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Combined inhibitory effect of nisin with EDTA against *Listeria monocytogenes* in soy-protein edible coating on turkey frankfurters stored at 4°C and 10°C

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ABSTRACT

Several food contamination outbreaks are linked to *Listeria monocytogenes*. More effective methods are needed to prevent the growth and recontamination of *L. monocytogenes* on ready-to-eat (RTE) food products. Therefore, the objectives of this study were to evaluate the inhibitory activities of nisin (10,000 IU/mL), EDTA (sodium Ethylenediaminetetraacetic acid: 1.6 mg/mL), and the combination of nisin (10,000 IU/mL) with EDTA 1.6 mg/mL either in brain-heart-infusion (BHI) media at 37°C for 72 h or in soy-protein edible coating on the surface of full-fat commercial turkey frankfurters against the cell populations of approximately 10⁶ colony forming units (CFU/mL) of *L. monocytogenes*. The surface-inoculated frankfurters were dipped into soy-protein film forming solutions with and without the addition of antimicrobial agents [(nisin (10,000 IU) or EDTA (0.16%) or the combination)] and stored at either 4°C or 10°C. The inhibitory effects of edible coatings were evaluated on a weekly basis for 45 d. The greatest inhibitory activities of 6 log cycle reductions of *L. monocytogenes* were found when nisin was combined with EDTA and eliminated 6 log cycles of *L. monocytogenes* in both systems. In the combined nisin (10,000 IU) with EDTA (0.16%) treatment, the *L. monocytogenes* population was reduced to undetectable levels after 15 h or 7 d incubation in BHI at 37°C or on turkey frankfurters stored at 4°C and 10°C, respectively. This research has demonstrated that the use of an edible film coating containing nisin with EDTA is a promising means of controlling the growth and recontamination of *L. monocytogenes* on RTE meat products.

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MEET THE STUDENT-AUTHOR

I grew up in Tulsa, Okla., and graduated from Union High School in 2002. I will graduate from the University of Arkansas in May 2006 with a B.S.A. in food science and a minor in Spanish. I have worked as a technical assistant in laboratories within my department for the past three years. I worked in the food safety lab last summer, which led me to this particular research project. Dr. Naval Hettiarachchy served as my research mentor and advisor. I also have served as vice president and now president of the Food Science Club. I play on the University of Arkansas' women's Ultimate Frisbee team. I have been involved with Campus Crusade for Christ while in college and am part of The Grove Church. I am a student member of the Institute of Food Technologists and Gamma Beta Phi honors fraternity. I really enjoy doing anything outside and traveling. I have loved my time at the University of Arkansas and the opportunities it has provided. After graduation I plan to work in the food industry to gain some experience in this chosen field. Someday I would like to work in foreign affairs in a food science-related field.



Emily Bennett

INTRODUCTION

Listeria monocytogenes is one of the foodborne pathogens that causes the highest mortality rate (20%) in people in the U.S. (CDC, 2003). Recontamination of ready-to-eat (RTE) poultry meat with *L. monocytogenes* has posed a major health concern to the general public and was re-addressed in a recent Food Safety and Inspection Service (FSIS) directive, published in the Federal Register, requesting new standards for the processing of these meat products (Federal Register, 2003). The Food and Drug Administration (FDA) is continually coordinating, conducting, and supporting research to evaluate the effectiveness of existing commercial treatments (e.g., post-packaging pasteurization, bacteriocins, irradiation, high pressure processing, and inhibitory compounds), and developing new technologies that can eliminate or prevent the growth of *L. monocytogenes* in RTE poultry and meat products (FDA/CFSAN 2003). These demands have led to a renewed interest in the use of antimicrobial combinations for effective control on foodborne pathogens.

Nisin is a bacteriocin produced by *Lactococcus lactis* sub sp. *Lactis* fermentation and is recognized as a safe

biological food preservative. Nisin acts upon the bacterial cell membrane by dissipation of proton motive force (PMF) through pore formation. The formation of pores by nisin leads to rapid removal of free amino acids, ATP, and cations from the cell (Montville et al., 2001). Nisin is effective in controlling a wide range of Gram-positive bacteria, including *L. monocytogenes* (Ko et al., 2001; Siragusa et al., 1999).

