



Growth of *Brochothrix thermosphacta* in ground beef following treatments with nisin in calcium alginate gels

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Sterilized, lean and adipose beef carcass tissues were inoculated with Brochothrix thermosphacta, left untreated (U), or treated with 100 µg/ml nisin (N), calcium alginate (A), or 100 µg/ml nisin immobilized in a calcium alginate gel (AN). Treated tissues were aseptically processed into ground beef and populations of B. thermosphacta and nisin activity were determined during refrigerated storage (4°C) at 0, 7, and 14 days. At day 0, bacterial populations of U- and A-treated ground beef were 3.24 and 3.17 log₁₀ CFU/g respectively. Ground beef treated with N exhibited populations of 2.80 log₁₀ CFU/g while AN significantly suppressed the organism to undetectable levels (<1.30 log₁₀ CFU/g) at day 0. In contrast to high nisin titers from AN-treated ground beef at day 0, nisin titers were undetectable in N-treated ground beef. By day 7, B. thermosphacta had grown to 7.18, 7.04, and 6.92 log₁₀ CFU/g in U-, A-, or N-treated ground beef, respectively, while AN-treated ground beef exhibited significantly different (P≤0.05) populations of 6.56 log₁₀ CFU/g. By day 7, nisin titers from AN-treated ground beef were considerably diminished. At day 14 of the study, all treatments exhibited bacterial populations >7 log₁₀ CFU/g and nisin titers were virtually undetectable in any of the ground beef samples. While the growth of B. thermosphacta could not be effectively suppressed for 14 days, the application of nisin in alginate gels to meat surfaces does afford some immediate protection against undesirable bacteria when these surfaces are processed into ground beef.

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Introduction

Edible films can be made from any one of a variety of compounds including pectins, starch derivatives, collagen, gelatin, proteins, polysaccharides, lipid, and seaweed extracts (Krochta et al. 1994, Ben and Kurth 1995).

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^aNames are 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.

The properties of edible films are similar to those of synthetic packaging films in that they act as barriers against moisture, oxygen, oil, and solute migration, improve mechanical handling of certain foods, retain volatile flavor compounds, and bind specific food additives (Ben and Kurth 1995).

Several studies have demonstrated that the incorporation of additives, such as food-grade antimicrobials, into edible coatings may provide additional barriers/hurdles to reduce the incidence of pathogenic or spoilage bacteria on the surfaces of meat or raw meat products (Meyer et al. 1959, Siragusa and Dickson 1992, 1993, Baron 1993, Cutter and

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Siragusa 1996, Fang et al. 1996). Siragusa and Dickson (1992, 1993) determined that the antimicrobial activity of lactic and acetic acids was greater when immobilized by calcium alginate gels than when the acids were applied alone. Previously, we have demonstrated that nisin activity was greater against the Gram-positive meat spoilage organism, *Brochothrix thermosphacta*, when immobilized in an edible gel than when applied directly to beef surfaces (Cutter and Siragusa 1996). Ultimately, the incorporation of antimicrobials with edible packaging materials may provide additional safety and shelf-life measures for raw meat products. The following study was conducted to determine if immobilization of nisin in an edible gel was a more effective delivery system for a bacteriocin into ground beef than direct application.

Materials and Methods

Bacterial cultures and inoculation of beef

Brochothrix thermosphacta ATCC 11509 was maintained in 75% glycerol at -20°C and propagated in trypticase soy broth (Troy Biologicals, Troy, MI, USA) supplemented with 0.5% yeast extract (Difco, Detroit, MI, USA) at 25°C for 18 h. The culture of *B. thermosphacta* was diluted 1:100 in buffered peptone water (BPW, Difco) prior to inoculation on beef tissues.

