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Incorporation of nisin into a meat binding system to inhibit bacteria on beef surfaces

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C.N. CUTTER AND G.R. SIRAGUSA. 1998. In two separate experiments, the bacteriocin, nisin, was incorporated into a commercially available meat binding system (Fibrimex®) and applied to meat surfaces as a way of inhibiting the meat spoilage organism, *Brochothrix thermosphacta* during extended refrigerated storage. In experiment 1, pre-rigor lean beef carcass tissue (BCT) was inoculated with *B. thermosphacta*, left untreated (U), treated with 10 μg ml^{-1} nisin (N), Fibrimex® (F) or Fibrimex® containing 10 μg ml^{-1} nisin (FN), held aerobically at 4 °C for up to 7 d, and populations of *B. thermosphacta* and nisin activity determined. Experiment 2 determined the effects of the same treatments but on post-rigor, frozen and thawed lean BCT that was inoculated, vacuum-packaged, and stored at 4 °C for up to 14 d. In both experiments, N- and FN-treated tissues exhibited significantly lower populations of *B. thermosphacta* compared to U- and F-treated tissues, for the duration of refrigerated storage. Nisin activity was detected up to 7 d in N- and FN-treated samples from experiment 1. However, activity was detected only to days 0 and 2 in FN- and N-treated samples, respectively, from experiment 2. These studies indicate that the addition of a bacteriocin to a meat binding system and application to meat surfaces may be useful in reducing undesirable bacteria in restructured meat products.

INTRODUCTION

Generally, small muscles of high quality or lean trimmings obtained from fabricated beef carcasses are processed into products with relatively low economic value such as sausages, meat snacks or raw ground products (Wijngaards and Paardekooper 1988). In the past, these muscle pieces and trimmings of high quality were tumbled with the addition of salts, phosphates or binding agents, and formed. Recently, a method has been developed for the preparation of restructured meat products (Paardekooper and Wijngaards 1988; Wijngaards and Paardekooper 1988). Known as Fibrimex® (F.N.A. Foods, Calgary, Alberta, Canada), this cold meat binding system results from an enzymatic reaction between the blood proteins, fibrinogen and thrombin, and the meat components, collagen and fibronectin (Wijngaards and Paardekooper 1988). Fibrimex® is approved in the USA for use in the meat industry as a means of adhering whole muscle or pieces of fresh meat, poultry, fish or seafood together into one piece. Regardless of the method used for compositing, external beef surfaces of muscles and trim used in restructured meat products may harbour pathogenic or spoilage bacteria which are then internalized. This observation illustrates the need to investigate methods for decontaminating external surfaces of meat or muscle destined for internalization in restructured meat products.

The use of bacteriocins, such as nisin, in food systems has been extensively documented (for a review, see Nettles and Barefoot 1993). Numerous reports have also addressed the addition of bacteriocins to intact or processed meat products as a means of inhibiting pathogenic or spoilage bacteria (Bell and De Lacy 1986; Chung *et al.* 1989; Vignolo *et al.* 1996). As an antimicrobial, the bacteriocin, nisin, has numerous

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[1]Names 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.

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advantages over other available antimicrobial compounds. Currently, it is a generally recognized as safe (GRAS) substance and approved for use in pasteurized processed cheese spreads or pasteurized liquid whole egg. Nisin is stable under refrigerated conditions, demonstrates heat stability, and is degraded by gut enzymes. The spectrum of activity of this compound not only includes Gram-positive pathogens such as *Clostridium botulinum, Staphylococcus aureus, Listeria monocytogenes* or *Bacillus cereus*, but also meat spoilage organisms (*Brochothrix thermosphacta, Lactobacillus* spp.). In these laboratories, it has previously been demonstrated that nisin can effectively suppress the meat spoilage isolate, *B. thermosphacta*, when the bacteriocin is applied directly to the meat surface (Cutter and Siragusa 1994a), immobilized in a calcium alginate gel (Cutter and Siragusa 1996b, 1997), or combined with vacuum packaging and refrigerated storage (Cutter and Siragusa 1996a).