EDTA is a safe, economical metal chelator, and it facilitates the activity of nisin by destabilization of the bacterial cell membrane. EDTA has been used in combination with other bacteriocins for enhancing antimicrobial activity. It effectively binds magnesium ions in the lipo-polysaccharide layer of a gram negative organism to produce microbial cells with increased susceptibility to antimicrobials like nisin. Research has shown the significant reductions of microbial populations of Gram positive and negative organisms when nisin was combined with EDTA (Hoffman et al., 2001; Stevens et al., 1991).

To date, limited information is available on nisin with EDTA combinations in antimicrobial, edible film coating to control *L. monocytogenes* on RTE meat products. Therefore, the objectives of this study were: (1) to evaluate the inhibitory activity of nisin, EDTA, and combina-

tion of nisin with EDTA in a laboratory medium at 37°C; and (2) to evaluate the inhibitory effects of these compounds incorporated into soy-protein coatings on turkey frankfurters against *L. monocytogenes* stored at 4°C and 10°C for 45 d.

MATERIALS AND METHODS

Brain-Heart-Infusion broth (BHI) was purchased from Becton Dickinson Microbiology Systems, Sparks, Md. The Listeria Selective Agar (Oxford formulation) was purchased from EM Science, Gibbstown, N.J. *Listeria monocytogenes* strain V7, serotype 1/2 a, (FDA) was provided by Dr. Johnson's Laboratory, Center for Food Safety and Quality Research, University of Arkansas, Fayetteville. Soy protein isolate (PRO-FAM® 974, protein content >90%) (ARDEX®) was obtained from Archer Daniels Midland, Decatur, Ill. Glycerol and analytical-grade sodium EDTA were purchased from Sigma Chemical Co., St. Louis, Mo. A commercial sample of nisin, Nisaplin®, was obtained from Alpin & Barrett Ltd. Trowbridge, Wilts, England. Whirl-Pak® bags (120 mL capacity, 7.5 cm x 18.5 cm) were purchased from National Account Service Company LLC®, Fort Atkinson, Wisc.

Preparation of turkey frankfurter samples

Commercial turkey frankfurters with a fat content of 21% were purchased from a local grocery store and cut into 1-cm cubes. Each cube was individually packaged in a Whirl-Pak® bag. These cubed samples were sterilized to eliminate any natural microbial flora by a linear electron accelerator at a 30-kGy dosage at Texas A & M University, College Station. The irradiated samples were kept in frozen storage in our laboratory facility until use.

Culture preparations

A pure culture of *L. monocytogenes* was taken from a frozen stock culture stored at -70°C and grown in 10 mL fresh BHI for 24 h at 37°C in an incubator. Following incubation, 10 µL of the culture were transferred into 10 mL fresh BHI and incubated for 18-24 h. The inoculum level of this culture (approximately 10⁶) was determined by decimal dilution in phosphate buffer saline (PBS at pH 7.0).

Evaluating inhibitory activity of nisin, EDTA, and the combination of nisin with EDTA against L. monocytogenes at 37°C

The antimicrobial activity of nisin, EDTA, and nisin with EDTA was tested against *L. monocytogenes* in BHI medium (approximately 10⁶ CFU/mL); treated cultures were incubated for 72 h at 37°C. To prepare microbial suspensions, a 10 µL loop of *L. monocytogenes* strain V7, serotype 1/2 a, (FDA) was taken from a frozen-stock cul-

ture stored at -70°C and grown in BHI for 24 h at 37°C in an incubator. Ten microliters of this pure culture were transferred into 10 mL fresh BHI and incubated for another 24 h prior to use. To prepare microbial pellets for tests, 1 mL of culture was centrifuged at 14,000 rpm for 20 min using an eppendorf centrifuge model 5415C (Brinkman Instruments, Inc. Westbury, N.Y.) at 14,000 rpm for 20 minutes. After centrifugation, the supernatant was discarded and the bacterial pellets were used to test the antimicrobial activity of the nisin (10,000 IU/mL); EDTA (1.6 mg/mL); or nisin (10,000 IU/mL) with EDTA (1.6 mg/mL). One milliliter of BHI solution containing these components was added to the pellets. A control sample consisted of bacterial pellets dissolved in 1 mL BHI, which contained no antimicrobial compounds. Triplicate samples were incubated at 37°C for 72 h on a rotary platform shaker at 250 rpm. Following incubation, *L. monocytogenes* was enumerated by serial dilutions in PBS, plating in duplicate on LSA, and incubating the plates 48 h at 37°C. The initial inoculum levels (approximately 10⁶ CFU/mL) used to inoculate test media were determined by serially diluting the control, microbial pellets dispersed in BHI, at 0 h in BHI, plating on Listeria Selective Agar (LSA), and incubating for 48 h at 37°C.

