Effect of nisin on yogurt starter, and on growth and survival of *Listeria monocytogenes* during fermentation and storage of yogurt

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SUMMARY

The effect of nisin on *Listeria monocytogenes* ATCC 7644 at different pH values was investigated in nonfat dry milk (NDM), in TSB and in yogurt. In TSB, nisin concentrations ranging from 10 to 200 RU/mL inhibited growth of *L. monocytogenes* at pH 6.0 and below but not at pH 6.8. A concentration of 10 RU/mL was inhibitory only at pH 4.5. In NDM, 50 RU/mL was inhibitory to the pathogen at pH 6.8 and below and activity increased as the pH decreased. In yogurt with added nisin (10 RU/mL), no *Listeria* survived at 24 hours during storage at refrigeration temperature (ca, 7 °C). The pathogen survived, however, 13 days of storage at the same temperature in control samples (without added nisin). Nisin inhibited the yogurt fermentation at a concentration higher than 50 RU/mL. Besides controlling *L. monocytogenes*, nisin addition may be recommended to prevent the excessive acidification and the wheying-off usually observed in yogurt when it approaches the end of its shelf life.

INTRODUCTION

Food-born outbreaks resulting from consumption of *Listeria*-contaminated foods continue to raise a major concern with regard to food safety. In the United States, *Listeria monocytogenes* was responsible for about 25% of the estimated food-borne-disease-related death (16). Milk and milk products are frequently incriminated (6, 8, 21). Consequently, considerable effort has been made to control the pathogen in dairy products. In this regard, the use of bacteriocins or bacteriocin-producing lactic acid bacteria as biological additives has been extensively studied (2, 4, 24). Among dairy products, yogurt received the least attention due to the fact that its high acidity and milk pasteurization were thought to be effective barriers to the growth of many pathogens including *L. monocytogenes*. It is now well established that the pathogen survives processing and storage of cultured milks including yogurt and other dairy products fermented with the same starter (20, 22, 23). According to De Buyser et al. (5), *L. monocytogenes* was responsible for 10 out of 64 outbreaks implicating dairy products among which 32.8% were made from pasteurized milk. Moreover, reported adaptation of the pathogen to acidity (9, 15) is warning us for its possible occurrence in low-acid foods.

Use of nisin as biological additive to yogurt to control sensitive pathogens and to extend the shelf-life has been suggested (24). However, such use is limited by the sensitivity of the starter culture to nisin which may impair the fermentation depending on the concentration of nisin and the strains used. The present work was carried out to investigate the effect of the nisin on *L. monocytogenes* during processing and storage of yogurt.

1. MATERIALS AND METHODS

1.1 Cultures

Listeria monocytogenes ATCC7644 was used in this study. It was grown on a slant of Trypticase Soy Agar (TSA, Biokar) and stored at 7 °C. Before each use, it was transferred to 10 mL of Trypticase Soy Broth [(TSB) (Bacto-Tryptone, 15 g; Bacto-soytone, 5 g; NaCl, 5 g; and distilled water, 1000 mL] and incubated 16 to 18 hours at 37 °C.

1.2 Starter culture

Commercial yogurt starter consisting of *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. Bulgaricus*) and *Streptococcus salivarius* subsp. *thermophilus* (*Str. thermophilus*) (1:1) (Redi-set, Hansen Laboratories, Inc. Milwaukee, WI) was used. The starter culture was activated in sterile NDM according to the manufacturer's recommendations.

1.3 Nisin

Nisaplin (37×10^6 RU/g) was purchased from Aplin and Barrett, Ltd., Trowbridge, England. Stock solution (37×10^3 IU/ml) was prepared by dissolving 0.1 g of nisin in 80 mL HCl, 0.02 mol/mL and holding at room temperature for 2 hours to complete dissolution. The volume was then made up to 100 mL with HCl, 0.02 mol/L and the solution

was filter-sterilized by passage through a 0.22-µm Millipore membrane and stored at -20 °C.