The objective of the following experiments was to determine if the bacteriocin, nisin, could be incorporated into a meat binding system and reduce bacteria on fresh or vacuum-packaged meat surfaces. The application of an antimicrobial, such as a bacteriocin, in Fibrimex® or similar products may be useful for reducing undesirable bacteria in restructured meat products made from whole muscles or trim that incorporate surface tissues into product interior.

MATERIALS AND METHODS

Bacterial cultures and inoculation of beef

Brochothrix thermosphacta ATCC 11509 was maintained in 75% glycerol at −20 °C and propagated for 18 h in trypticase soy broth (TSB; Troy Biologicals, Troy, MI, USA) at 26 °C.

For experiment 1, lean tissue (cutaneous trunci) from the outer surfaces of pre-rigor (15 min post exsanguination) beef carcasses was obtained from a local cow/bull processing plant, placed in plastic bags, stored in insulated carriers to prevent rapid cooling, transported to the Roman L. Hruska U.S. Meat Animal Research Center (MARC), and used within 2 h of slaughter. Prior to experiment 1, the cutaneous trunci was trimmed to fit onto sterile trays. For experiment 2, post-rigor, frozen, and thawed lean tissue (cutaneous trunci) was cut to 7·5 cm × 7·5 cm. For both experiments, the surfaces were surface sterilized by u.v. light (Cutter and Siragusa 1994b). Sterility was monitored by individually sampling pieces of uninoculated tissue using the enumeration procedures described below. Overnight cultures of *B. thermosphacta* were diluted 1:1000 in sterile physiological saline (pH 7·0) to obtain a viable cell population of approximately 6 $\log_{10}$ cfu ml^{-1}. The inoculum was aseptically sprayed onto the tissue with a hand-held sprayer (Ace Hardware) and left undisturbed for 15 min, at 25 °C, prior to applying treatments. Initial bacterial populations of approximately 3–4 $\log_{10}$ cfu cm^{-2} were obtained using this procedure.

Bacteriocin preparation and activity assays

Purified nisin (Ambicin™, Applied Microbiology, New York, USA) was solubilized in distilled water, filter-sterilized (0·2 μm Acrodisc™, Gelman Sciences, Ann Arbor, MI, USA), added to sterile distilled water for a final stock concentration of 1 mg ml^{-1} (pH 6·0), and stored at −20 °C. Prior to experiments, the stock solution was thawed to 25 °C and diluted 1:100 in sterile distilled water or fibrinogen (Fibrimex®) solution for a final nisin concentration of 10 μg ml^{-1} used throughout the study.

Spot assays were performed on lawns of *B. thermosphacta* to monitor bacteriocin activity in solutions containing nisin, as well as stomachates, as previously described (Siragusa 1992). Samples of stomachates (10 μl) from all treated tissues were used in spot assays. In addition to stomachates, Fibrimex® coatings and purge (liquid found in vacuum-packaged meats) were assayed for nisin activity. Fibrimex® coatings formed on F- and FN-treated tissues were aseptically scraped from the vacuum package and placed onto a lawn of *B. thermosphacta*. Samples of the purge (10 μl) from U- and N-treated samples were used in spot assays as described above. All plates were incubated for 18 h at 26 °C.

Treatments

After inoculation with *B. thermosphacta*, tissue surfaces were left untreated (U), or treated with Fibrimex® (F), nisin (N) or Fibrimex®-nisin (FN). In both experiments, F, N and FN treatments were performed at 25 °C by spraying the compounds evenly over the tissues with a sterile hand-held spray bottle and allowed to remain undisturbed for 5 min; F samples were spray-treated with 7 ml fibrinogen (Fibrimex®), followed by spray treatments with 3 ml thrombin (Fibrimex®), N samples were spray-treated with 7 ml nisin (10 μg ml^{-1} concentration) and 3 ml sterile distilled water, and FN samples were spray-treated with 7 ml fibrinogen-nisin (final nisin concentration, 10 μg ml^{-1}) and 3 ml thrombin. Following treatments in experiment 1, tissues were loosely covered with sterile aluminium foil and stored at 4 °C. Treated tissues from experiment 2 were vacuum-packaged in u.v.-treated, suitably-sized 3·2 ml nylon/copolymer bags with an oxygen transmission rate at 23 °C of 52 cc m^{-2} (Hollymatic, Countryside, IL, USA), vacuum-sealed using a Hollymatic model LV 10 G, and stored at 4 °C.

