, Lauric Acid, and EDTA

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ABSTRACT

Bacterial growth during food transport and storage is a problem that may be addressed with packaging materials that release antimicrobials during food contact. In a series of five experiments, EDTA, lauric acid (LA), nisin, and combinations of the three antimicrobial agents were incorporated into a corn zein film and exposed to broth cultures of Listeria monocytogenes and Salmonella Enteritidis for 48 h (sampled at 2, 4, 8, 12, 24, and 48 h). Four experiments used starting cultures of 10⁶ CFU/ml in separate experiments tested against each bacterium; the fifth experiment examined the inhibitory effect of selected antimicrobial agents on Salmonella Enteritidis with an initial inoculum of 10⁴ CFU/ml. L. monocytogenes cell numbers decreased by greater than 4 logs after 48 h of exposure to films containing LA and nisin alone. No cells were detected for L. monocytogenes (8-log reduction) after 24-h exposure to any film combination that included LA. Of all film agent combinations tested, none had greater than a 1-log reduction of Salmonella Enteritidis when a 10⁴ CFU/ml broth culture was used. When a 10⁴ CFU/ml of Salmonella Enteritidis initial inoculum was used, the films with EDTA and LA and EDTA, LA, and nisin were bacteriostatic. However, there was a 3-log increase in cells exposed to control within 24 h. The results demonstrate bacteriocidal and bacteriostatic activity of films containing antimicrobial agents.

Direct addition of antimicrobials results in some loss of activity at the surface because of leaching into the food core, enzymatic activity, and reaction with other food components such as lipids and proteins (5, 9, 17). Listeria monocytogenes is a concern in food with extended shelf life because of the ability to tolerate salt, pH changes, inadequate pasteurization, and refrigerated temperatures (2, 3). If an antimicrobial can be released from the package during an extended period, the activity can be extended into the transport and storage phase of food distribution.

Non–food grade antimicrobial polymers are available, several of which use Triclosan (18), silver (1), and quaternary ammonium salts (18). In the United States, the number of approved antimicrobial agents for food contact are limited at present and much of the work has focused on using organic acids and bacteriocins. Ming et al. (11) coated cellulose casings with pediocin to inhibit the growth of L. monocytogenes on cooked meats. Salmonella Typhimurium–inoculated poultry skin was reduced by 0.4 to 2.1 logs after exposure to nisin-coated polymer films (12). Siragusa et al. (20) incorporated nisin into polyethylene-based films and observed 1.4-log reduced growth of Brochothrix thermosphacta on vacuum-packaged beef surfaces compared with films not impregnated with nisin. Nisin activity is restricted to gram-positive bacteria (17, 20) but can be active against gram-negative bacteria when combined with chelators and surfactants (21, 22). Larsen (10) found that 15 mM EDTA was effective against Escherichia coli when used in corn zein films with different nisin concentrations. Lauric acid (LA) at concentrations of 4% (400 mg/ml) and 8% (800 mg/ml) in corn zein films effectively inhibited Lactobacillus plantarum (16). Nisin, at various concentrations alone and with other antimicrobial agents incorporated into films, was effective against various organisms, including L. plantarum, L. monocytogenes, E. coli, and Salmonella sp. (4, 10, 15). The study objective was to determine the effect of corn zein films impregnated with nisin, LA, and EDTA on L. monocytogenes and Salmonella Enteritidis.

MATERIALS AND METHODS

Five experiments were conducted to fulfill the objective. Experiments 1 and 2 compared EDTA, LA, and nisin (singly) in corn zein films for inhibiting L. monocytogenes and Salmonella Enteritidis, respectively. Experiments 3 and 4 compared the combinations of EDTA and LA; EDTA and nisin; and EDTA, LA, and nisin in corn zein films for inhibiting the same two bacteria, respectively. Experiment 5 tested lower inoculum levels (10⁴ CFU/ml) of Salmonella Enteritidis in a growth medium exposed to films containing LA; EDTA and LA; and EDTA, LA, and nisin.