Lean and adipose tissues from the outer surfaces of post rigor (24 h post-mortem) beef carcasses were obtained from the Roman L. Hruska US Meat Animal Research Center (RLHUSMARC) abattoir, vacuum packaged, and stored at -20°C . Frozen tissues were transported to the Linear Accelerator Facility at Iowa State University (Ames, IA, U.S.A.) where they were sterilized by electron beam (dose ranged from 32.3–39.4 kGy; 10 meV, 10 kW, 2.80 fpm), kept frozen, and transported back to RLHUSMARC. Frozen, irradiated tissue was thawed overnight at 5°C and then brought to room temperature (25°C) on the day of the experiment. Lean and adipose tissues were aseptically trimmed of other

extraneous tissue and weighed to obtain c. 425 g of lean and c. 75 g adipose. The individual pieces of lean and adipose tissues were placed onto a sterile tray, the suspension of *B. thermosphacta* was brushed onto the tissue with a sterile 2-inch wide paint brush (Dorsa et al. 1996), and the tissue pieces were incubated for 60 min, 5°C to allow for bacterial attachment and for the tissue to harden before grinding.

Bacteriocin preparation and treatments

Purified nisin (AmbicinTM, Applied Microbiology, NY, USA) was solubilized in distilled water, filter sterilized ($0.2\text{ }\mu\text{m}$ AcrodiscTM, Gelman Sciences, Ann Arbor, MI, USA), added to sterile distilled water for a final stock concentration of 1 mg/ml (pH 6.0), and stored at -20°C . Prior to experiments, the stock solution was thawed to 25°C and diluted 1:10 in sterile distilled water or sterile alginate solution for a final nisin concentration of 100 $\mu\text{g/ml}$ used throughout the study.

One percent (w/v) high viscosity sodium alginate (pH 7.0; Sigma Chemical Co., St. Louis, MO, USA) and 90 mM calcium chloride (pH 7.0; CaCl_2 , Sigma) were prepared in distilled water and sterilized by autoclaving. Lean and adipose tissues inoculated with *B. thermosphacta* were separated into four batches and treated as follows: untreated (U); treated with 25 ml of alginate solution and cross linked with 25 ml of CaCl_2 (A); treated with 25 ml of 100 $\mu\text{g/ml}$ nisin (N); treated with 25 ml of alginate solution containing 100 $\mu\text{g/ml}$ nisin and cross linked with 25 ml of CaCl_2 (AN). All treatments were performed at 25°C by spraying evenly over the tissues with a hand-held spray bottle (Ace Hardware). A grinder (Jupiter Model 863, Germany) fitted with a $1/4$ " plate was used to grind individual batches of U-, A-, N-, and AN-treated beef tissues. Ground beef was collected in a sterile polyethylene stomacher bag (Spiral Biotech, Bethesda, MD, USA), tied off, and stored at 4°C until sampled.

Bacterial enumeration and nisin assays

At 0 (within 30 min of grinding), 7, or 14 days

of refrigeration, three 25-g samples were taken from each batch of U-, A-, N-, and AN-treated ground beef. Each sample was stomached for 2 min (Stomacher 400, Tekmar, Inc., Cincinnati, OH, USA) in a Sterefil™ Stomacher bag (Spiral Biotech) with 25 ml of buffered peptone water (BPW, pH 7.0; BBL, Cockeysville, MD, USA) containing 0.1% Tween 20 (Fisher, St. Louis, MO, USA), serially diluted in BPW, and spiral-plated (Model D Spiral Plater; Spiral Biotech) in duplicate on trypticase soy agar (TSA; Difco, Detroit, MI, USA) supplemented with 0.5% yeast extract (Difco). Plates were enumerated with the CASBA IV image analyser (Spiral Biotech) after incubation for 36 h at 25°C. The lowest level of detection of *B. thermosphacta* was $1.3 \log_{10}$ CFU/cm² using spiral plating procedures.

Spot assays were performed on lawns of *B. thermosphacta* to monitor bacteriocin activity in all solutions containing nisin and ground beef samples (Siragusa 1992). Residual nisin activity in ground beef samples was determined by two methods. For the first method, 10 µl of stomached samples were spotted directly onto a lawn of *B. thermosphacta* (Siragusa 1992). For the second method, 10 µl of the supernatant was obtained from ground beef samples subjected to a modified acid-boil procedure (Cutter and Siragusa 1996) and used in spot assays (Siragusa 1992). All plates were incubated overnight at 26°C. All samples exhibiting activity during initial nisin assays were stored at -20°C for 48–72 h, thawed to 25°C, titered to extinction on lawns of *B. thermosphacta*, and the reciprocal of the highest dilution exhibiting activity was recorded (Siragusa 1992). After enumeration procedures and nisin assays, stomachates from each treatment, batch, and/or replication were stored at -20°C.

