Research Note

Influence of pH, Salt, and Temperature on Nisin Resistance in Listeria monocytogenes†

ELAINE C. P. DE MARTINIS,1 ALLISON D. CRANDALL,2 ALEJANDRO S. MAZZOTTA,2 and THOMAS J. MONTVILLE2*

1Department of Foods and Nutrition, Universidade de São Paulo, São Paulo, Brasil; and 2Department of Food Science, New Jersey Agricultural Experiment Station, Cook College, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08903-0231, USA

ABSTRACT

The influence of pH (5.0, 5.5, and 6.0), salt (0.5, 2.0, and 3.5%) and temperature (10, 20, and 30°C) on the frequency of nisin resistance in Listeria monocytogenes Scott A was evaluated. At 20 and 30°C, resistance frequencies of around 1 in 106 were obtained regardless of salt concentration or pH. At 10°C the frequency of nisin resistance dropped with decreasing pH and decreasing salt concentration. At pH 5.5 and 0.5% NaCl it became impossible to generate nisin-resistant isolates. Low salt (2 to 3.5%) appeared to play a protective role, allowing L. monocytogenes to better survive nisin at low temperature (10°C).

Key words: Listeria monocytogenes, nisin, resistance

Nisin, a bacteriocin produced by certain strains of Lactococcus lactis, is a promising antimicrobial agent for food. Nisin has been approved by the World Health Organization as a preservative in the food industry and has enjoyed international use for approximately 45 years in a wide variety of food products such as milk, cheese, canned vegetables, and bakery products (5, 10). As the only bacteriocin currently approved for food use in the United States, nisin is generally recognized as safe to inhibit Clostridium botulinum in certain pasteurized cheese spreads (7).

The ability of nisin to inhibit the food-borne pathogen Listeria monocytogenes has attracted a great deal of interest (1, 2, 12). L. monocytogenes is a particularly challenging pathogen to control in food, because it is quite tolerant of high levels of sodium chloride and can grow at a relatively low pH. For example, L. monocytogenes can grow in medium containing 10% NaCl at pH 7 and 25°C (14), survive in 20% NaCl for 8 weeks at 4°C (17), and initiate growth at a pH as low as 4.39 at 30°C (8). Additionally, this pathogen can survive and grow at refrigeration temperatures (21). While nisin has the potential to serve as a useful biopreservative against L. monocytogenes, its effectiveness would be compromised by the development of resistance in this bacterium.

Both Harris et al. (9) and Ming and Daeschel (15) have investigated nisin resistance in L. monocytogenes under favorable growth conditions (brain heart infusion agar at a neutral pH, incubated at 37°C). Under these conditions nisin resistance occurs at a frequency of around 10-6. In addition, Harris et al. (9) also looked at the separate effects of NaCl and pH on nisin resistance. They found an additional 2-log-unit reduction in the resistance frequency at pH 5.5 compared to that at pH 6.5. The effectiveness of nisin was slightly enhanced by 2.5% NaCl at low concentrations of nisin (40 to 400 IU/ml). However, there are currently no data on how these traditional food-preservation hurdles act in combination to influence the efficacy of nisin. Our goal was to assess the combined effect of salt, pH, and temperature on the effectiveness of nisin against L. monocytogenes.

MATERIALS AND METHODS

Bacterial strain and culture conditions

L. monocytogenes Scott A stock culture was maintained at -80°C in Trypticase soy broth without dextrose (BBL Microbiology Systems, Cockeysville, MD) supplemented with 0.6% yeast extract (Difco Laboratories, Detroit, MI) and 0.5% glucose (Fisher Scientific Company, Pittsburgh, PA) (TSBYEG) containing 20% glycerol. Working cultures were made as slants on TSBYE supplemented with 1.5% Bacto-agar (Difco), stored at 4°C, and transferred bimonthly for 6 months before a new working culture was made. Inocula were grown at 30°C.

