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Population Reductions of Gram-Negative Pathogens Following Treatments with Nisin and Chelators under Various Conditions

CATHERINE N. CUTTER* and GREGORY R. SIRAGUSA

United States Department of Agriculture, Agricultural Research Service,† Roman L. Hruska U.S. Meat Animal Research Center, P.O. Box 166, Clay Center, Nebraska 68933 USA

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ABSTRACT

When used in combination with chelating agents (EDTA, EGTA, citrate, phosphate), the bacteriocin nisin is effective for reducing populations of gram-negative bacteria in vitro. This study examined parameters (buffers, temperature presence of divalent cations) that affect nisin inhibition of *Escherichia coli* O157:H7 and *Salmonella typhimurium*. Approximately 7 $\log_{10}$ colony-forming units (CFU) per ml of *E. coli* and *S. typhimurium* were treated in PBS or MOPS buffers containing 50 µg/ml of purified nisin, alone or in combination with 500 mM lactate, 100 mM citrate, 50 mM EDTA, and 1% (wt/vol) sodium hexametaphosphate (pH 7.0) at 37°C for 60 min or 5°C for 30 min. Surviving bacterial populations were compared to untreated controls (buffers without nisin). Data indicated that treatments with nisin in buffers resulted in reductions of 4.30 and 2.30 $\log_{10}$ CFU/ml of *E. coli* and *S. typhimurium*, respectively, as compared to untreated controls. Population reductions ranging from 2.29 to 5.49 $\log_{10}$ CFU/ml were observed when cells were treated with nisin and chelator combinations at either 37°C for 60 min or 5°C for 30 min. The addition of magnesium and calcium to buffers with nisin decreased inhibition. Data obtained from spectrophotometric experiments indicated that treatments were causing the release of cellular constituents. However, transmission electron microscopy (TEM) analyses were inconclusive, since cellular membranes did not appear to be disrupted.

Key words: *Escherichia coli* O157:H7, *Salmonella typhimurium*, nisin, chelators

The bacteriocin nisin is a 3,500-Da peptide produced by *Lactococcus lactis* subsp. *lactis* that inhibits gram-positive organisms, including the pathogens *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* (*9*). Although nisin was once thought to inhibit only gram-positive bacteria, researchers have found that the spectrum of nisin activity can be extended to gram-negative bacteria, including a variety of pathogens. Stevens et al. (*13*) reported that when gram-negative cells were treated with 20 mM EDTA and 50 µg/ml of nisin in a complex phage buffer and incubated at 37°C for 60 min, populations of *Salmonella* spp., *Enterobacter aerogenes*, *Shigella flexneri*, *Citrobacter freundii*, and *Escherichia coli* O157:H7 were reduced by 3 to 6 $\log_{10}$ colony-forming units (CFU) per ml. Treatments with nisin and 20 mM EGTA, citrate, or phosphate at 30 to 42°C also enhanced the activity of nisin towards gram-negative bacteria (*12*). EDTA is thought to chelate magnesium ions from the lipopolysaccharide (LPS) layer of the outer membrane of gram-negative bacteria (*11*). It has been suggested that alterations in the LPS layer may cause the outer cell surface to become more permeable, thereby allowing the bacteriocin access to the cytoplasmic membrane where nisin-mediated inactivation occurs (*12*).

Methods to substantially reduce or inhibit gram-negative bacteria by food-grade compounds are of considerable interest to the food industry, since there are both public health and economic concerns. This study was undertaken to determine if treatments with nisin and other food-grade chelators could reduce populations of two gram-negative pathogens, *E. coli* O157:H7 and *S. typhimurium*, and to examine parameters (ie, temperature, buffers, presence of divalent cations) affecting nisin activity against the organisms. Further investigation of nisin and chelator combinations against pure cultures under various conditions is required before such treatments can be utilized effectively in food systems.

MATERIALS AND METHODS

Bacterial strains

Salmonella cholerasuis subsp. *cholerius* serotype *typhimurium* ATCC 14028 (*S. typhimurium*) and *Listeria monocytogenes* Scott A were obtained from the American Type Culture Collection (ATCC, Rockville, MD). *Escherichia coli* O157:H7 was obtained from the Food Research Institute (Madison, WI). Cultures were maintained in 75% glycerol at –20°C and propagated in tryptic soy broth (TSB)(Troy Biologicals, Troy, MI) at 37°C for 18h.

†*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*

Nisin buffers, and chelators

Nisin (Ambicin, Applied Microbiology, NY) was stored at 4°C. Final concentrations of 50 µg/ml of nisin were used throughout this study. Nisin activity was monitored by using *L. monocytogenes* Scott A in spot assays as described previously (*1*).

The following buffers were used: 100 mM 3-[N-morpholino]propanesulfonic acid (MOPS)(Sigma Chemical Co., St. Louis, MO) and 1 or 10 mM phosphate-buffered saline (10 mM PBS contains 530 mg NaCl, 13 mg KH_2PO_4, 77 mg Na_2HPO_4, and 13 mg KCl per liter; pH 7.2). Additional cell buffers were composed of 10 mM PBS, 50 mM (hydroxymethyl) aminomethane (Tris)(Sigma), or 100 mM MOPS with 1 mM $CaCl_2$ (Sigma) and 4 mM $MgSO_4$ (Sigma). After the addition of compounds (chelators or nisin) to buffers, all solutions were adjusted to pH 7.0 with 10 M HCl or 0.1 M NaOH, filter sterilized (0.45 µm Acrodiscs, Gelman Sciences, Ann Arbor, MI), stored at –20°C until needed, and tempered to 25°C prior to use.