Film formation. The corn zein films were formed using the casting method described by Gennadios and Testin (6). Corn zein (6.75 g) (F-4000 regular grade, Freeman Industries, Tuckahoe, N.Y.) was dissolved in 40.6 ml of 95% ethanol with stirring. Glycerin (1.9 ml) (Fisher Scientific, Fairlawn, N.J.) was added as a plasticizer and the mixture was heated slowly to a boil. The mix-
ture was boiled 5 min to help reduce gas bubbles in the films during casting. For antimicrobial-incorporated films, antimicrobial agents were mixed with 10 ml of the film solution in glass tubes just before casting. A mortal and pestle were used to reduce the particle size of the EDTA and nisin, then the fine particles were dispersed in the film solution using a sterile glass rod. LA was dissolved into the film solution. Five milliliters of the film mixtures were pipetted into level petri dishes (100-mm diameter by 15-mm depth) and allowed to dry at room temperature (23 to 25°C) overnight. After casting, five measurements were made on each sample using an electronic micrometer (Model 49–60, Testing Machines, Inc., Amityville, N.Y.), and the mean thickness was calculated to the nearest 0.005 mm.

**Antimicrobial agents.** EDTA (J.T. Baker Inc., Phillipsburg, N.J.), the acid form of LA (J.T. Baker), and nisin (Sigma Chemical Co., St. Louis, Mo.) were added separately to the three glass tubes containing 10 ml of film mixture at concentrations of 5.58, 40, and 0.0375 mg/ml. The final concentration of each antimicrobial compound per film was 27.9, 200, and 0.188 mg for EDTA, LA, and nisin, respectively. Films with combinations of the antimicrobial agents were also formed using the concentrations stated: EDTA and LA, EDTA and nisin, LA and nisin, and all three together. With each test, a control film was also formed with no antimicrobial agents added.

**Nisin activity.** The nisin was reported to be 2.5% pure, with the remaining components being listed as sodium chloride and milk solids. The activity of the nisin was measured using an arbitrary unit (AU) dilution method. A total of 1.5 mg of the stock nisin was mixed in 1 ml of distilled, deionized water. A 10-μl aliquot was taken from this 1.5-mg/ml solution and diluted at 1: 1, 1:2, 1:4, and 1:8; then 10 μl of these were spotted on a lawn of *L. plantarum*. The lowest dilution showing a clear zone was at 1: 4; therefore, 10 μl of the 1.5-mg/ml solution was equated to 4 AU. This converts to 400 AU/ml of the 1.5-mg/ml solution or 400 AU/1.5 mg of the stock nisin. Calculation of activity is therefore 266.6 AU/mg of stock nisin or 2.67 × 10^5 AU/g.

**Enumeration.** *L. monocytogenes* ATCC 15313 and *Salmonella Enteritidis* phage type 13 resistant to nalidixic acid (Southeastern Poultry Research Laboratory, Athens, Ga.) were used. *L. monocytogenes* was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) and incubated aerobically for 16 h at 37°C. Two 10-ml tubes of *L. monocytogenes* were then centrifuged for 20 min in 1,500 × g, decanted, washed with 0.1% peptone (Difco), centrifuged 20 min, and decanted. The two pellets of organisms were placed into 99 ml of peptone water to obtain an inoculum of approximately 10^6 CFU/ml. The 10^6-CFU/ml count was verified by serial dilutions of the inoculum suspended in peptone water and plating in BHI agar. Fifteen milliliters of the inoculum were added to each of the petri plates containing the films. The film with no added antimicrobials was used as a control. The plates were put on an orbital shaker and rotated at room temperature (23°C) at 50 rpm. After 0, 2, 4, 8, 12, 24, and 48 h, 0.1-ml samples were taken from the petri dishes, diluted, and plated in duplicate on BHI agar. The plates were incubated at 37°C in an aerobic chamber for 48 h. The number of colonies on each plate was counted and reported as CFU/ml.

