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Decontamination of beef carcass tissue with nisin using a pilot scale model carcass washer

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Nisin spray treatments were tested for controlling Gram-positive bacteria attached to beef carcass surface tissue using a pilot scale model carcass washer. Sections of lean and adipose tissues were inoculated with approximately 4 $\log_{10}$ cfu cm^{-2} of Brochothrix thermosphacta, Carnobacterium divergens *or* Listeria innocua. *Following 28°C water or nisin sprays, bacterial populations were enumerated immediately and after incubation for 24 h at 4°C. Spray treatments with water did not significantly alter the bacterial populations at day 0 or 1 (< 1 $\log_{10}$ reduction). However, nisin spray treatments (5000 AU ml^{-1}) reduced populations by 1·79 to 3·54 $\log_{10}$ cfu cm^{-2} at day 0 and by 1·97 to 3·60 $\log_{10}$ cfu cm^{-2} at day 1. This study demonstrates that spray washing is an effective means of applying bacteriocins and that these compounds may be useful as sanitizers of red meat carcasses.*

Introduction

The bacteriocin, nisin, inhibits Gram-positive bacteria including the pathogens *Clostridium botulinum, Staphylococcus aureus, Listeria monocytogenes* and *Bacillus cereus* (Hurst 1983, Harris et al. 1992, Ray 1992, Nettles and Barefoot 1993). Numerous reports have examined the use of nisin to inhibit spoilage and pathogenic bacteria in foods and beverages including milk (Jung et al. 1992), cheese (Jones 1973, Somers and Taylor 1987), cottage cheese (Benkerroum and Sandine 1988), yogurt (Gupta and Prasad 1988), eggs (Delves-Broughton et al. 1992), brandy (Henning et al. 1986), and canned soups or vegetables (Gibbs and Hurst 1991). Several other reports have addressed the addition of nisin to various meat products. The bacteriocin has been incorporated into ham models (Houben and Krol 1985, Rayman and Hurst 1981), combined with nitrite in vacuum-packaged bacon (Calderon et al. 1985, Taylor and Somers 1985) and frankfurters (Taylor et al. 1985), used with other preservatives in cured meat products (Bell and DeLacy 1987, Taylor et al. 1985), and used directly on beef muscle (Chung et al. 1989) to inhibit undesirable bacteria. The efficacy of nisin varied between studies presumably due to inherent microbial populations associated with the product, the presence of nisin-resistant bacteria, the unequal

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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distribution of nisin throughout the product, the interference of endogenous compounds, or the binding of nisin to meat components (Bell and DeLacy 1986, Ray 1992, Nettles and Barefoot 1993).

Presently, food grade sanitizers, such as organic acids (FSIS 1992) and trisodium phosphate (Giese 1993), are used for decontamination of beef and poultry carcasses in commercial processing plants. Given the potential use of nisin as a biopreservative in various food products and the stability of the bacteriocin on beef under refrigerated conditions (Chung et al. 1989), the following study was undertaken to determine the effectiveness of spray washing beef carcass surface tissue with nisin to inhibit Gram positive bacteria.

Materials and Methods

Bacterial cultures

Brochothrix thermosphacta ATCC 11509, *Carnobacterium divergens* ATCC 35677 and *Listeria innocua* ATCC 33090 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). *L. innocua* LA1 was obtained from the Roman L. Hruska US Meat Animal Research Center (RLHUSMARC) culture collection. All strains were maintained in 75% glycerol at –20°C. *L. innocua* strains were propagated in tryptic soy broth (TSB; Troy Biologicals, Troy, MI, USA) at 37°C for 18 h. *B. thermosphacta* was propagated in TSB at 25°C for 18 h. *C. divergens* was propagated in lactobacilli MRS broth (Troy Biologicals, Troy, MI, USA) at 30°C for 18 h.