Food-grade chelators were added to 100 mM MOPS or 1 mM or 10mM PBS buffers for final concentrations of 500 mM DL-lactate (vol/vol)(Sigma), 100 mM citrate (anhydrous; Sigma), 50mM disodium EDTA (Fisher Scientific Co., Kansas City, MO), and 1% hexametaphosphate (HMP; wt/vol, sodium polyphosphate)(FMC, Norwalk, CT).

Treatments

From an overnight culture, *E. coli* O157:H7 or *S. typhimurium* 14028 cells were inoculated into fresh media, grown to approximately 7 $\log_{10}$ CFU/ml (OD_{600} ca. 0.1), centrifuged (15 min,1,480 × g, 5°C), and resuspended in the original volume of the respective buffer. In the first experiment, 1 ml of cell suspension was added to individual microcentrifuge tubes, centrifuged (16,000 × g,10 min, 25°C), and the cell pellet resuspended in 1 ml of the following solutions: (a) buffer, (b) buffer and nisin, (c) buffer and chelator, or (d) buffer, chelator, and nisin. After the addition of a solution, cells were incubated under two conditions: 37°C for 60 min or 5°C for 30 min. Additional experiments involving treatments of nisin and chelators in buffers (10 mM PBS, 100 mM MOPS, 50 mM Tris) with magnesium sulfate and calcium chloride were carried out similarly, but incubation was for 60 min at 37°C. After incubation, cells were centrifuged, washed with the respective buffer, serially diluted in 0.1% buffered peptone water (BPW)(BBL, Cockeysville, MD), and plated in duplicate on trypticase soy agar (TSA) (Troy Biologicals, Troy, MI) using a Model D Spiral Plater (Spiral Biosystems Instruments, Bethesda, MD). Numbers of bacteria were determined after incubation for 36 h at 37°C.

Spectrophotometric determinations

Bacteria were grown to an optical density of 0.1 (600 nm) and centrifuged for 15 min at 5°C and 1,480 × g. Cells were washed twice by resuspension in equal volumes of 0.1% peptone (pH 6.8), and centrifuged for 7 min at 16,000 × g and 25°C. Cells were resuspended in equal volumes of (a) 1 mM PBS buffer, (b)1 mM PBS buffer with nisin, (c) 1 mM PBS buffer with chelators, or (d) 1 mM PBS buffer with nisin and chelators and incubated at 37°C for 1 h and 6 h. Following incubation, cell suspensions were centrifuged for 7 min at 16,000 × g and 25°C to pellet the cells. The absorbance of the cell-free supernatants were determined at 260 and 280 nm with a Shimadzu spectrophotometer.

Electron microscopy studies

Following U.V. absorbance experiments after 6 h at 37°C, the remaining cell pellets were fixed in 0.25% glutaraldehyde in 0.2 M cacodylate buffer at 25°C for 18 h and prepared for transmission electron microscopy (*6*) with phosphotungstic acid-negative staining.

Calculations and statistical analyses

Least squared means of bacterial populations ($\log_{10}$ CFU/ml) were calculated from three experimental replications. Statistical data analysis (Analysis of Variance, ANOVA) was performed using the General Linear Models procedure of SAS (*10*). Inoculum counts were used as a covariant to normalize data from treatment replications. Log reduction factors (LRF) were calculated as the difference between populations of cells in buffer (untreated) and cells treated with chelator, nisin, or nisin and chelator combinations (LRF= log CFU/ml untreated –log CFU/ml treated). The probability level was $P \leq 0.05$, unless otherwise noted.

RESULTS

Treatments

When all treatments (nisin, alone or in combination with chelators in 10 mM PBS or 100 mM MOPS buffers), were examined against *E. coli* O157:H7 (Fig. 1), only treatment had a significant effect ($P \leq 0.0001$). Of the treatments, EDTA and nisin resulted in a greater LRF (5.42) than HMP and nisin (3.74), lactate and nisin (4.47), citrate and nisin (4.32), or nisin alone (4.30). Even in buffer, LRFs were associated with lactate (1.80) and citrate (1.47) treatments. There were no statistical differences between buffer type (MOPS, PBS) or conditions (37°C, 60 min or 5°C, 30 min), nor were 2- and 3-way interactions observed for analyses of *E. coli* O157:H7 data.

In contrast, statistical analyses for *S. typhimurium* demonstrated that in addition to the effects of treatment and buffer ($P \leq 0.0001$), 2-way interactions of treatment by buffer ($P \leq 0.04$) and buffer by condition ($P \leq 0.0008$) occurred. Of the treatments examined (Fig. 2), EDTA with nisin resulted in the greatest LRF (4.53), compared to lactate with nisin (4.25), citrate with nisin (4.06), HMP with nisin (1.88), or nisin alone (2.31). Treatments with lactate (LRF=0.44) and citrate (LRF=0.38) did not significantly alter bacterial populations. The 2-way interaction of treatment by buffer (Fig. 3) indicated that when *S. typhimurium* was treated with chelators plus nisin in PBS buffer, resulting populations were significantly lower ($P \leq 0.0001$) than comparable populations in MOPS buffer alone. When the interaction of buffer by condition was examined, the greatest reductions of bacteria also were observed when treatments were performed in PBS buffer under either condition (data not shown).