Bacterial enumeration

A 25 cm^2 piece was aseptically excised from the untreated or treated tissues at 0, 1, 2 and 7 days of refrigeration for

experiment 1 or 0, 1, 2, 7 and 14 days of refrigeration for experiment 2. All individual samples were stomached for 2 min (Stomacher 400, Tekmar, Inc., Cincinnati, OH, USA) in a Sterefil™ Stomacher bag (Spiral Biotech, Bethesda, MD, USA) 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). Each stomachate was serially diluted in BPW and either spiral-plated (Model D Spiral Plater; Spiral Biotech) in duplicate or spread-plated in quadruplicate on STAA agar base (Oxoid) supplemented with 1·5% glycerol (Sigma), 50 μg ml^{-1} thallium acetate (Sigma), 500 μg ml^{-1} streptomycin sulphate (Sigma) and 50 μg ml^{-1} cycloheximide (Sigma). Plates were enumerated with CASBA IV image analyser (Spiral Biotech) after incubation for 48 h at 26 °C.

Calculations and statistical analyses

After enumeration, bacterial populations were converted to $\log_{10}$ cfu cm^{-2}. Least squared means (LSM) of bacterial populations ($\log_{10}$ cfu cm^{-2}) were calculated from six experimental replications for each experiment. Analysis of Variance was performed using the General Linear Models procedure of SAS (1988). Inoculum counts were used as a covariant to normalize data between treatment replications. Statistical significance was defined as $P \leqslant 0{\cdot}05$, unless otherwise noted.

RESULTS

In experiment 1, populations of *B. thermosphacta* remaining on tissues subjected to N and FN treatments were statistically similar and virtually undetectable ($<0{\cdot}50$ $\log_{10}$ cfu cm^{-2}) for the 7 days of aerobic, refrigerated storage (Table 1). On all days examined, populations of *B. thermosphacta* from N- and FN-treated tissues were significantly different from U- and F-treated tissues, which were not significantly different from one another. Bacteriocin assays performed on meat stomachates demonstrated that nisin was active in N and FN samples through day 7 (Table 2).

In experiment 2, bacterial populations of *B. thermosphacta* were effectively suppressed following treatments with N or FN (Table 3) and vacuum-packaged, refrigerated storage for up to 7 days. Populations of N- or FN-treated tissues were not significantly different from each other except at day 1, but both treatments were significantly different than U- and F-treated tissues. By day 14, bacterial populations had increased slightly on N- and FN-treated tissues, but were significantly different than U- and F-treated tissues. Bacteriocin assays performed on meat stomachates demonstrated that nisin was active in N and FN samples only through days 2 and 0, respectively (Table 2). Nisin activity from Fibrimex® coatings through day 14 indicated that the bacteriocin was still active (data not presented). Similarly, purge samples from N-treated tissues up to 14 days also demonstrated nisin activity (data not presented).

Table 1 Experiment 1. Populations of *Brochothrix thermosphacta* on lean beef tissue following treatments with nisin (10 μg ml^{-1}), Fibrimex® or Fibrimex®-nisin (10 μg ml^{-1}) and aerobic storage at 4 °C

Populations of *Brochothrix thermosphacta* ($\log_{10}$ cfu cm^{-2})				
Treatment	Day 0	Day 1	Day 2	Day 7
Untreated	4·89[a]	4·98[a]	5·42[a]	8·01[a]
Fibrimex®	3·62[a]	4·02[a]	5·70[a]	8·44[a]
Nisin	0·00[b]	0·00[b]	0·23[b]	0·30[b]
Fibrimex®-nisin	0·00[b]	0·00[b]	0·00[b]	0·00[b]

[a,b,ab,bc,c] Different letters within column denote statistically significant differences between treatments ($P \leqslant 0{\cdot}05$). Each value is the mean of six replications.