Inoculation of beef carcass tissue

Surface lean and adipose beef carcass tissues (BCT) from the outer surfaces of post rigor beef carcasses were obtained from the RLHUSMARC abattoir, trimmed to 7·5 cm × 7·5 cm, sterilized by ultraviolet light, and stored at –20°C, as described previously (Cutter and Siragusa 1994). Early stationary phase cultures were diluted 1 : 100 in sterile physiological saline (pH 7·0) to obtain a viable cell density of approximately 9 $\log_{10}$ cfu ml^{-1}. After thawing to room temperature, the fascia of individual pieces of lean and adipose BCT were placed in a sterile weigh boat (14 cm × 14 cm) containing 10 ml of the bacterial suspension, incubated for 15 min, 25°C, allowed to drip, and subjected to spray treatments with water or nisin.

Bacteriocin preparation

Nisin (Ambicin, Applied Microbiology, New York, USA) was prepared in distilled water, filter sterilized (0·2 μm Vacucap, Gelman Sciences, Ann Arbor, MI, USA), and added to sterile distilled water for a final concentration of 5000 activity units ml^{-1} (pH 6·0), as determined by the manufacturer. This solution was used immediately after preparation. Sterile distilled water (pH 6·5) was stored at 28°C until needed.

Bacteriocin spray treatments and experimental design

The CAPER unit (pilot scale model carcass washer), located at RLHUSMARC was used to apply water or nisin as described previously (Cutter and Siragusa 1994, DeZuniga et al. 1991). Operation parameters for the CAPER unit were as follows: spray nozzle oscillation speed, 80 cycles min^{-1}; chain speed, 14 m min^{-1}; nozzle pressure, 60 psi; flow rate, 4·2 l min^{-1}; nozzle distance from sample, 17·8 cm; temperature of solutions, 28°C. Immediately after spray treatments with either water or nisin, a 5 cm × 5 cm (25 cm^2 total surface area) section was aseptically excised from the spray treated BCT, placed into a Sterefil Stomacher bag (Spiral BioTech, Bethesda, MD, USA), and stored at 4°C. Day 0 samples were prepared for bacterial enumeration within 1 h. Additional 25 cm^2 sections of BCT were incubated at 4°C for 24 h (day 1) in stomacher bags to prevent contamination and dehydration of the BCT, and then prepared for bacterial enumeration. BCT inoculated with bacteria but not subjected to spray treatments (untreated samples) were handled similarily. After excising the 25 cm^2 section, remaining pieces of control and treated BCT were used to assess surface pH values (flat electrode, Corning Instruments, Corning, NY, USA) at day 0 and again after storage at 4°C, 24 h (day 1).

Bacterial enumeration

Each 25 cm^2 piece of untreated or spray treated BCT from day 0 or day 1 was pummeled for 2 min (Stomacher 400, Tekmar, Inc., Cincinnati, OH, USA) in 50 ml of a

buffer consisting of buffered peptone water (BPW; pH 7·0; BBL, Cockeysville, MD, USA) with 0·1% Tween 20 (Fisher) using the stomacher bags. Stomachates were taken from within the filter of the stomacher bags, serially diluted in 2% BPW, and plated on tryptic soy agar (TSA, Troy Biologicals, Troy, MI, USA) or MRS agar (Troy Biologicals, Troy, MI, USA) using a Model D Spiral Plater (Spiral Biotech, Bethesda, MD, USA). Plates were enumerated after incubation for 24 h at 37°C for *L. innocua*, for 24 h at 30°C for *B. thermosphacta*, and after 48 h at 30°C under flowing carbon dioxide (8%) for *C. divergens*.

Nisin sensitivity to pronase has been documented previously (Carminati et al. 1989). In a preliminary laboratory experiment, 100 μl of a 20 mg ml^{-1} (1400 proteolytic units) stock of Pronase™ protease (pH 7·0; Calbiochem, San Diego, CA) was spread plated onto agar media to inactivate nisin remaining in stomachates. Bacterial populations were enumerated on media with and without the enzyme and compared (data not presented). Results indicated that bacterial populations grown on enzyme treated media were not numerically different than populations grown on non-enzyme treated media. Because residual nisin did not interfere with enumeration, enzymatic inactivation of nisin was not included in the experimental design of this study.