Preparation of soy-protein edible film coating containing nisin, EDTA, or the combination of nisin with EDTA

Soy-protein film-forming solution was prepared according to the procedure of Eswaranandam et al. (2004). The film-forming solution was prepared by dissolving 10 g soy protein isolate into 90 mL water followed by the addition of 3.5 g glycerol to the mixture. To ensure thorough mixing, the film-forming solution was stirred with a magnetic stir bar for 30 min. This solution was heated with continuous stirring at 85°C for 30 min in a water bath. The solution was cooled to room temperature, and the antimicrobials including nisin (N) (1 g), EDTA (0.16 g) and the combinations of nisin (1 g) + EDTA (0.16 g) were added. The resulting solutions were mixed for 30 min and used to coat the meat samples.

Inoculation and coating of meat samples

One-centimeter cut cubes of defrosted, irradiated, full-fat commercial turkey frankfurters were individually dipped for 1 min into the culture broth containing approximately 10⁶ CFU/mL of *L. monocytogenes*. The pieces (each weighing approximately 1 g) were removed from the culture broth, allowed to drip free of excess inoculum, and allowed to dry for 30 min under a laminar hood with blowing air. A total of 192 [triplicates (3) of each type of coating (4) per temperature (2) per day (8)] pieces were coated with the different coating treatments by dipping them into film-forming solutions for 1

min. Coating treatments were performed in triplicates and included four treatments: SPI (soy protein coat without antimicrobials); SPI+N; SPI+EDTA; and SPI+N+EDTA. Each of the four treatments was subjected to two temperatures (4°C and 10°C), and *L. monocytogenes* was enumerated at d 0, 7, 14, 21, 28, and d 45. Following the film coating, the frankfurters were allowed to drip dry. Two types of positive controls were used. One consisted of inoculated frankfurter pieces without the SPI coating (Lm control) and the other consisted of pieces with the SPI coating but without the addition of antimicrobials. The treated pieces were placed individually into sterile Whirl-Pak® bags and refrigerated at 4°C and 10°C for 45 d.

Bacterial count during storage at d 0, 7, 14, 21, 28, and d 45

Bacterial counts at different storage periods were determined to test the ability of nisin, EDTA, and their combination incorporated in soy-protein film coatings in killing or inhibiting the growth of *L. monocytogenes*. Triplicates of the 1-cm cubes of meat in Whirl-Pak® bags stored at 4°C and 10°C were used. Phosphate buffer saline (PBS) diluents were added to make a 10-fold dilution. The samples were stomached for 2 min, decimally serially diluted with 0.1% PBS, and surface-plated in duplicate onto plates of LSA for the enumeration of *L. monocytogenes*. Plates were incubated at 37°C. After 48 h, colonies were counted and CFU/mL was calculated.

Statistical analysis

All values are reported as means of three experimental replications, performed in duplicate. Analysis of variance was conducted using general linear model, a procedure of the Statistical Analysis System (SAS 8.2, SAS Institute, Cary, N.C. 2000). The least significant difference procedure (student t-test) was used to compare means, significant mean differences among treatments, and treatment combinations. Significant differences were determined at $P < 0.05$.

RESULTS AND DISCUSSION

Antimicrobial activities of nisin, EDTA, or the combination of nisin with EDTA in BHI broth medium at 37°C

As shown in Fig. 1, it was observed that the initial population of *L. monocytogenes* as the control was about 6.7 logs of CFU/mL and it grew to 9.1 logs of CFU/mL after 72 h at 37°C in BHI medium. Neither nisin nor EDTA alone was ineffective against the growth of *L. monocytogenes*. The cell populations were 8.6 and 8.2 logs of CFU/mL, respectively, after 72 h incubation.

The nisin treatment initially controlled the population of *L. monocytogenes* by a log of 3.4 CFU/mL. After 6 h of incubation, the populations were similar to the

control. The initial reduction of *L. monocytogenes* by nisin (10,000 IU) might be due to its inhibitory activity. With prolonged incubation, nisin might have lost its activity against *L. monocytogenes* or the *L. monocytogenes* cells might have become resistant to nisin activity (Fig. 1). The nisin resistance in *L. monocytogenes* has been demonstrated in previous studies and it is a complex phenomenon (Crandall et al., 1998).