1.4 Effect of Nisin on *L. monocytogenes* ATCC 7644

1.4.1 Varying pH with constant nisin concentration

Three series of test tubes containing 9 mL of reconstituted NDM (100 g/L) each were used. The tubes of each series were adjusted to different pH values (6.8, 5.5 and 4.5) with citric acid. To the first series (test), nisin was added to a final concentration of 50 RU/mL along with 0.1 mL of *L. monocytogenes* ATCC 7644 overnight (16 to 18h) culture (ca, 10^6 cell/mL). To the second series, only *Listeria* was added to the same concentration. The third series was a negative control to test the sterility of the medium; neither nisin nor the microorganism was added. Cultures were incubated at 37 °C and the growth of *L. monocytogenes* was monitored by plate count on TSA at 0, 4, 7, 24 and 48 hours.

1.4.2 Varying pH and nisin concentration

A series of test tubes containing 9 to 10 mL of TSB each were used. The tubes of each series were adjusted to different pH values (6.8, 6.0, 5.5 and 5.0) with citric acid. For each pH, a different nisin concentration was used (10, 50, 100, and 200 RU/mL). Tubes were inoculated with 0.1 mL of an overnight *Listeria* culture (16 to18 hours) and incubated at 37 °C. Negative and positive controls were prepared as described above. Growth of the pathogen was monitored by O.D. determinations at 600 nm at 0, 2, 4, 24 and 48 hours.

1.5 Effect of Nisin on Yogurt Fermentation

As yogurt starter is reported to be sensitive to nisin (12, 13, 24) this preliminary experiment was carried out to determine the concentration of nisin which can be used without affecting significantly normal yogurt processing and acid production.

Yogurt trials were conducted as follows: Nonfat dry milk was added to raw milk to adjust fat and dry matter contents to approximately 17 and 150 g/L, respectively. After thorough mixing, pasteurization was performed in water bath at 80 °C (internal temperature) for 30 min. Of the pasteurized milk, 100 milliliters were dispensed in plastic cups and inoculated with the yogurt starter activated as described above to the level of 20 mL/L. Nisin was added to the cups to a final concentration of 10, 50 or 100 RU/mL. A sample not containing nisin was used as a control. The containers were incubated at 43 °C until coagulation (6 to 7 h), then transferred to the refrigerator (ca, 7 °C). The pH and acidity were measured every hour until coagulation then at 24 h. pH measurements were done with a Crison pH meter using an Ingold combination electrode. Acidity was measured by titration with NaOH, 0.1 mol/L in presence of 10 g/L phenolphthalein solution.

2.6. Effect of Nisin on *Listeria monocytogenes* in Yogurt

Two series of yogurt cups were prepared as described above. Nisin was added to one of them to a final concentration of 10 RU/mL. The second was a positive control. All cups were inoculated with 0.1 L (ca, 10^5 RU/mL) of an overnight *Listeria* culture. Samples were incubated at 43 °C until coagulation. They were then stored in the refrigerator (ca, 7 °C) and sampled for plate count on ASLM (1) at 1, 2, 3, 13 and 15 days.

2.7 Statistical analysis

At least two replicates of each trial were done and each determination was repeated twice. The mean and standard deviation of each determination were calculated. Student t-test and analysis of variance (P = 0.05) were used to analyze changes in pH, acidity and Listeriae cell numbers in NDM, TSB and yogurt samples.

3. Results and discussion

3.1 Effect of Nisin on *L. monocytogenes* at Different pH Values

Growth of *L. monocytogenes* in NDM at different pH values in absence or presence of 50 RU/mL of nisin is shown in Table 1. It may be seen that numbers of *L. monocytogenes* increased at pH 6.8 in the controls (without nisin), while they decreased steadily in test samples containing 50 RU/mL of nisin. Similar results were obtained at pH 5.5. At pH 4.5, a decrease in *Listeria* counts was observed in both test and control samples; however, the pathogen was eliminated from the test samples within 24 hours while, in the control, few cells were still viable even at 48 hours. The same behavior was observed at pH 5.0 (Table1). Similar results were previously reported in TSB but with a higher nisin concentration (e.g 37.10^2 RU/mL) (Benkerroum Sandine). These data suggest that 50 RU/mL of nisin is effective in the control of *L. monocytogenes* in milk and dairy products. A concentration of 10 to 500 RU/g has been recommended in food preservation in general (7).

