ELSEVIER
International Journal of Food Microbiology 71 (2001) 159–168
INTERNATIONAL JOURNAL OF
Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Efficacy of nisin in combination with protective cultures against *Listeria monocytogenes* Scott A in tofu

U. Schillinger [a,*], B. Becker [a], G. Vignolo [b], W.H. Holzapfel [a]

[a] *Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition, Haid- und Neustr. 9, D-76131 Karlsruhe, Germany*
[b] *Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, Tucumán, Argentina*

Received 12 November 2000; received in revised form 11 March 2001; accepted 23 June 2001

Abstract

Nisin can be used as a biopreservative to control growth of *Listeria monocytogenes* in various minimally processed foods. Tofu is an example of a non-fermented soybean product, which may allow growth of *Listeria* at refrigeration temperatures and in which nisin may be applied to prevent multiplication of *Listeria*.

The efficacy of nisin against *Listeria* may be compromised by the emergence of spontaneous nisin-resistant mutants. Exposure of *L. monocytogenes* Scott A to nisin in a culture medium or in a food product results in an initial reduction of *Listeria* population which is followed by regrowth of survivors to nisin during further incubation. In vitro studies using Standard I Nutrient broth showed that *Enterococcus faecium* BFE 900-6a and *Lactobacillus sakei* Lb 706-1a used as protective cultures in combination with nisin were able to suppress proliferation of *Listeria* cells not killed by nisin at 10 °C. Growth and bacteriocin production of these two strains and a third protective culture, *Lactococcus lactis* BFE 902 was also observed in soymilk and tofu at 10 °C.

Inoculation studies with tofu prepared with nisin and protective cultures showed that lower amounts of nisin are required for an effective inhibition of *L. monocytogenes* Scott A when either *E. faecium* BFE 900-6a or *Lc. lactis* BFE 902 are used in addition. The combination of nisin with these bacteriocinogenic lactic acid bacteria (LAB) resulted in a complete suppression of listerial growth in homemade tofu stored at 10 °C for 1 week. *Lb. sakei* Lb 706-1a was less effective and did not prevent a slight increase of *L. monocytogenes* Scott A numbers during storage. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Nisin; *Listeria monocytogenes*; Protective cultures; Tofu; Soymilk

1. Introduction

Nisin is the best known and studied bacteriocin produced by lactic acid bacteria (LAB). It is the only bacteriocin that has been approved as a food additive in Europe and which has achieved GRAS (generally recognized as safe) status in the United States (Delves-Broughton, 1990; Food and Drug Administration, 1998). Its current use is directed primarily towards inhibiting outgrowth of *Clostridium* and *Bacillus* spores in the production of processed cheeses, in canned vegetables and in various pasteurised dairy products (Delves-Broughton et al.,

* Corresponding author. Tel.: +49-721-6625-461; fax: +49-721-6625-453.
E-mail address: Ulrich.Schillinger@bfe.uni-karlsruhe.de (U. Schillinger).

0168-1605/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0168-1605(01)00612-2

1996). Nisin, however, is active against a broad spectrum of Gram-positive bacteria including *Listeria monocytogenes*. The inhibition of *Listeria* by nisin has been demonstrated in culture media as well as in different foods such as cottage cheese (Ferreira and Lund, 1996), ricotta-type cheeses (Davies et al., 1997), fresh pork sausages (Scannell et al., 1997), cold-smoked salmon (Nilsson et al., 1997) and ice cream (Dean and Zottola, 1996).

Strains of *L. monocytogenes* vary greatly in their sensitivity to nisin (Ukuku and Shelef, 1997; Rasch and Knochel, 1998) and even in sensitive populations of *Listeria* some cells are resistant or tolerate certain concentrations of nisin and are able to survive and to multiply in the presence of this bacteriocin (Harris et al., 1991). Various studies using laboratory media showed that addition of nisin to a culture of *L. monocytogenes* resulted in a rapid decrease of viable counts followed by regrowth of surviving bacteria to population levels similar to those of the untreated cells (Mohamed et al., 1984; Richard, 1996; Schillinger et al., 1998, Ukuku and Shelef, 1997).