The combination of nisin with EDTA had effective inhibitory activity against *L. monocytogenes* in BHI medium at 37°C. In combination, the initial count of 6.7 logs CFU/mL was reduced to an undetectable level after 15 h of incubation. In combination, EDTA might have enhanced the activity of nisin against *L. monocytogenes*. Similarly, Brannen et al. (1990) also demonstrated that the combined activity of nisin with EDTA was more potent compared to nisin alone against *L. monocytogenes*. There were no survivors detected after 15 h of incubation in BHI medium at 37°C (Fig. 1).

Inhibitory effects of nisin, EDTA, and the combined effect of nisin with EDTA against L. monocytogenes on full-fat commercial turkey frankfurters stored at 4°C and 10°C

Tables 1 and 2 show the effect of nisin (10,000 IU); EDTA (0.16%); and the combination of nisin (10,000 IU) with EDTA (0.16%) against *L. monocytogenes* on full-fat turkey commercial frankfurters at 4°C and 10°C for 45 d respectively. The control without the addition of EDTA or nisin in the SPI coating showed the initial population of *L. monocytogenes* at 6.3 log CFU/g grew to 7.5 and 7.9 log CFU/g after 45 d at 4°C and 10°C respectively. EDTA (0.16%) alone did not have any significant inhibitory activity against *L. monocytogenes* on frankfurters at both 4°C and 10°C for 45 d ($P < 0.05$). The cell populations were similar to the controls and were 7.9 and 8.2 log CFU/g at 4°C and 10°C respectively on d 45 when coated with EDTA-incorporated SPI on turkey frankfurters.

Nisin (10,000 IU) treatment had significant influence on the populations of *L. monocytogenes* for the first 7 d on frankfurters at 4°C and 10°C. The population of *L. monocytogenes* declined by 1.4 and 1.7 log cycles of CFU/g at 4°C and 10°C, respectively, in comparison with the *L. monocytogenes* control. After a 7 d, the population increased and was similar to the control containing no antimicrobials: up to 7.3 and 7.2 log CFU/g at 4°C and 10°C respectively on d 45. The sensitivity of *L. monocytogenes* towards nisin activity might have reduced during extended, refrigerated storage conditions. Janes et al. (2002) also found nisin incorporated into corn-zein coatings did not have significant ($P < 0.05$) inhibitory effects after 24 d of storage at 4 and 8°C.

In combination, nisin (10,000 IU) with EDTA (0.16%) dramatically reduced the *L. monocytogenes* pop-

ulation at both 4°C and 10°C. The initial population of approximately 6 log CFU/mL was reduced to undetectable levels at d 7 at both 4°C and 10°C. Furthermore, no survivors were detected after a 7 d until 45 d at both temperature-storage conditions.

Results indicate that the usage of combined nisin (10,000) with EDTA (0.16%) was more highly effective than nisin (10,000 IU) or EDTA (0.16%) alone against *L. monocytogenes*. This combination can improve the efficacy and eliminate practical problems associated with the use of nisin against *L. monocytogenes* in meat preservation systems. Further studies are required to enhance the combined effects of nisin with EDTA against *L. monocytogenes* on other food products that are more susceptible to *L. monocytogenes* contamination.

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Table 1. Combined effect of nisin with EDTA on inhibition of *L. monocytogenes* on full-fat commercial turkey frankfurters stored at 4° C.

Treatment ^z	<i>L. monocytogenes</i> population (Mean ± SE) Log CFU/g					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 45
Lm control	6.4± 0.1 ^a ^y	6.1± 0.2 ^a	6.5±0.1 ^b	7.0 ± 0.2 ^b	7.1± 0.1 ^b	7.5± 0.1 ^b
SPI	5.9± 0.1 ^b	6.3± 0.1 ^a	7.4±0.3 ^a	7.9± 0.0 ^a	8.4± 0.1 ^a	8.3 ± 0.0 ^a
SPI+ N	6.4± 0.1 ^a	4.7± 0.1 ^b	5.2±0.1 ^c	5.5± 0.0 ^c	6.2± 0.0 ^c	7.2± 0.1 ^c
SPI+ EDTA	6.3± 0.0 ^a	6.3± 0.0 ^a	6.7± 0.1 ^b	7.1 ± 0.1 ^b	8.2± 0.1 ^a	8.2± 0.1 ^a
SPI+ N+EDTA	6.3± 0.0 ^a	0.0± 0.0 ^c	0.0± 0.0 ^d	0.0± 0.0 ^d	0.0± 0.0 ^d	0.0± 0.0 ^c