A second experiment was conducted to determine if the buffers were acting as chelators. Bacterial cells were treated with 100 mM MOPS, 10 mM PBS, or 50 mM Tris buffer containing magnesium sulfate and calcium chloride, with and without nisin, at 37°C for 60 min. Treatments with buffers alone or in combination with the divalent cations did not result in statistically significant population reductions (Fig. 4). Treating cells with buffer and nisin resulted in significant population reductions. However, upon addition of divalent cations to buffers with nisin, the cations appeared to reduce nisin activity towards the pathogens.

Spectrophotometric experiments

In addition to calculating reductions of bacterial populations from plate count data, spectrophotometric experiments were performed to monitor the release of cellular components during treatments. Since it was found that the extent of inactivation by 1 mM PBS was less than that by 10 mM PBS

Figure 1. *Effect and significance* ($P \leq 0.0001$) *of treatments with nisin and chelators, in either buffer, on* E. coli *O157:H7. *Denotes log reduction factor (LRF: CFU/ml control – CFU/ml treated).* [a, ab, b, c, d] *Denote statistically significant differences between populations.*

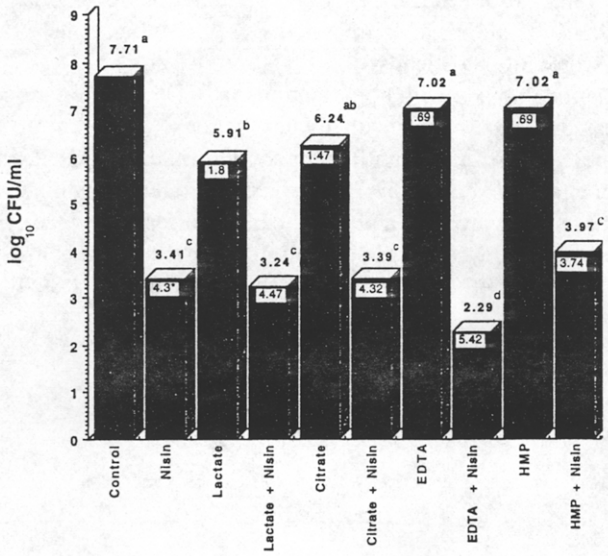
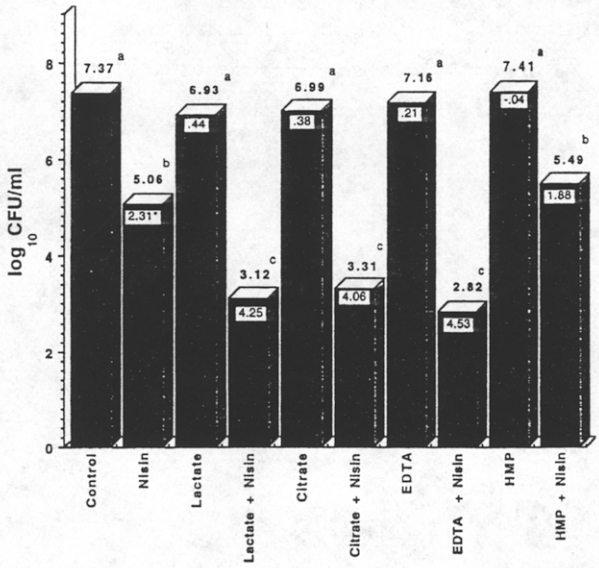


Figure 2. *Effect and significance* ($P \leq 0.0001$) *of treatments with nisin and chelators, in either buffer, on* S. typhimurium *ATCC 14028. *Denotes log reduction factor (LRF: CFU/ml control –CFU/ml treated).* [a, b, c] *Denote statistically significant differences between populations.*



(data not shown), 1 mM PBS was used throughout these experiments. Data from absorbance readings of cell supernatants at 260 nm for each organism are presented in Fig. 5 and 6. Except for treatments with lactate, absorbance readings were 2 to 4 times greater for cells treated with a combination of buffer, nisin, and chelators than for cells treated with buffer alone, buffer and nisin, or buffer and chelators. Similar patterns were observed for absorbance readings at 280 nm (data not shown). The release of cellular components when cells were treated with buffer and nisin, or combinations of buffer, nisin, and chelators, suggests some degree of membrane damage or cell lysis.

Electron Microscopy

Transmission electron microscopy (TEM) of cells following treatments with citrate and nisin in 1 mM PBS revealed some condensation within the cytoplasm of the bacterial cells (Fig. 7a, 8a). This observation was not detected in bacterial cells treated with buffer alone (Fig. 7b, 8b). With the exception of some cell ghosts, no other visible abberations were detected in electron micrographs of cells treated with nisin, chelators, or nisin and chelator combinations.