Nisin activity assays

Determination of nisin activity from solutions and stomachates was performed using methods described previously (Siragusa 1992, Siragusa and Cutter 1993). For either well diffusion or spot assays, *L. innocua* strains, and *B. thermosphacta* were propagated in TSB at 37°C, 18 h and 25°C, 18 h, respectively while *C. divergens* was grown in MRS broth at 30°C, 18 h. Each broth culture was diluted 1:10 in phosphate buffered saline (pH 7·0) and 100 μl of the dilution was added to 8 ml of TSB or MRS semisoft agar (0·75% w/v) for a final concentration of approximately 6 $\log_{10}$ cfu ml^{-1} of target organisms. This suspension was overlaid on a standard agar plate (TSA or MRS) and allowed to solidify for 15 min, 25°C. For the well diffusion assay, 6 wells were made on each solidified plate with a sterile cork borer (4 mm) and 20 μl of serially diluted bacteriocin solutions were added to each well. For the spot assays, 20 μl of filter sterilized (0·2 μm Acrodiscs, Gelman Sciences, Ann Arbor, MI, USA) stomachates were spotted directly onto a lawn of *B. thermosphacta*, and allowed to dry (15 min, 25°C). All TSA plates were incubated for 18 h at respective temperatures to allow for growth of the target organisms; MRS plates were incubated at 30°C, 24 h under flowing carbon dioxide (8%). Clear zones of inhibition were considered positive for nisin activity. Sensitivity to nisin was determined as AU/ml (reciprocal of highest dilution; Siragusa 1992, Siragusa and Cutter 1993).

Calculations and statistical analyses

Least squared means (LSM) of bacterial populations were calculated from three experimental replications. One-way statistical analysis (Analysis of Variance, ANOVA) was performed using the General Linear Models procedure of SAS (SAS Institute 1982) or InStat (GraphPad Software, San Diego, CA, USA). Inoculum counts were used as a covariant to normalize data from treatment replications. Log reduction factors (LRF) were calculated as differences between populations of untreated and treated tissues (LRF = log cfu cm^{-2} untreated – log cfu cm^{-2} treated). LSM values calculated from surface pH data for both tissue types were analyzed by ANOVA using General Linear Models procedure of SAS. The probability level was $P \leq 0{\cdot}05$, unless otherwise noted.

Results

Bacteriocin assays

Prior to spray treatments, the four organisms were assayed by the well diffusion method to determine the concentration of nisin used in this study. *B. thermosphacta* was the most sensitive to nisin (minimum inhibitory concentration, MIC = 5 AU ml^{-1}) while *L. innocua* and *C. divergens* were the most resistant (MIC = 5000 AU ml^{-1}). Because of the resistance demonstrated by *L. innocua* and *C. divergens* in bacteriocin assays, 5000 AU ml^{-1} of nisin was used for spray treatments in this study.

Spot assays of filter sterilized stomachates demonstrated faint zones of inhibition, indicative of some nisin activity, in all of the samples spray treated with

the bacteriocin at day 0. However, nisin activity was not detected at day 1. Nisin activity was not found in meat controls or water treated samples at either day as determined by spot assays.

Spray treatments with water and nisin

The experiment was a 4 (organism) × 2 (tissue type) × 2 (treatments; water and nisin) × 2 (days 0 and 1) factorially arranged, completely randomized design. When examined across all organisms, treatments and tissues, bacterial populations at day 0 (3·17 $\log_{10}$) were significantly different ($P \leq 0{\cdot}0004$) than day 1 (3·60 $\log_{10}$). Spray treatments with nisin resulted in populations (1·74 $\log_{10}$) that were significantly different ($P \leq 0{\cdot}001$) than water treated (3·91 $\log_{10}$) or untreated BCT (4·46 $\log_{10}$). The 2-way interaction of organism × day was also significant ($P \leq 0{\cdot}01$). No significant 3-way interactions were observed in SAS analyses.

Figure 1 illustrates the results of spray washing with water or nisin against *B. thermosphacta* attached to lean and adipose BCT. Overall, LRFs of approximately 2 to 3·6 $\log_{10}$ were observed following treatments with nisin. LRFs of <0·6 $\log_{10}$ were observed when BCT was spray treated with water. With the exception of adipose at day 0, all nisin treatments were statistically different ($P \leq 0{\cdot}05$) than either water or untreated tissues; none of which were statistically different ($P \geq 0{\cdot}05$) from one another.