Determination of nisin resistance frequencies

Agar plating media at the appropriate pH and salt concentrations were prepared by solidifying TSBYE containing 0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO) with 1.5% Bacto-agar. The pH was adjusted to 5.5, 6.0, or 6.5 with 2 N HCl (Fisher) and NaCl (Fisher) was added at 0, 1.5, or 3% prior to
autoclaving. Given that TSBYEG contains 0.5% NaCl, the final NaCl concentrations were 0.5, 2.0, and 3.5%. Nisin (Aplin & Barret Ltd., Trowbridge, UK) dissolved in 0.02 N HCl with 0.75% NaCl (pH 2) was autoclaved separately and aseptically added to the sterile media to a final nisin concentration of 100 IU/ml. This concentration was used because it produces an intermediate level of lethality, allowing both increases and decreases in resistance to be observed. Control plates were prepared with an equal volume of nisin diluent.

A 50-μl aliquot from a sample containing approximately 10⁹ CFU/ml of mid-log-phase L. monocytogenes Scott A cells per ml was deposited on the plates with a spiral plater (Model D, Spiral Biotech, Inc., Bethesda, MD). Plates were incubated at 10, 20, and 30°C. Plates incubated at 10°C were enclosed in plastic bags to prevent evaporation losses over the long incubation. Colonies were counted with a Laser Bacteria Colony Counter (Model 500A, Spiral Biotech, Inc) after 48 h for plates incubated at 30°C, after 2 to 4 days at 20°C, and after colonies were well formed (or a maximum of 30 days) at 10°C.

Nisin resistance frequencies were calculated by dividing the number of colonies formed at a given pH, NaCl, and temperature combination in the presence of nisin by the number formed in the absence of nisin at that temperature. The experiment was carried out in duplicate with mean values reported.

**MIC determination**

The MIC of nisin was determined following the manufacturer’s procedures for the spiral plater mentioned above. Briefly, a 10⁴ IU of nisin per ml stock solution was spiral-plated onto agar plates and the plates were incubated at 4°C for 30 min to allow the nisin to be absorbed by the agar. Radial streaks of L. monocytogenes culture were deposited onto the surface of the agar with a sterile swab. After incubation at 10, 20, and 30°C, the spiral gradient endpoints were measured as the distance from the endpoint of growth to the center of the plate. The deposition factors and the MICs were calculated in accordance with the instructions provided in the user guide (18). Some experiments were also carried out using TSBYEG agar without added Tween 20 or containing 0.1% Tween 40, 60, 65, 80, or 85, or 1% lecithin.

**RESULTS AND DISCUSSION**

Salt concentration and pH had little effect on the ability of L. monocytogenes to grow and form colonies in the presence of 100 IU of nisin per ml at 20 and 30°C (Figure 1, A and B). Under these conditions, around 1 in 10⁸ cells were nisin resistant. It was somewhat surprising to find that decreased pH had little effect on the resistance frequency, as Harris et al. (9) have reported obtaining an additional 2-log-unit reduction in cell number at pH 5.5 compared to cell number at pH 6.5 in the presence of 100 IU of nisin per ml at 37°C.

It should be pointed out that the nature of the acidulant and not only the final pH may be of importance in the inhibition of L. monocytogenes. Farber et al. (6) reported that hydrochloric acid was less effective in inhibiting the growth of L. monocytogenes at 4 and 30°C than organic acids. However, Harris et al. (9) found that lactic and hydrochloric acid were similarly effective in reducing the number of survivors in the presence of nisin.

Only when the incubation temperature was further reduced to 10°C (Figure 1C) was the frequency of nisin resistance influenced by the NaCl concentration and pH. The lower temperature may be functioning as an additional barrier to growth. At pH 6.5 with either 2 or 3.5% salt, the resistance frequency remained around 10⁻⁵, similar to that seen under all conditions at both 20 and 30°C. Reduction of
the salt concentration to 0.5%, however, reduced this frequency to around $10^{-8}$. No nisin-resistant isolates could be generated at salt concentrations of 0.5% and pH 5.5.