Figure 3. *Effect and significance* ($P \leq 0.0001$) *of treatment by buffer (10 mM PBS and 50 mM MOPS) for* S. typhimurium *14028.*

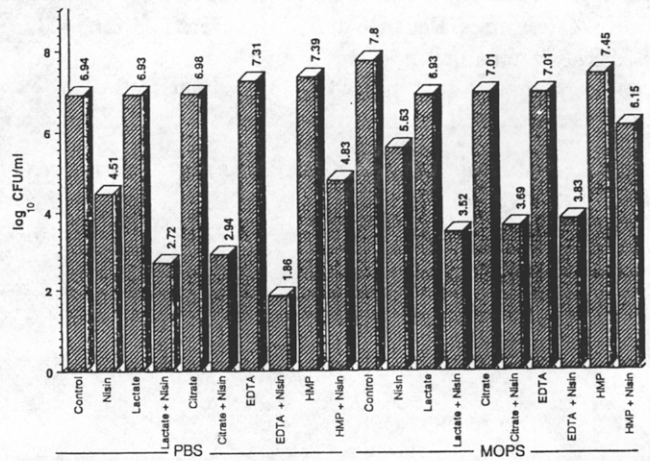
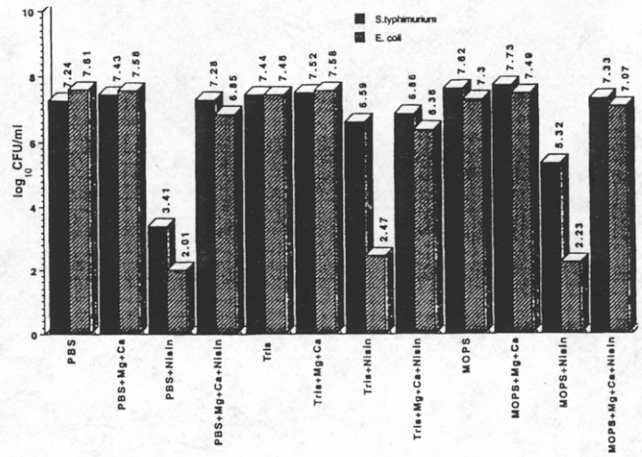


Figure 4. *Effect and significance* ($P \leq 0.0001$) *of treatment by organism for* E. coli *O157:H7 and* S. typhimurium *ATCC 14028 following experiments with divalent cations (37°C, 60 min).*