In view of these results and considering the fact that in practice there is a tendency to minimize the amount of an additive in food preservation, this experiment was carried out in TSB using different combinations of pH and nisin concentrations. At pH 6.8, no concentration was inhibitory to *L. monocytogenes*; no significant difference ($P > 0.05$) between O.D. reached after 48 hours in samples containing up to 200 RU/mL and the control, was observed (Fig 1, A). At pH 6.0, 5.5 and 5.0, however, 50, 100 and 200 RU/mL were all effective (Figures 1B, 1C and 1D). A concentration of 10 RU/mL had no significant ($P > 0.05$) effect on *L. monocytogenes* at pH 6.8 and 5.5 but was significantly ($p<0.05$) inhibitory at pH 5.0. These data show that much less nisin is needed to control *L. monocytogenes* in low pH than in high pH systems either because nisin is more effective at low pH, as has been shown earlier (11), or because of an additive effect of acidity and nisin action. Indeed nisin activity is pH dependent. Henning et al., (10) reported that nisin activity is completely lost at neutral pH

Table 1: Effect of pH and added nisin (50 RU/mL) on growth of *Listeria monocytogenes* (log CFU/mL) in reconstituted nonfat dry milk incubated at 37 °C.

	PH							
	6.8		5.5		5.0		4.5	
Hours	+nisin	- nisin	+ nisin	- nisin	+ nisin	-nisin	+nisin	-nisin
0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0
4	6.7(0.29)[1]	7.5(0.53)	6.4(0.59)	6.7(0.56)	5.6(0.82)	6.2(0.16)	4.7(0.37)	5.2(0.70)
7	7.8(0.18)	8.9(0.29)	6.6(0.66)	7.1(0.26)	5.7(0.26)	5.8(0.48)	4.5(0.38)	4.7(0.29)
24	3.8(0.63)	9.5(0.41)	2.8(0.55)	6.9(0.42)	3.3(0.53)	5.8(0.77)	0.0(0)	2.8(073)
48	1.5(0.50)	9.5(0.29)	1.2(0.30)	8.3(0.33)	0.0(0)	2.4(0.43)	0.0(0)	0.8(0.85)

Values are means of four determinations.
[1]Standard deviations in parenthesis.

due to a decrease in its solubility. Wei and Norman (25) investigated the effect of pH on nisin solubility and found that it decreases exponentially from pH 2.0 to 6.0 and that it is almost insoluble around pH 8.0. However, we showed in previous work (2) that a nisin concentration of 37.10^2 RU/mL is effective against *L. monocytogenes* at pH7.0 in TSB. Moreover, Mohamed et al., (17) showed that nisin is active against *L. monocytogenes* at pH 7.4 and that only 32 RU/ml were necessary to inhibit this strain at 37 °C. They also showed that the sensitivity of this strain decreases with the temperature of incubation. A nisin concentration of 256 RU/ml was required to inhibit the same strain at 22 °C at the same pH. This amount was 16-fold reduced at pH 5.5 at the same temperature. Decrease in nisin activity at pH values above 7.0 may also be due to a partial inactivation of the bacteriocin with subsequent reduction of the initial concentration or to the polymerization of the molecule (12). In fact, nisin activity was completely and irreversibly lost after 4 days of storage at pH 7.0 and ambient temperature (unpublished data). Our results show that nisin action at a given pH depends on the concentration and the medium used. In effect, 50 RU/mL resulted in more than 4 log units reduction in *Listeria* counts in NDM at pH 6.8 after 48 hours; while, 200 RU/mL had only a slight effect on the pathogen in TSB at the same pH (Table 1 and Figure 1, respectively).

3.2 Effect of Nisin on Yogurt Fermentation

Figure 2 shows the decrease in the pH and the increase in acidity during fermentation of yogurt containing different concentrations of nisin. A nisin concentration of 50 RU/mL or less had no noticeable effect on yogurt fermentation. The pH dropped and the acidity increased in the same way as in the control. Milk coagulation in all samples was normal; it occurred from 6 to 7 hours and the curd was firm and without syneresis. However, in the samples containing 100 RU/mL of nisin, fermentation was greatly retarded and the curd had an abnormal, viscous body. After 15 days, the titratable acidity and the pH of yogurt containing 100 RU/mL of nisin averaged 0.6% and 4.5, respectively, while in the control, acidity was about 0.9% and pH 4.0. This result is due to the fact that nisin is inhibitory to the yogurt starter as was shown herein and elsewhere (12, 13). Several strains of lactobacilli have been tested for their sensitivity to nisin and found that the MIC values ranged between 35 and 100 RU/mL for *Lb. delbueckii* subsp. *bulgaricus* strains at optimal growth conditions and when an inuculum of 10 mL/L was used. These values doubled for an inoculum of 20 mL/L (13). As matter of fact, a moderate delay in yogurt acidification may be suitable in yogurt technology since in the conventional process the product usually develop too much acidity towards the end of the storage and wheys-off. According to Bayoumi (3), nisin addition to the level of 50 AU/ml prevents such defect resulting in 7 days increase of the shelf-life without affecting the sensory characteristics.