DISCUSSION

For the first time, it has been demonstrated that nisin can be incorporated into a cold meat binding system and applied to the surface of beef carcass tissue and have sustained antimicrobial activity, as indicated by reductions in bacterial populations during aerobic or vacuum-packaged refrigerated storage. While this system is not used specifically in restructured meat products, the results suggest that if antimicrobial compounds such as bacteriocins are combined with a cold meat binding system and applied to intact meat/muscle surfaces, the shelf-life and/or microbiological stability of restructured raw meat products may be improved.

The decrease in nisin activity exhibited in this study and others (Bell and DeLacy 1986; Chung *et al.* 1989; Cutter and Siragusa 1996a,b, 1997) is not fully understood. When bacteriocins are applied directly to a meat surface, there are several possibilities for diminished activity, including adsorption of nisin onto meat proteins or lipid particles, or assays that are not sensitive enough to detect the bacteriocin in samples (Tramer and Fowler 1964; Bell and DeLacy 1986). While bacteriocin activity from nisin-treated tissues diminished over time in the present study, bacterial populations remained suppressed compared to untreated controls, indicating that nisin was still active for the duration of the experiment.

Previously, it had been demonstrated that nisin could be immobilized in calcium alginate gels on beef surfaces and incorporated into ground beef (Cutter and Siragusa 1996a,b, 1997). As applied in that study, calcium alginate was used solely as a delivery system for the antimicrobial and did not impart any additional value or binding properties to the meat

Table 2 Presence or absence of nisin activity from stomachates obtained from untreated beef carcass tissue, beef carcass tissue treated with Fibrimex®, nisin (10 μg ml^{-1}) or Fibrimex®-nisin (10 μg ml^{-1}) and refrigerated storage (4 °C)

	Experiment 1[a] (Day)				Experiment 2[b] (Day)				
Treatment	0	1	2	7	0	1	2	7	14
Untreated	–[c]	–	–	–	–	–	–	–	–
Fibrimex®	–	–	–	–	–	–	–	–	–
Nisin	+[d]/6	+/6	+/4	+/5	+/3	+/2	+/3	–	–
Fibrimex®-nisin	+/6	+/6	+/6	+/1	+/4	–	–	–	–

[a] Following treatment, samples were stored aerobically at 4 °C.
[b] Following treatment, samples were vacuum-packed and stored at 4 °C.
[c] Bacteriocin activity was not detected in stomachates.
[d] Bacteriocin activity was detected in stomachates; number of positive samples from six replications.

Table 3 Experiment 2. Populations of *Brochothrix thermosphacta* on lean beef tissue following treatments with nisin (10 μg ml^{-1}), Fibrimex® or Fibrimex®-nisin (10 μg ml^{-1}) and vacuum-packaged storage at 4 °C

	Populations of *Brochothrix thermosphacta* ($\log_{10}$ cfu cm^{-2})				
Treatment	Day 0	Day 1	Day 2	Day 7	Day 14
Untreated	3·58[a]	2·58[a]	2·78[a]	3·23[a]	4·56[a]
Fibrimex®	3·29[a]	2·82[a]	2·90[a]	4·03[a]	5·17[a]
Nisin	0·48[b]	0·00[c]	0·00[b]	0·00[b]	1·94[b]
Fibrimex®-nisin	0·00[b]	1·02[b]	0·50[b]	0·46[b]	2·18[b]

[a,b,c] Different letters within column denote statistically significant differences between treatments ($P \leqslant 0{\cdot}05$). Each value is the mean of six replications.

product. The use of Fibrimex® as a delivery system for antimicrobials to meat surfaces and possibly, to further processed or restructured meat products, may be more acceptable than calcium alginate, given its binding properties, edible nature and current approved use in meat products.

The addition of the bacteriocin, nisin, to a meat binding system may provide an added antimicrobial advantage to restructured raw meat products that incorporate surface tissues into product interior, or as a delivery system for antimicrobials to meat surfaces.

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