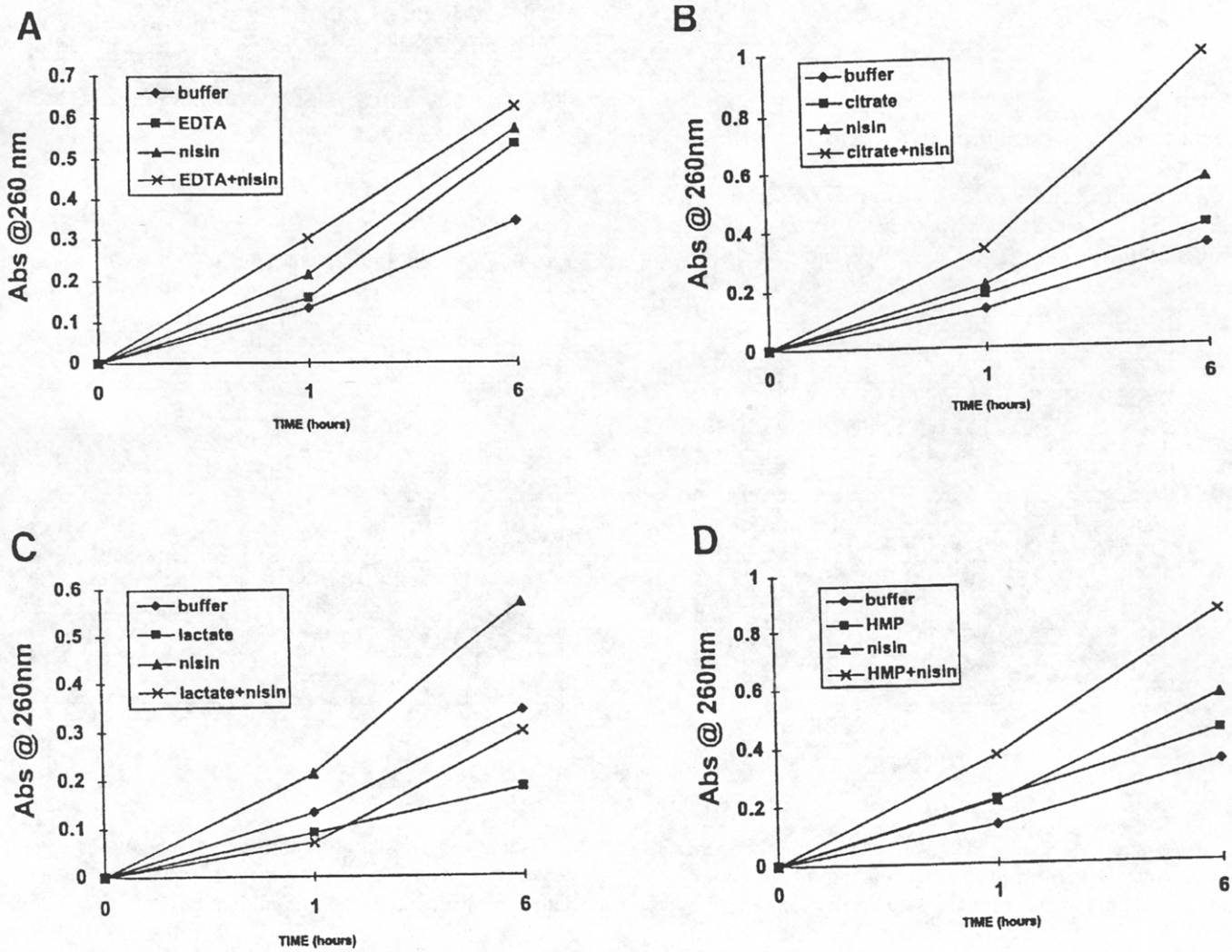


DISCUSSION

Other researchers have examined the effect of nisin and various food-grade chelators against gram-negative bacteria at 37°C in vitro *(2, 11–13)*. In their studies, nisin and chelators were combined in a complex phage buffer consisting of Tris-HCl, gelatin, magnesium sulfate, calcium chloride, and sodium chloride (pH 7.2). In this study, food-grade chelators were combined with 50 μg/ml nisin in simple buffers (100 mM MOPS, 50 mM Tris, and 10 mM PBS, pH 7.0). At these concentrations, the buffers alone did not reduce bacterial populations (data not shown). However, when cells were treated with buffers containing nisin, LRFs ranging from 2 to 4 $\log_{10}$ CFU/ml occurred. The magnitude of reduction diminished as lower concentrations of buffer were used (data not shown). The effectiveness of nisin diminished when magnesium sulfate and calcium chloride were added to the buffers containing nisin, suggesting that MOPS, PBS, and Tris buffers may be acting as chelators. If they bound magnesium and calcium from the surrounding solution, the buffers could not effectively chelate divalent cations from the LPS layer of the gram-negative cells. Because the cations were not removed, the LPS layer remained intact and nisin could not penetrate the outer membrane *(13)*. It has been demonstrated previously that the use of magnesium and calcium with nisin in a complex phage buffer *(11, 12, 13)* does not interfere with results. However, neither this study nor previous studies have addressed the effects of the individual cations or other compounds (e.g., gelatin, sodium chloride) found in the phage buffer on nisin and chelator combination efficacy.

Stevens et al. *(12)* previously demonstrated that nisin and phosphate combinations resulted in population reductions; therefore, our results with a phosphate buffer and nisin and concurrent reductions of the pathogens and release of cellular components are not unexpected. In general, combinations of bacteriocin and chelators in 10 mM PBS buffer resulted in greater population reductions of *S. typhimurium* as compared to 100 mM MOPS buffer. However, populations of *E. coli* O157:H7 remaining after treatments with combinations in PBS were not statistically different from *E. coli* O157:H7 populations treated with combinations in MOPS buffer. As indicated by experiments with divalent cations, MOPS buffer may be capable of chelating. Treatments with nisin and MOPS buffer against *E. coli* O157:H7 resulted in reductions similar to those seen with nisin and Tris or PBS buffers. However, *S. typhimurium* was not as susceptible to similar treatments with MOPS. In other studies, researchers have demonstrated

Figure 5. *U.V. absorbance (260 nm) experiments performed on* E. coli *O157:H7 after treatments with nisin and chelators in 1 mM PBS at 37°C for 1 and 6 h. Each point represents an average taken from 3 readings. A. Data from treatments with EDTA. B. Data from treatments with citrate. C. Data from treatments with lactate. D. Data from treatments with HMP.*