3.3 Effect of nisin on *L. monocytogenes* in yogurt:

Figure 3 shows the behavior of *L. monocytogenes* in yogurt in the presence and absence of nisin during storage at 7 °C. Although a significant decrease in *Listeria* counts was observed in yogurt without nisin, the pathogen survived manufacture and 13 days of storage at 7 °C. However, in yogurt containing 10 RU/mL of nisin, no *Listeria* was found in 1-ml samples at or after 24 hours. Survival of pathogens in fermented dairy products, in spite of the antagonistic effect of lactic acid bacteria used as starter cultures is well documented (4, 5, 6). In yogurt and in other dairy products fermented with the same starter [e.g. *Lb. bulgaricus*, and *Str. thermophilus* (ST:LB::1:1)] such as some cultured milks and Feta cheese, *L. monocytogenes* survives manufacture and storage (20, 21). Papageorgiou and Marth (18) showed that *L. monocytogenes* could survive the manufacture and more than 90 days of storage at 4 °C in Feta cheese. This bacterium survived in cultured milk fermented with STLB and in yogurt for 1 to 12 weeks and 1 to 12 days, respectively (19).

The same authors showed that survival of *L. monocytogenes* in yogurt depended on the size of *Listeria* and starter culture inocula, the final pH reached, the temperature and duration of the fermentation, and *Listeria* strain. Shaack and Marth (22) showed that *L. monocytogenes* survives only between 9 to 15 hours during the actual fermentation process of yogurt. However, in a typical yogurt fermentation of 4 to 6 hours, *Listeria* was able to grow during fermentation and then survive during storage at 4 °C. Lammerding and Doyle (14) also could recover *L. monocytogenes* from yogurt after 7 days of storage at 4 °C, although the initial inoculum was relatively low (about 32 x 10^2 CFU/mL).

4. Conclusion

These results confirm that food processors should not solely rely on pasteurization and fermentation to insure full protection and safety of fermented milk products. Some food grade additives may also be used in addition along with Good Manufacture Practices. Nisin proved to be effective in controlling the growth of *L. monocytogenes* in both cottage cheese (2) and yogurt (the present study). For the latter, the amount of nisin used should inhibit the pathogen without harming significantly the yogurt starter. In our case, a nisin concentration of up to 50 RU/mL had no adverse effect on the yogurt starter we used (e.g Rediset). Moreover, nisin addition to yogurt may play a beneficial role in keeping yogurt acidity from dropping too low during storage and thus prevents deterioration of its sensory attributes and extends its shelf-life. Such use is of more interest in developing countries where yogurt is rarely stored at refrigeration temperature when marketed. Nisin (50 RU/mL) was added to reconstituted NDM previously adjusted to different pH values along with *L. monocytogenes* ATCC 7644 (ca., 10^6 cell/mL). The experiment was conducted against a positive (only *Listeria* was added) and a negative (without added nisin or *Listeria*) controls. Growth of *L. monocytogenes* ATCC 7644 was monitored at 37 °C by plate count on TSA at 0, 4, 7, 24 and 48 hours.

REFERRENCES

[1] Al-Zoreky, N and Sandine, W.E. Highly selective medium for isalation of *Listeria monocytogenes* from food. Appl. Environ. Microbiol., **56** (1990) 30145-30157

[2] Benkerroum, N., and Sandine, W. E. Inhibitory action of nisin against *Listeria monocytogenes*. J. Dairy Sc., **71** (1988) 3237-3245.

[3] Bayoumi S., Nisin and Yogurt manufacture. Chem. Microbiol. Technol. Lebensm. 13 (1991) 65-69.