Bacteriocin stability experiment

Three frozen (-20°C, 14 days) stomached samples taken from untreated ground beef at day 0 and containing no nisin activity, were thawed 18 h at 5°C. Two ml of each sample were transferred to sterile centrifuge tubes and centrifuged for 15 min, 3500 rpm, 5°C. From each of the three samples, the resulting

supernatants (volume=1.8 ml) were transferred to individual, sterile tubes. The remaining pellets were resuspended in 1.8 ml of physiological saline (pH 7.2). To each of the supernatants and resuspended pellets, 200 µl of a 100 µg/ml concentration of nisin were added and mixed (final nisin concentration, 10 µg/ml). At days 0, 1, 2, 3, 4, 5, 6, 7, 14, and 21 of refrigerated storage, all pellet and supernatant samples were assayed for nisin activity on lawns of *B. thermosphacta* (Siragusa 1992).

Calculations and statistical analyses

The experiment was a 4 (treatments) × 3 (day) factorially arranged, completely randomized design. After enumeration, bacterial populations were converted to $\log_{10}$ CFU/g. Least squared means (LSM) of bacterial populations ($\log_{10}$ CFU/g) were calculated from six experimental replications. Analysis of variance (ANOVA) was performed using the general linear models (GLM) procedure of SAS (SAS Institute, ver. 6.06.01, 1989, SAS Inst., Inc., Cary, NC, 1982). Inoculum counts were used as a covariant to normalize data between treatment replications. Statistical significance was defined as $P \leq 0.05$, unless otherwise noted.

Nisin titers were converted to the reciprocal of the highest dilution exhibiting activity and these values were used for analyses. LSM of nisin titers from stomachates or acid-boil samples were calculated from six experimental replications. ANOVA was performed using the GLM procedure of SAS. The probability level for population or nisin titer data was $P \leq 0.05$, unless otherwise noted.

Results and Discussion

Prior to the examination of the methods described in this study, we carried out experiments to determine if immobilization of nisin in calcium alginate beads and application of these beads to ground beef could be used to reduce undesirable bacteria (data not presented). While nisin activity was detectable in the beads, delivery of the nisin-immobilized beads to ground beef was ineffective

for reducing *B. thermosphacta*. Subsequent research in our laboratories has demonstrated that immobilization of nisin in calcium alginate gels and application to the surfaces of lean and adipose beef carcass tissues was effective for reducing *B. thermosphacta* up to 14 days of refrigerated storage (Cutter and Siragusa 1996). Since the surfaces of beef can be incorporated into ground beef during the fabrication process, the present study investigated the feasibility of using surface tissues treated with calcium alginate to deliver nisin to ground beef.

As indicated by the data presented in Table 1, similar bacterial populations (c. 3 log₁₀ CFU/g) of *B. thermosphacta* were observed for untreated (U) and alginate-treated (A) ground beef at day 0. Nisin-treated (N) ground beef exhibited bacterial populations (2.8 log₁₀ CFU/g) that were statistically different from U and A and were statistically different from the alginate-nisin-treated (AN) ground beef (1.33 log₁₀ CFU/g). The differences between the populations of the controls (U, A) and the populations of bacteriocin-treated (N, AN) ground beef at day 0 indicate that nisin affects an immediate reduction of the test organism. Based on our earlier study with surface tissues (Cutter and Siragusa 1996), it is possible that the organism was effectively reduced by N or AN prior to processing into ground beef.

By day 7, bacterial populations of N- and AN-treated ground beef had increased, with N-treated tissues exhibiting similar populations (c. 7 log₁₀ CFU/g) of *B. thermosphacta* as the U and A controls (Table 1). Finally, by

day 14, the AN-treated ground beef increased to a population of >7 log₁₀ CFU/g, which was similar to U, A, and N-treated ground beef. It is apparent from these data that immobilization of nisin in an alginate gel (AN) sufficiently suppressed the growth of *B. thermosphacta* in ground beef up to 7 days of refrigerated storage.