The higher resistance frequencies in the presence of salt were at first surprising. However, Cole et al. (4) have reported that, at lower temperatures, low concentrations of salt provide a slight protective effect, allowing better *L. monocytogenes* survival at lower pH values than in the absence of additional salt. Furthermore, in modeling the growth of *L. monocytogenes* in different combinations of pH and salt concentrations, they predicted the optimum salt concentration for *L. monocytogenes* growth at 10°C to be around 2 to 2.5%. As is consistent with these findings, salt concentrations of around 2% appear to play a protective role in our system, allowing more nisin-resistant colonies to form in the presence of nisin at low temperature.

Next we asked the question whether the colonies which formed in the presence of nisin were resistant to nisin, or whether they were simply survivors. Five colonies which formed at 20°C on plates containing nisin and 3.5% NaCl at pH 5.5 were picked, grown overnight in nisin-free TSBYE and their MICs of nisin determined. Cultures from four of the colonies were resistant to nisin, growing at all nisin concentrations assayed; the nisin MICs were higher than 10^4 IU/ml. For the fifth colony the nisin MIC was 84 IU/ml, suggesting an intermediate level of resistance between the wild-type strain (nisin MIC, 18 IU/ml) and the nisin-resistant colonies. Ming and Daeschel (15) also obtained nisin-resistant colonies after a single exposure to nisin. Both Ming and Daeschel (15) and our group (13) have shown that when mutants are isolated after multiple exposures to higher nisin concentrations, the resistance remains stable after multiple passages through nisin-free media.

In the course of these experiments, we realized that nisin had a greatly reduced antilisterial activity in agar unless Tween 20 was present. All of the Tween series tested andlecithin decreased the MIC approximately 10-fold at 10, 20, and 30°C. Historically, most investigators have added Tween 20 to their media when assaying for nisin. This practice dates back to the original Tramer and Fowler (19) assay which includes Tween 20, perhaps because it is included in selective media for the growth of lactobacilli. Its inclusion is thought to enhance diffusion and eliminate the need for an overnight refrigerated prediffusion step. Joosten and Nuñez (11) have suggested that Tween functions by inhibiting adsorption to surfaces. However, an alternative explanation is that Tween 20 sensitizes the cells to nisin. In some microorganisms, Tween can influence the structure and function of the cell membrane. When grown in a medium with Tween 80, *Streptococcus mutans* cells contain less *n*-palmitate and more octadecanoate (18:1), changes which suggest an increase in the fluidity of lipids (20). Additionally, membrane permeabilities to various compounds are increased in *Pseudomonas aeruginosa* cells (3) and mycobacterial cells (16) grown in medium with Tween 80. Although a Tween alone is not inhibitory to *L. monocytogenes* (12), perhaps it sufficiently disrupts the fluidity of the cytoplasmic membrane, allowing nisin to more easily kill the cell. In that case, all of the published reports on the antilisterial activity of nisin in media which contain Tween are really about the effect of nisin and Tween.

The use of nisin as the sole preservative for a food product would probably be unwise, as multiple exposures of a pathogen to nisin would greatly increase the probability of generating stable resistant mutants. However, as we have demonstrated, coupling nisin with several other common food preservation strategies greatly reduces the frequency at which resistance arises. In applications utilizing nisin at low temperatures, food processors should be aware of the potential protective effect of low concentrations of salt. The mechanism of this protection is currently unknown. Rather than functioning as a “silver bullet” of food preservation, nisin will probably be best used as an additional hurdle in a multiple-hurdle preservation strategy.

**ACKNOWLEDGMENTS**

Research in the authors’ laboratory and preparation of this manuscript were supported by state appropriations, U.S. Hatch Act Funds and U.S. Department of Agriculture CSRS NRI Food Safety Program (no. 94-37201-0994). We acknowledge the financial assistance of Aplin and Barrett Ltd. (Trowbridge, England) supporting E. M. ’s visit to our laboratory.

**REFERENCES**

bacteria and the specific resistance response of *Listeria monocytogenes* Scott A. J. Food Prot. 56:944–948.