[4] Benkerroum, N., Oubel, H., Zahar , M., Dlia, S., Filali-Maltouf, A., Isolation of a bacteriocin-producing *Lactococcus lactis* subsp. *lactis* and application to control *Listeria monocytogenes* in Moroccan Jben, J. Appl. Microbiol., **89** (2000) 960-969.

[5] De Buyser M-L., Dufour B., Maire M., Lafarge V., Implication of milk and milk products in food-borne diseases in France and in different industrialized countries, Int. J. Food Microbiol., **67** (2001) 1-17.

[6] Donnely, C.W., Concerns of microbial pathogens in association with dairy foods. J. Dairy Sci., **73** (1990) 1656-1661.

[7] Eapen,K C., Sandaran, R., Vijaraghavan, P. K., The present status on the use of nisin in processed foods. J. Food Sci. Technol., 20 (1983) 231-240.

[8] Farber J.M., Peterkin P.I., *Listeria monocytogenes*, a food-borne pathogen, Microbiol. Rev., **55** (1991) 133-158.

[9] Gahan, C.G.M., O'Driscoll, B., Hill, CAcid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. Appl. Environ. Microbiol., **62** (1996) 3128-3132.

[10] Henning, S., Metz, R. and Hammes, W. P., New aspects for the application of nisin to food products based on its mode of action. Int. J. Food Microbiol., **3** (1986) 135-141.

[11] Hurst A., Nisin: a review. *In*: Perlman D. and. Laskin A. I (Eds.), Advances in Microbiology, Academic Press, New York, 27 (1981) 85-123

[12] Kumar N., Prasard D.N., Effect of incubation period on inhibitory action of nisin in skim milk against lactic and non-lactic microorganisms. Microbiol. Alim. Nut., **12** (1994) 163-164

[13] Kumar N., Prasard D.N. Factors affecting the inhibitory action of nisin against *Lactobacillus* and *Bacillus* spp. Microbiol. Alim. Nut., **12** (1994) 155-161.

[14] Lammerding, A.M., Doyle, M.P., Evaluation of enrichment procedures for recovering *Listeria monocytogenes* from dairy products. Int. J. Food Microbiol., **9** (1989) 249-268

[15] Mazzotta A. S., Thermal inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juice, J. Food Prot., **64** (2001) 315-320.

[16] Mead, P.S., Slutsker L., Dietz V., McCaig L.F., Bresee J.S., Shapiro C., Griffin P.M., Tauxe R.V., Food-rlated illness and death in the United states. Emerg. Inf. Dis. **5** (1999) 607-625.

[17] Mohamed, G. E., Seaman, A. and Woodbine, M. Food antibiotic nisin comparative effect on *Erysipelothrix* and *Listeria*. In: Woodbine M. (Ed), Antimicrobials and Agriculture. Butterworth, London, pp. 435-442 (1984).

[18] Papageorgiou D. K., Marth E. H., Fate of *Listeria monocytogenes* during the manufacture, ripening and storage of Feta cheese. J. food Prot., **52**, 82-87 (1989)

[19] Piccinin, D.M. and Shelef, L.A., Survival of *Listeria monocytogenes* in Cottage cheese. J. Food Prot., **58** (1995) 128-131

[20] Ribeiro, S.H.S. and Carminati, D. Survival of *Listeria monocytogenes* in fermented milk and yogurt: effect of pH, lysosyme content and storage at 4 °C. Sc. Alim., **16** (1996) 175-185

[21] Rocourt, J., Risks factors for listeriosis. Food Control., **7** (1996) 195-202**.**

[22] Schaack, M. M. and Marth, E. H. Survival of *Listeria monocytogenes* in refrigerated cultured milks and yogurt. J. Food Prot., **51** (1988) 848-852

[23] Schaak, M. M. and Marth, E. H. Behavior of *Listeria monocytogenes* in skim milk and in yogurt mix during fermentation by thermophilic lactic acid bacteria. J. food Prot., **51** (1988) 607-614 .

[24] Vandenbergh R.A., Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS. Microbiol. Rev. **12** (1993) 221-238.

[25] Wei, L., Norman, H. J., Some chemical and physical properties of nisin, a small protein antibiotic produced by *Lactococcus lactis*. Appl. Environ. Microbiol., **56** (1990) 2551-2558.