Previous studies in our laboratory using nisin in combination with a bacteriocin-producing protective culture showed that growth of nisin-tolerant subpopulations of *L. monocytogenes* was suppressed by nisin-resistant bacteriocinogenic strains of *Lactobacillus sakei* and *Enterococcus faecium* (Schillinger et al., 1998).

The purpose of this study was to determine if the efficacy of nisin against *Listeria* in a food system can be increased by the addition of an appropriate protective culture. A highly perishable soybean product, tofu was chosen to study the effect of combinations of nisin with three different bacteriocinogenic LAB against *L. monocytogenes* Scott A.

2. Materials and methods

2.1. Preparation of nisin solution

Nisaplin, a commercial product of nisin containing 10^6 IU/g, was provided by Aplin and Barret (Trowbridge, UK). Stock solutions of nisin were prepared by dissolving an appropriate amount of nisaplin in 0.02 N HCl. The solutions were subjected to heating for 5 or 10 min and kept at −20 °C until use.

2.2. Bacterial strains and culture media

Lb. sakei Lb 706-1a, a nisin-resistant mutant of *Lb. sakei* Lb 706 (Schillinger et al., 1998), *E. faecium* BFE 900-6a, a nisin-resistant mutant of *E. faecium* BFE 900 (Schillinger et al., 1998) and *Lactococcus lactis* BFE 902 (Franz et al., 1997) were used as model strains of bacteriocinogenic LAB. *L. monocytogenes* Scott A was used as target organism. *Lb. sakei* DSM 20017 was used as indicator strain for the determination of bacteriocin activity. LAB were grown in MRS broth (Merck, Darmstadt, Germany) at 30 °C and *L. monocytogenes* was propagated in Standard I Nutrient broth (Merck) at 30 °C. The cultures were maintained as frozen stocks at −20 °C in 15% glycerol.

2.3. Determination of bacteriocin activity

Bacteriocin activity was estimated by using an agar spot assay as described by Schillinger et al. (1993). Briefly, 10-μl volumes of twofold serial dilutions of the bacteriocin-containing samples were spotted onto the surface of a Standard I Nutrient agar plate overlaid with 10 ml MRS soft agar (0.7%), which had been seeded with approximately 10^7 cfu of the indicator bacteria. The assay plates were incubated at 30 °C for 24 h. One activity unit was defined as the reciprocal of the highest dilution yielding a definite zone of inhibition on the indicator lawn and had to be multiplied by 100 to obtain the activity units per ml (AU/ml).

For detection of bacteriocin activity in tofu, 10-g tofu was homogenised with 90-ml quarter-strength Ringers solution in a stomacher bag and 10-μl volumes were spotted onto the indicator lawns. Alternatively, small pieces of the tofu were placed onto agar plates, which had been overlaid with soft agar and inoculated with the indicator strain. Clear zones around the tofu pieces indicated the presence of bacteriocin(s).

2.4. Inhibition of L. monocytogenes Scott A by nisin and LAB in Standard I Nutrient broth

Nisin (1000 IU) was added to 10-ml Standard I Nutrient broth at pH 7.5 and this medium was inoculated with *L. monocytogenes* Scott A at an initial level of 10^6 cfu/ml and with *Lb. sakei* Lb 706-1a or *E. faecium* BFE 900-6a at about 10^5 or 10^6 cfu/ml. Incubation was at 10 °C for 7 days. Bacterial viable counts were determined at particular time intervals using Standard I Nutrient agar for aerobic mesophilic bacteria, MRS agar for the LAB and PALCAM *Listeria* selective agar (Merck) for *Listeria*. All experiments were done at least in duplicate.