Similar results were observed when *C. divergens* was subjected to spray treatments with either water or nisin (Fig. 2). Specifically, spray treatments with water resulted in LRFs of 1 $\log_{10}$ or less, while nisin treatments affected LRFs of 2·8 $\log_{10}$ or greater. Despite slight bacterial population increases over the 24 h period, nisin spray treatments effected LRFs of 3 $\log_{10}$ or greater on either tissue.

Consistent with results obtained for *B. thermosphacta* and *C. divergens*, spray treatments with water resulted in LRFs of < 1 $\log_{10}$ for *L. innocua* LA1 and ATCC 33090. Populations of water treated *L. innocua* LA1 were statistically different than untreated controls at day 0 (Fig. 3); however, after 24 h,

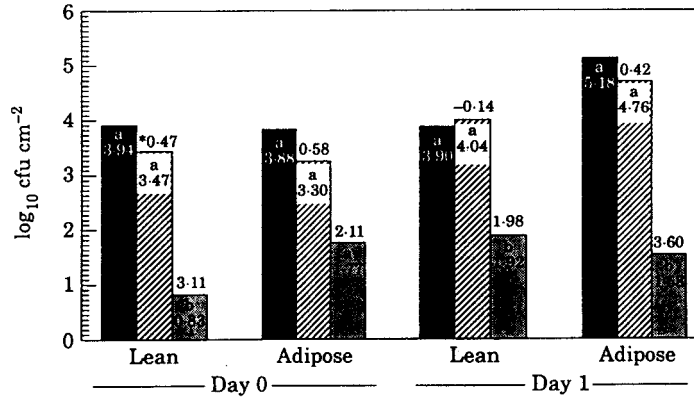

Fig. 1. Following spray treatments with water or nisin (5000 AU ml^{-1}), populations of *Brochothrix thermosphacta* ATCC 11509 attached to lean or adipose beef carcass tissue (BCT) were enumerated (days 0 and 1). Least square means (LSM) of bacterial populations are presented within each column. *Denotes log reduction factor (LRF). One-way ANOVA was performed for each tissue at each day. [a,b]Denotes statistical differences ($P \leq 0{\cdot}05$) between treatments. ■, untreated; ▨, water; ▩, risin.

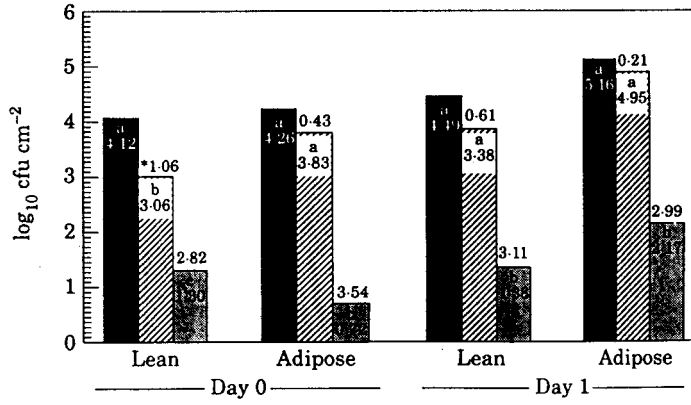

Fig. 2. Following spray treatments with water or nisin (5000 AU ml^{-1}), populations of *Carnobacteria divergens* ATCC 35677 attached to lean or adipose BCT were enumerated (days 0 and 1). LSM of bacterial populations are presented within each column. *Denotes LRF. One-way ANOVA was performed for each tissue at each day. [a,b]Denotes statistical differences ($P \leq 0{\cdot}05$) between treatments. ■, untreated; ▨, water; ▩, nisin.

populations were not statistically different. Populations of *L. innocua* ATCC 33090 were not statistically different ($P \geq 0{\cdot}05$) from untreated BCT at either day (Fig. 4). LRFs for *L. innocua* LA1 and 33090 were not as numerically substantial as those seen for *B. thermosphacta* or *C. divergens*. Additionally, the populations of *L. innocua* attached to either tissue did not differ numerically over 24 h, whereas *B. thermosphacta* and *C. divergens* demonstrated some growth (> 1 $\log_{10}$) when attached to adipose BCT.