^z Lm control: Inoculation of *L. monocytogenes* without coating. SPI: Soy protein coating without Nisin/EDTA, SPI+ N = Nisin (10,000 IU/g) incorporated soy protein coating, SPI+N+EDTA: Nisin (10,000 IU/g) + EDTA 0.16% incorporated soy protein coating.

^y All means were measurements of three experiments in duplicates. Means within a column followed by same superscript are not significantly different (p<0.05). Minimum detection limit was 100 CFU/mL.

Table 2. Combined effect of nisin with EDTA on inhibition of *L. monocytogenes* on full-fat commercial turkey frankfurters stored at 10° C

Treatment ^z	<i>L. monocytogenes</i> population (Mean ± SE) Log CFU/g					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 45
Lm control	6.5± 0.0 ^a ^y	6.1±0.1 ^b	5.7±0.0 ^b	7.1± 0.3 ^b	7.5± 0.4 ^b	7.9± 0.1 ^a
SPI	5.9± 0.5 ^a	7.8 ± 0.0 ^a	7.5 ± 0.1 ^a	7.3± 0.2 ^b	7.9± 0.0 ^b	8.1± 0.1 ^a
SPI+ N	6.3± 0.1 ^a	4.4± 0.1 ^c	5.4± 0.1 ^b	5.8± 0.1 ^c	7.7± 0.1 ^c	7.3± 0.3 ^b
SPI+ EDTA	6.2± 0.1 ^a	7.7± 0.1 ^a	7.5± 0.2 ^a	8.1± 0.2 ^a	7.8± 0.1 ^a	7.9± 0.1 ^a
SPI+ N+EDTA	6.2± 0.1 ^a	0.0± 0.0 ^d	0.0± 0.0 ^c	0.0± 0.0 ^d	0.0± 0.0 ^d	0.0±0.0 ^c

^z Lm control: Inoculation of *L. monocytogenes* without coating. SPI: Soy protein coating without Nisin/EDTA, SPI+ N = Nisin (10,000IU/g) incorporated soy protein coating, SPI+N+EDTA: Nisin (10,000IU/g) + EDTA 0.16% incorporated soy protein coating.

^y All means were measurements of three experiments in duplicates. Means within a column followed by same superscript are not significantly different (p<0.05). Minimum detection limit was 100 CFU/mL.

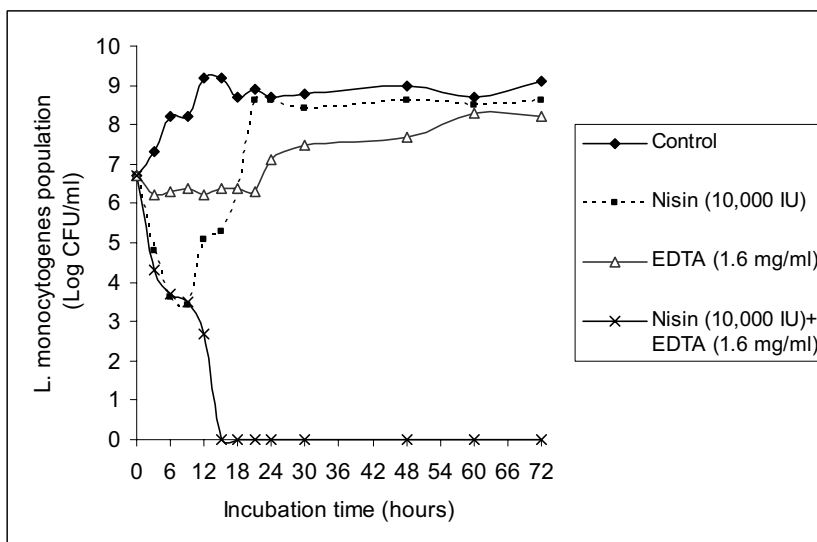


Fig. 1. Inhibitory effects of nisin, EDTA, and the combination of nisin with EDTA against *L. monocytogenes* in BHI broth medium at 37°C. (Values are means of three determinations.)