Nisin assays from N-treated ground beef were virtually undetectable for the duration of the experiment, regardless of assay method employed (Fig. 1). Nisin titers from AN-treated ground beef were substantially greater and statistically different than N-treated ground beef using either assay method during the 14 day incubation. Based on our results, the acid-boil method appeared to recover higher nisin activity than did the direct spot assay of stomachates. Other researchers have demonstrated that nisin is liberated from meat components using the acid-boil procedure (Tramer and Fowler 1964, Bell and DeLacy 1986). As the nisin titer and plate count data demonstrate, the decrease in nisin activity was concurrent with an increase in bacterial growth. We have previously demonstrated a similar relationship between titers and bacterial growth when immobilized nisin was applied to lean or adipose beef surfaces (Cutter and Siragusa 1996).

It is well known that the ability of nisin to effectively suppress undesirable bacteria in foods is dependent upon several factors, including presence of protein or fat particulates (Jung et al. 1992). Therefore, we conducted a bacteriocin stability experiment to determine if components (e.g. endogenous proteases) of the ground beef could be responsible for decreased nisin activity over time. Even after prolonged, refrigerated storage of nisin in both the supernatant and pellet of ground beef stomachates, nisin activity remained stable (data not presented). Since this experiment was carried out with physiological saline, it was not possible to determine if nisin bound to components associated with the pellet, since salt interferes with nisin adsorption in meat products (Bell and DeLacy 1985). The loss of nisin activity associated with meat components is not clearly understood and is the focus of an ongoing investigation in our laboratories.

Table 1. Populations of *Brochothrix thermosphacta* in ground beef following treatments with alginate, nisin (100 µg/ml), and alginate containing nisin (100 µg/ml) and refrigerated storage up to 14 days

Treatment	Bacterial populations of <i>B. thermosphacta</i> (log ₁₀ CFU/g)		
	Day 0	Day 7	Day 14
Untreated	3.24 ^a	6.92 ^a	7.21 ^a
Alginate	3.17 ^a	7.04 ^a	7.30 ^a
Nisin	2.80 ^b	7.18 ^a	7.32 ^a
Alginate-Nisin	1.33 ^c	6.56 ^b	7.44 ^a

^{a-c}Denote statistical differences between treatments within columns.

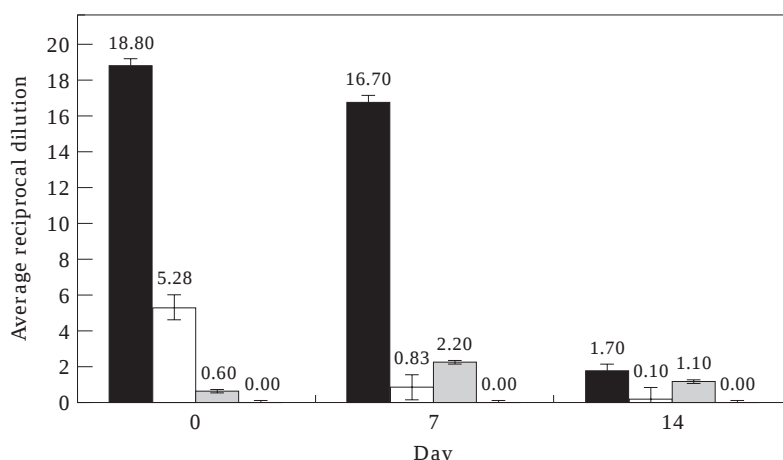


Figure 1. Nisin titres taken from ground beef samples treated with nisin and alginate containing nisin, and performed by spotting stomachates directly onto a lawn of *Brochothrix thermosphacta* or subjecting the samples to a modified acid-boil procedure and spotting supernatants directly onto a lawn of *Brochothrix thermosphacta*. ■=Alginate/Nisin—acid-boil, □=Alginate/Nisin-stomachate, ▒=Nisin-acid-boil, ▨=Nisin-stomachate.

To our knowledge, we have presented the first report in which a bacteriocin is immobilized in an edible gel and incorporated into a processed, raw beef product. While the growth of *B. thermosphacta* could not be effectively suppressed for 14 days, our results suggest that application of nisin in alginate gels to meat surfaces does afford some immediate protection against undesirable bacteria when these surfaces are processed into ground beef.

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