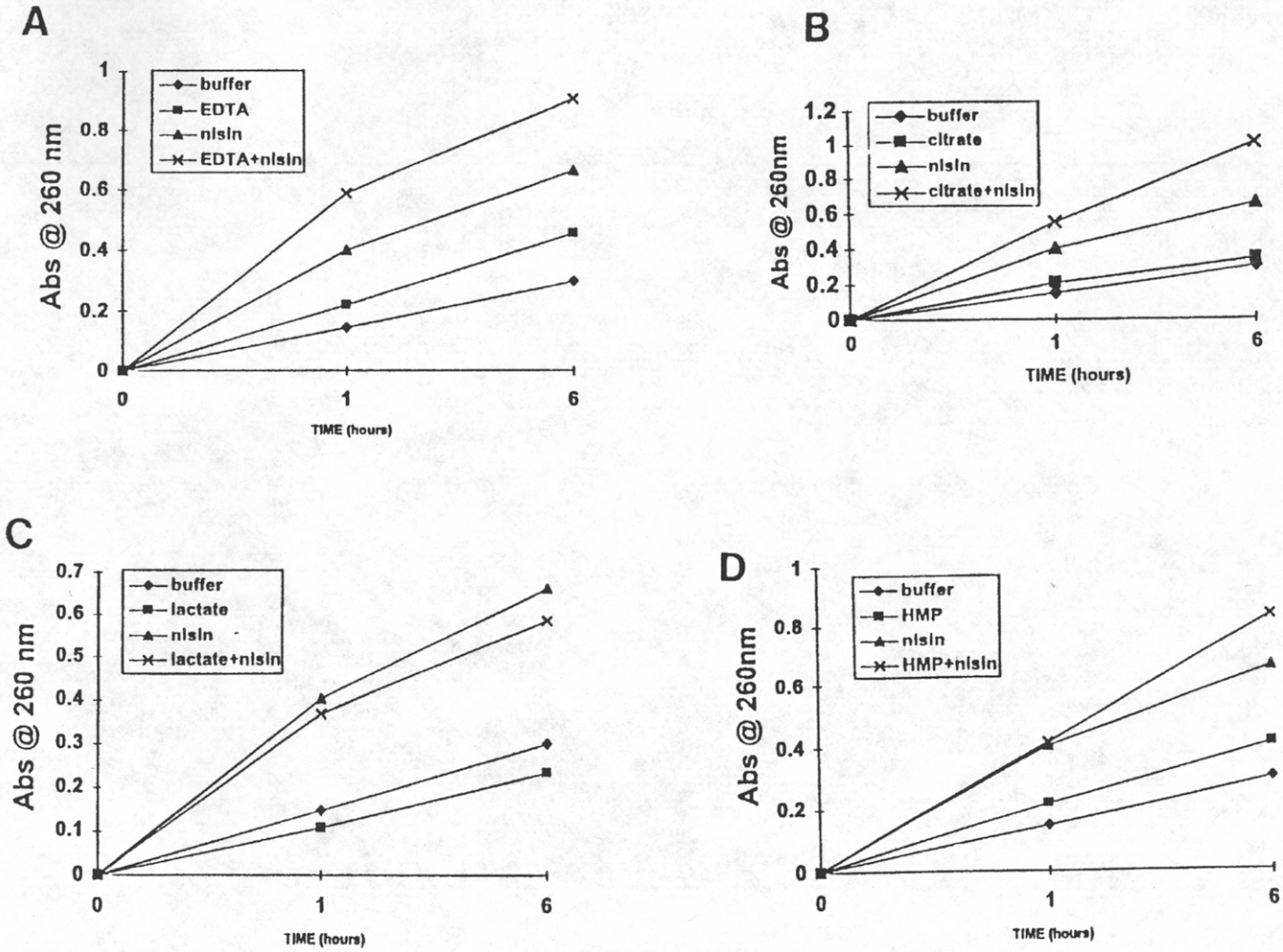
that Tris, a primary amine, is able to increase the permeability of the outer membranes of gram-negative bacteria and enhance the activity of various antimicrobial compounds (*7, 8, 15, 16*). As demonstrated in this study, Tris buffer may not only chelate divalent cations, but may enhance nisin activity against gram-negative bacteria in solution.

In earlier experiments, EDTA, EGTA, citrate, and phosphate at a concentration of 20 mM were used in combination with nisin against various gram-negative bacteria (*11, 12*). In this study, the food-grade compounds 500 mM lactate and 1% sodium HMP, as well as higher concentrations of EDTA (50 mM) and citrate (100 mM), were examined for the ability to enhance nisin activity against *E. coli* O157:H7 and *S. typhimurium*. Presently, there is no report addressing the efficacy of HMP and lactate in combination with nisin against gram-negative bacteria. Other researchers have demonstrated that sodium HMP makes the outer membrane of gram-negative bacteria permeable, thereby allowing hydrophobic antibiotics or detergents to effectively inhibit the organisms (*15*). The results from this study demonstrated that treatments with HMP and nisin caused, the release of cellular components and population reductions compared to buffer controls. However, treatments with 1% HMP were not as effective as nisin in buffers, or combinations of nisin with other chelators in buffers. Treatments with lactate and nisin resulted in population reductions comparable to combinations of nisin with citrate. Of the chelators examined in earlier studies, 20 mM EDTA, when combined with nisin, was consistently the most effective for reducing populations of gram-negative bacteria (*11, 12*). As demonstrated in this study, even at 50 mM concentrations in MOPS or PBS buffers, EDTA and nisin resulted in significant population reductions. Despite the higher amounts of chelators used in this study, such concentrations may be necessary to remove exogenous divalent cations associated with food systems and still be effective enough to disrupt the LPS layer of gram-negative spoilage or pathogenic bacteria.

In addition to examining treatments at 37°C for 60 min (*2, 11–13*), treatments were efficacious under refrigerated conditions (5°C for 30 min). The data from this study and previous research (*2, 11–13*) demonstrate that bacteriocin and chelator combinations effectively reduce populations of gram-negative bacteria at temperatures from 5 to 42°C.

While treatments with buffer or buffer and chelators caused the release of some cellular components, the extent of release was not as great as that in treatments with buffer and nisin or buffer, nisin, and chelator. Plate count data demon-

Figure 6. *U.V. absorbance (260 nm) experiments performed on* S. typhimurium *ATCC 14028 after treatments with nisin and chelators in 1 mM PBS at 37°C for 1 and 6 h. Each point represents an average taken from 3 readings. A. Data from treatments with EDTA. B. Data from treatments with citrate. C. Data from treatments with lactate. D. Data from treatments with HMP.*



strated that significant LRFs occurred when cells were treated with these same solutions. Nisin-induced cell damage and concurrent population reductions also have been reported previously (*11*).