The same tests were performed using *Salmonella Enteritidis* resistant to nalidixic acid grown in Trypticase soy broth (Difco) at 37°C for 16 h. Shaking was necessary and only one tube (pellet) of *Salmonella Enteritidis* in 99 ml of peptone water was needed to obtain 10^6 CFU/ml. Also, 3.0 ml of nalidixic acid (Sigma) per 200 ml of media was added to the Trypticase soy agar when plat-

**RESULTS AND DISCUSSION**

Film thickness ranged from 0.61 to 0.81 mm, with an average of 0.68 ± 0.06 mm. The addition of antimicrobial compounds had no effect on the film thickness or the variation of film thickness compared with films with no added compounds.

**Effects of EDTA, LA, and nisin-impregnated film effects on *L. monocytogenes*.** The control film without antimicrobial agents and the films containing EDTA caused no change (*P* > 0.05) in *L. monocytogenes* populations after 48 h of exposure (Fig. 1). This was not surprising since EDTA is a chelating agent that enhances the effect of other antimicrobials but has little antagonistic effect itself (21, 22). LA and nisin had significant antibacterial effects
(P < 0.0001). LA reduced the population of L. monocytogenes by 0.1 log (CFU/ml) at 2 h, 0.8 log at 12 h, 2.9 logs at 24 h, and to 5.0 logs after 48 h. Padgett et al. (16) reported films containing LA alone did not have a significant effect on L. plantarum when using a zone of inhibition method, but LA reduced the population of L. plantarum by 5 logs in 6 h when the film was in contact with a liquid broth. The film containing nisin reduced L. monocytogenes population by 1.0 log at 2 h and 5.5 logs after 48 h. Nisin concentrations in heat-pressed corn zein films as low as 0.1 mg/g inhibited LA containing LA films alone did not have a significant effect on L. plantarum, P. L. plantarum, L. monocytogenes, and L. monocytogenes sp. culture was used, the nisin treatment was applied directly in the previous study rather than in a film, and different concentrations of antimicrobials were used (lower concentration of nisin, higher concentration of EDTA). Broth cultures exposed to corn zein film containing EDTA and LA and EDTA, LA, and nisin had lower populations than cultures exposed to other films after 48 h; however, these differences were not significant from a practical standpoint.

At 2, 4, and 8 h, Salmonella Enteritidis log counts were 7.93, 7.99, and 8.13 CFU/ml, respectively. The mean log reduction values were not different (P > 0.05) from 8.18 CFU/ml at 12 h to 8.19 CFU/ml at 48 h. The results suggest no bacteriocidal effect on Salmonella Enteritidis by the films containing single antimicrobial agents. For some antimicrobial agents, the outer membrane lipopolysaccharide portion of gram-negative bacteria is difficult to penetrate. When EDTA disrupts the lipopolysaccharide layer, there is an increase in cell permeability (22), allowing nisin to disrupt cell activity and eventually resulting in cell lysis. It was concluded that EDTA and nisin in this study needed to be in simultaneous contact with the bacterial culture to occur; therefore, only the main effects for treatment and the main effects for time were compared. Salmonella Enteritidis displayed little growth during exposure to the control film up to 48 h. The effect of the control film was not different (P > 0.05) from the EDTA- and nisin-containing films (Table 1). The results of the present study were similar to those found by Stevens et al. (21), in which EDTA and nisin (not incorporated into films) were tested against Salmonella Typhimurium with no inhibition. The corn zein film containing LA displayed no increase in culture population, and although different (P ≤ 0.05) from the other film treatments, LA should be considered ineffective in reducing Salmonella Enteritidis under these conditions. The effect of the control film was not different (P > 0.05) from that of the films containing EDTA and nisin or LA and nisin. Stevens et al. (21) reported that after 1 h of direct exposure to 50 μg/ml of nisin and 20 mM EDTA, Salmonella Enteritidis Puerto Rico no. 1 was reduced 3.6 logs. The results of the present study may differ from those reported by Stevens et al. (21) because a different Salmonella sp. culture was used, the nisin treatment was applied directly in the previous study rather than in a film, and different concentrations of antimicrobials were used (lower concentration of nisin, higher concentration of EDTA). Broth cultures exposed to corn zein film containing EDTA and LA and EDTA, LA, and nisin had lower (P ≤ 0.05) populations than cultures exposed to other films after 48 h; however, these differences were not significant from a practical standpoint.