pH data

Surface pH data demonstrated that significant differences ($P \leq 0{\cdot}0001$) ex-

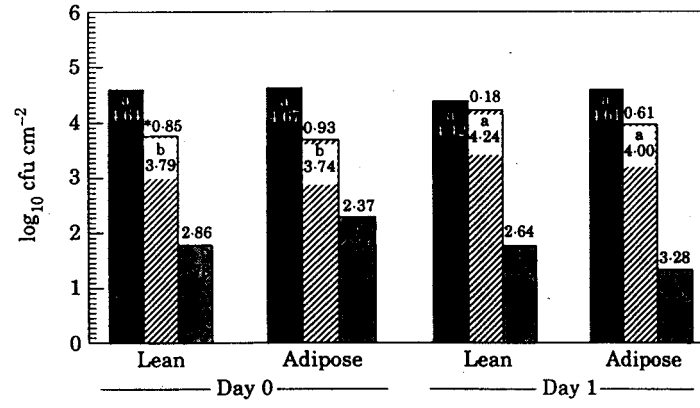

Fig. 3. Following spray treatments with water or nisin (5000 AU ml^{-1}), populations of *Listeria innocua* LA1 attached to lean or adipose BCT were enumerated (days 0 and 1). LSM of bacterial populations are presented within each column. *Denotes LRF. One-way ANOVA was performed for each tissue at each day. [a,b]Denotes statistical differences ($P \leq 0{\cdot}05$) between treatments. ■, untreated; ▨, water; ▩, nisin.

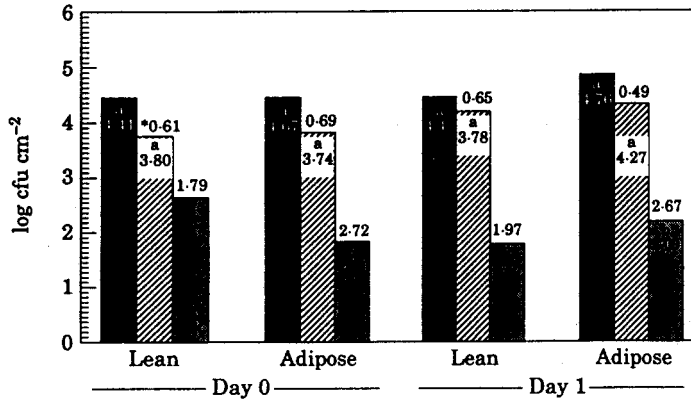

Fig. 4. Following spray treatments with water or nisin (5000 AU ml^{-1}), populations of *Listeria innocua* ATCC 33090 attached to lean or adipose BCT were enumerated (days 0 and 1). LSM of bacterial populations are presented within each column. *Denotes LRF. One-way ANOVA was performed for each tissue at each day. [a,b]Denotes statistical differences ($P \leq 0{\cdot}05$) between treatments. ■, untreated; ▨, water; ▩, nisin.

isted between the surface pH values of the two tissues, regardless of treatment or day. However, spray treatments with water or nisin did not alter the surface pH values of either lean or adipose BCT ($P \geq 0{\cdot}05$).

Discussion

Despite the available research regarding the effectiveness of nisin in various food products, in the United States, it is approved for use only as a direct food ingredient in processed cheese spreads (FDA 1988). Given the spectrum of its inhibitory activity and stability, nisin should find uses in additional food products (Nettles and Barefoot 1993). The use of nisin in red meat products has been documented, including one report that examined the use of nisin with unprocessed red meat. Chung et al. (1989) found that after soaking pieces of beef in nisin solutions, *S. aureus* and *L. monocytogenes* were initially reduced by approximately 1·35 $\log_{10}$ and 2·45 $\log_{10}$, respectively. After 4 weeks at 5°C, reductions of 0·92 $\log_{10}$ were demonstrated for *S. aureus* and 1·49 for *L. monocytogenes* (Chung et al. 1989). To date, there are no reports that address the application of bacteriocins to red meat by spray washing. In this study, we demonstrate that nisin can be applied to red meats by means of spray washing and that some bacterial populations can be reduced 3 $\log_{10}$ units or by 99·9%.