While U.V.-absorbing cellular components were detected in the supernatants after treatments, TEM analyses did not indicate that membranes were disrupted. The appearance of cell ghosts could be attributed to the presence of dead cells in the initial suspensions or to artifacts that occurred during processing of the cell pellets. Only treatments with nisin and citrate in 1 mM PBS appeared to affect *E. coli* O157:H7 and *S. typhimurium* internally, as indicated by condensation of cytoplasmic material. Cherrington et al. (*3–5*) observed that when *E. coli* K12 was treated with formic or propionic acids (pH 5.0), cytoplasmic constituents appeared to clump, β-galactosidase was released, and RNA, DNA, protein, lipid, and cell-wall syntheses were slowed or inhibited. It was concluded that visible clumping of cellular constituents was due to an irreversible denaturation of acid-labile protein and DNA, resulting from lowered cytoplasmic pH (*5*). In this study, since all treatments were performed at neutral pH, denaturation of protein and DNA due to cytoplasmic pH changes was unlikely. However, nisin-mediated inactivation due to destabilization of the cytoplasmic membrane, the efflux of amino acids, disruption of proton motive force, and in some cases cell lysis (*9*) could affect internal pH. With the exception of cell lysis, such abberations associated with the Gram-negative bacteria examined in this study may not be detected by TEM. Further research will be required to determine why cytoplasmic clumping occurs after treatments with nisin and citrate under the conditions described.

Given that the concentrations of lactate, citrate, HMP, EDTA, or simple buffers used in this study effectively enhanced nisin, and that treatments could be performed under

Figure 7. *A. Electron micrograph (31,000×) of* E. coli *O157:H7 treated with citrate and nisin in 1 mM PBS buffer, 37°C, 6 h. B. Electron micrograph (31,000×) of* E. coli *O157:H7 treated with 1 mM PBS buffer, 37°C, 6 h. Scale: 1 micron (μ) = 3.1 cm.*

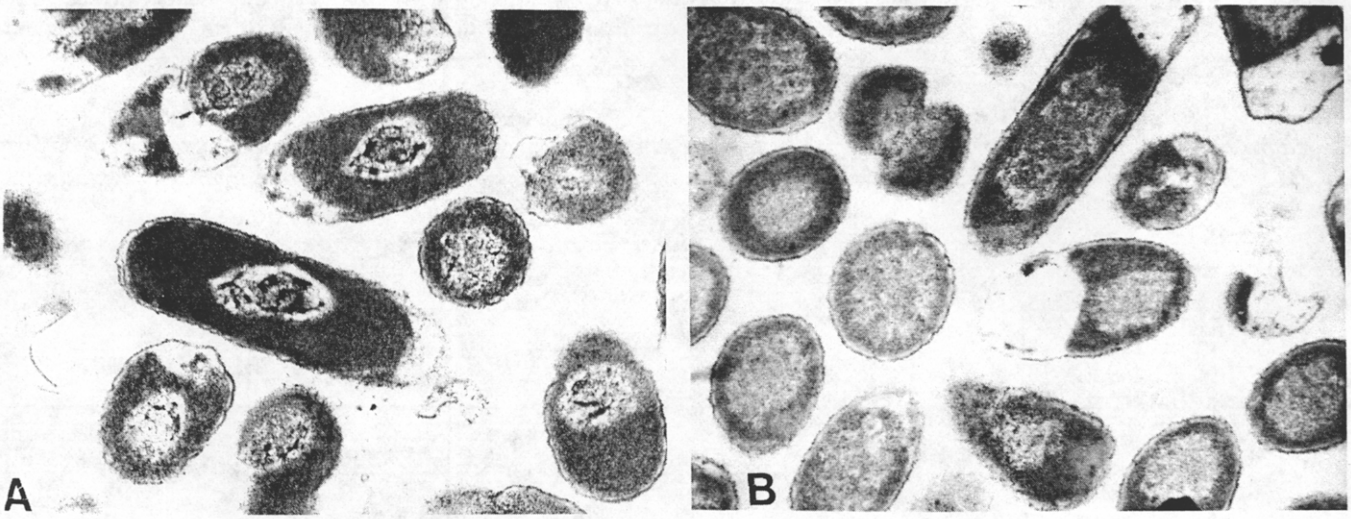
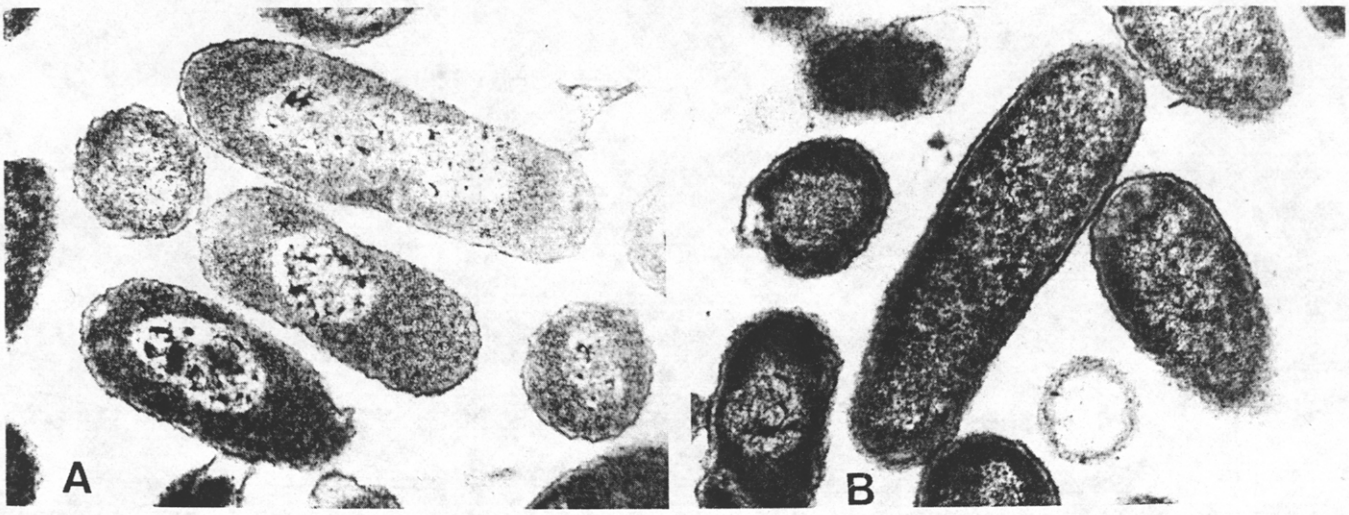