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Table 1. Effect of different antimicrobial films exposed to Salmonella Enteritidis suspended in 0.1% peptone water

<table>
<thead>
<tr>
<th>Antimicrobial treatment</th>
<th>Mean log counts (CFU/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>8.14 AB</td>
</tr>
<tr>
<td>EDTA</td>
<td>8.11 B</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>8.02 c</td>
</tr>
<tr>
<td>Nisin</td>
<td>8.16 A</td>
</tr>
<tr>
<td>EDTA and lauric acid</td>
<td>7.89 d</td>
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<tr>
<td>EDTA and nisin</td>
<td>8.02 c</td>
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<tr>
<td>Lauric acid and nisin</td>
<td>8.02 c</td>
</tr>
<tr>
<td>EDTA, lauric acid and nisin</td>
<td>7.82 e</td>
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a n = 36; SE = 0.02. b Means having the same superscript are not significantly different (P > 0.05).
FIGURE 3. Effects of LA; EDTA and LA (EL); EDTA and nisin (EN); and EDTA, LA, and nisin (All) in corn zein films on a 10^4 CFU/ml Salmonella Enteritidis starting population. Asterisk indicates P value; n = 6; SEM = 0.01.

have a killing effect. A statistically significant increase in the Salmonella Enteritidis population was observed at and after 8 h; however, these are again fractions of log values and are not noteworthy from a practical standpoint.

Effect of film impregnated with LA; EDTA and LA; EDTA and nisin; and EDTA, LA, and nisin on a 10^4 CFU/ml Salmonella Enteritidis population. The starting 10^8 CFU/ml population would not be expected to increase in cell density much beyond the 10^8 level; therefore, the bacteriostatic properties of these treatments at lower initial inoculation levels were evaluated in experiment 5. The most inhibitory antimicrobial treatments from the 10^8-CFU/ml experiment were used with a 10^4-CFU/ml Salmonella Enteritidis inoculum.

For all of the film samples, Salmonella Enteritidis grew from 0 to 48 h (Fig. 3). The control and LA films did not differ (P > 0.05) in cell growth, with Salmonella Enteritidis cell numbers increasing from 10^4 CFU/ml to 10^10 CFU/ml. With the EDTA and nisin film, Salmonella Enteritidis cell numbers increased 4 logs after 48 h. The EDTA and LA and the EDTA, LA, and nisin films held the increase in cell numbers to 1 log after 48 h, which was 5 logs less than the control film at 48 h. Natrajan and Sheldon (12) reported a 0.4- to 2.1-log reduction of Salmonella Typhimurium for inoculated broiler skin exposed to polymer films coated with nisin formulations. In a separate experiment, these researchers obtained up to a 4.6-log reduction by broiler drumsticks coating with agar or alginate gels containing a nisin mixture (13). The presence of moisture at the meat surface was determined to be beneficial for the efficacy of nisin formulations (12, 13).

Temperature, pH, time, growth medium, and film properties may have affected the antimicrobial activity of the agents tested. For example, nisin may be less effective in corn zein films because of binding with the corn proteins. Also, nisin is less stable and soluble at higher pH ranges (8). The film solution pH was approximately 4.6, whereas the cell solution on the films had a pH of 6.0 to 7.0. However, the nisin incorporated into the films was very effective against L. monocytogenes, which suggests that it was not binding (or had very little binding) with the corn proteins of the films and was stable and active at the pH ranges used in this study. The application of package-based biocides to reduce postprocess growth of food pathogens has promise. The application of antimicrobial films might allow for migration of the antimicrobial to the film surface and therefore a continued antimicrobial effect at the food surface during extended exposure. Direct addition of antimicrobials to food will result in an immediate reduction of bacterial populations but direct addition may not address the recovery of injured cells or the growth of cells that were not destroyed by direct addition. Thus, antimicrobial films may have applications for both fluid and semisolids foods by inhibiting bacterial growth hours and days after packaging. The growth and death rates of bacteria will vary for each growth medium; therefore, conclusions on how antimicrobial films will perform with a food product must be determined for each food application.

REFERENCES