In vitro experiments with well diffusion assays demonstrated that a nisin concentration of 5000 AU ml^{-1} was sufficient to inhibit approximately 6 $\log_{10}$ cfu ml^{-1} of the meat spoilage organisms, *C. divergens* and *B. thermosphacta*, as well as two strains of *L. innocua*. When nisin was applied to surface BCT with 4 $\log_{10}$ cfu cm^{-2} attached bacteria, none of the four organisms was completely inhibited. The inoculum levels used in this study may not represent the true microbial load of fresh beef carcasses. If higher inoculum levels are present, bacteria may not be as effectively inhibited by nisin. The results from this study also demonstrate that bacteria attached to meat surfaces appear more resistant to nisin than

non-attached cells. Presently, it is not understood how bacterial attachment to a complex menstrua interferes with the efficacy of bacteriocins, but such information may be useful for the identification of other approaches to sanitizing with these or other compounds.

Although exhibiting nisin resistance in well diffusion assays, *C. divergens* appeared as sensitive to the bacteriocin when attached to surface BCT as did *B. thermosphacta*. Populations of *C. divergens* attached to adipose BCT and *B. thermosphacta* attached to lean BCT, were reduced from approximately 4 to 0·72 $\log_{10}$ and 3·90 to 0·83 $\log_{10}$, respectively. In both instances, population reductions of approximately 99·9% were observed with nisin spray treatments. The reductions associated with nisin treatments of *C. divergens* and *B. thermosphacta* attached to BCT are encouraging. Sanitizing beef carcasses with nisin may be a useful method to inhibit spoilage bacteria and therefore, extend the shelf-life of red meat.

L. monocytogenes attached to beef is sensitive to nisin activity (Chung et al. 1989). Earlier well diffusion assays performed in our laboratories indicated that *L. monocytogenes* was more sensitive to nisin than the *L. innocua* strains used in this study (unpublished data). Given that spray treatments with nisin reduced bacterial populations of *L. innocua* LA1 and ATCC 33090 attached to BCT by approximately 2 $\log_{10}$ cfu cm^{-2}, populations of *L. monocytogenes* or other Gram positive pathogens found on beef carcasses may be effectively reduced by similar treatments with nisin.

Overall, water spray treatments afforded reductions of ≤ 1 $\log_{10}$ against all the organisms attached to BCT. Slight reductions can be attributed to the physical removal of bacteria as a result of the force of the water spray. Other studies have documented reductions associated with water washing (Cutter and Siragusa 1994, DeZuniga et al. 1991). Surface pH data also indicated that the nisin solution (pH 6·5) did not significantly alter the pH of the tissues after application. Therefore, the observed population reductions can be attributed to the inhibitory activity of nisin, and not to the effects of pH and/or spray washing.

Bacteriocin assays performed in this study demonstrated that some nisin activity was detected on BCT after spray washing, but that the activity diminished after 24 h. Other researchers have encountered difficulties when recovering nisin from meat surfaces (Bell and DeLacy 1986). Speculative explanations for lack of nisin recovery are degradation of the protein by endogenous proteases associated with the surface of red meat, adsorption of nisin onto meat proteins or lipid particles, or assays that are not sensitive enough to detect the bacteriocin in samples (Bell and DeLacy 1986, Nettles and Barefoot 1993, Ray 1992). In this study, nisin activity was not detected on BCT samples 24 h after application. However, bacterial populations remained supressed, as compared to untreated controls, indicating that nisin was still active.

Spray washing with antimicrobial compounds such as organic acids or trisodium phosphate has been approved in commercial meat and/or poultry processing plants as a means of sanitizing or decontaminating carcasses. This study demonstrated that spray treatments with other antimicrobial compounds, such as bacteriocins, may not only extend the shelf-life of post rigor beef, but these compounds also may improve the microbiological safety of beef carcasses by suppressing the growth of spoilage or pathogenic bacteria.

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