Figure 8. *A. Electron micrograph (38,925×) of* S. typhimurium *ATCC 14028 treated with citrate and nisin in 1 mM PBS buffer, 37°C, 6 h. B. Electron micrograph (38,925×) of* S. typhimurium *ATCC 14028 treated with 1 mM PBS buffer, 37°C, 6 h. Scale: 1 micron (μ) = 3.9 cm.*



refrigerated conditions, this information could be used to apply nisin and chelators to a variety of foods for the inhibition of pathogenic or spoilage gram-negative bacteria.

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REFERENCES

1. Barefoot, S. F., and T. R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45: 1808–1815.
2. Blackburn, P., J. Polak, S. Gusik, and S. D. Rubino. 1989. Nisin compositions for use as enhanced, broad range bacteriocins. International Patent Application no. PCT/US89/02625; International Publication No. WO89/12399. Applied Microbiology, Inc., New York.
3. Cherrington, C. A., M. Hinton, and I. Chopra. 1990. Effect of short chain organic acids on macromolecular synthesis in *Escherichia coli*. J. Bacteriol. 68:69–74.
4. Cherrington, C. A., M. Hinton, G. R. Pearson, and I. Chopra. 1991. Inhibition of *Escherichia coli* K12 by short-chain organic acids: lack of evidence for induction of the SOS response. J. Appl. Bacteriol. 70:156–160.
5. Cherrington, C. A., M. Hinton, G. R. Pearson, and I. Chopra. 1991. Short chain organic acids at pH 5.0 kill *Escherichia coli* and *Salmonella* spp. without causing membrane perturbation. J. Appl. Bacteriol. 70:161–165.
6. Glauert, A. M. 1984. Fixation, dehydration and embedding of biological specimens: practical methods in electron microscopy, vol. 3, part 1. North-Holland Publishing Co., Amsterdam.
7. Hancock, R. E. W. 1984. Alterations in outer membrane permeability. Ann. Rev. Microbiol. 38:237–264.
8. Irvin, R. T., T. J. MacAlister, and J. W. Costerton. 1981. Tris (hydroxymethyl) aminomethane buffer modification of *Escherichia coli* outer membrane permeability. J. Bacteriol. 145:1397–1403.
9. Nettles, C. G., and S. F. Barefoot. 1993. Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria. J. Food Prot. 56:338–356.
10. SAS Institute. 1982. Statistical Analysis System. Cary, NC.
11. Stevens, K. A., N. A. Klapes, B. W. Sheldon, and T. R. Klaenhammer. 1992. Antimicrobial action of nisin against *Salmonella typhimurium* lipopolysaccharide mutants. Appl. Environ. Microbiol. 58:1786–1788.
12. Stevens, K. A., N. A. Klapes, B. W. Sheldon, and T. R. Klaenhammer. 1992. Effect of treatment conditions on nisin inactivation of gram-negative bacteria. J. Food Prot. 55:763–766.
13. Stevens, K. A., B. W. Sheldon, N. A. Klapes, and T. R. Klaenhammer. 1991. Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. Appl. Environ. Microbiol. 57:3613–3615.
14. Vaara, M. 1992. Agents that increase the permeability of the outer membrane Microbiol. Rev. 56:395–411.
15. Vaara, M., and J. Jaakkola. 1989. Sodium hexametaphosphate sensitizes *Pseudomonas aeruginosa*, several other species of Pseudomonas, and *Escherichia coli* to hydrophobic drugs. Antimicro. Agents Chemother. 33: 1741–1747.
16. Voss, J. G. 1967. Effects of organic cations on the gram-negative cell wall and their bactericidal activity with ethylenediamine-tetra-acetate and surface active agents. J. Gen. Microbiol. 48:391–400.