2.5. Inoculation experiments with soymilk and tofu

2.5.1. Preparation of soymilk and homemade tofu

Soybeans (125 g) were soaked in 300-ml water overnight at 4 or 10 °C. Soaked beans (250 g) mixed with 600-ml water were ground using a Waring blender (Model 38BL41, New Hartford, USA). The liquid and solid portions were separated by filtration through a sieve and a cheesecloth. The resulting soymilk was used for both inoculation experiments and the preparation of tofu. For the inoculation experiments, soymilk was filled into 20-ml tubes that were autoclaved prior to inoculation with the test organisms.

For the preparation of tofu, soymilk was heated to boiling for 30 min and then 6 ml 1 M $MgSO_4$ were added. Nisin stock solutions (2.5, 5 or 10 ml) of 10 000 IU/ml were added. The mixture was stirred for some minutes and then left to set for about 10 min. The resulting curd was put into a pressing form lined with cheesecloth. A pressing board was placed on top and weighted with a load for about 10 min to extrude the whey. The warm tofu was sliced into four blocks and then into smaller cubes. After cooling to room temperature, the tofu was used directly for the inoculation experiments.

2.5.2. Inoculation experiments with soymilk

A 10-ml of soymilk was inoculated with appropriate dilutions of overnight cultures of *Lb. sakei* Lb 706-1a, *E. faecium* BFE 900-6a and *Lc. lactis* BFE 902 and then incubated at 10 °C. MRS broth was inoculated in the same way. Viable counts were determined at selected time intervals. The experiment was repeated twice.

2.5.3. Inoculation experiments with commercial tofu

Fresh tofu packaged in plastic cartons was purchased from a local supermarket in Karlsruhe. The tofu cake (450 g) was aseptically cut into cubes of approximately $5 \times 5 \times 5$ mm using a sterile knife and portions of 100 g were placed into sterile flasks covered with aluminium foil.

Tofu was inoculated with *Listeria* or protective cultures by adding 200 ml of a bacterial suspension prepared from overnight cultures (4 to 5×10^8 cfu/ml). The inoculum was prepared by adding 2 ml of a 10^{-3} dilution of *L. monocytogenes* Scott A or 20 ml of undiluted *Lb. sakei* Lb 706-1a, *E. faecium* BFE 900-6a or *Lc. lactis* BFE 902 to 200 ml quarter-strength Ringers solution. For the tofu samples to be inoculated with both *L. monocytogenes* and protective cultures, 20 ml of overnight cultures of *Lb. sakei* Lb 706-1a, *E. faecium* BFE 900-6a or *Lc. lactis* BFE 902 were added to the *Listeria* inoculum. After 30-min incubation of tofu with theses inocula, the bacterial suspensions were decanted.

For the experiments with nisin, the tofu was kept at 10 °C for another 30 min. Then 200 ml of a nisin solution of 3000 IU/ml was added to the inoculated tofu cubes and was decanted after 30 min. For the control samples, nisin solution was replaced by sterile quarter-strength Ringers solution. The tofu samples were kept at 10 °C for at least 5 days and analysed for *Listeria* and LAB populations at selected times.

For the bacteriological examination, 10-g portions of the tofu were aseptically transferred to a stomacher bag and 90 ml of quarter-strength Ringers solution added. The sample was homogenised with a stomacher (Model Mix-1, Meintrup-Labortechnik, Lähden, Germany) for 2 min and appropriate dilutions were plated in duplicate onto Standard I Nutrient, MRS and PALCAM *Listeria* selective agar. Aerobic mesophilic bacterial numbers were determined on Standard I Nutrient agar after incubation for 24 h at 30 °C. *L. monocytogenes* was counted on PALCAM-*Listeria* selective agar after 48 h of incubation at 30 °C. MRS agar incubated for 48 h at 30 °C was used to enumerate the LAB. pH of the tofu

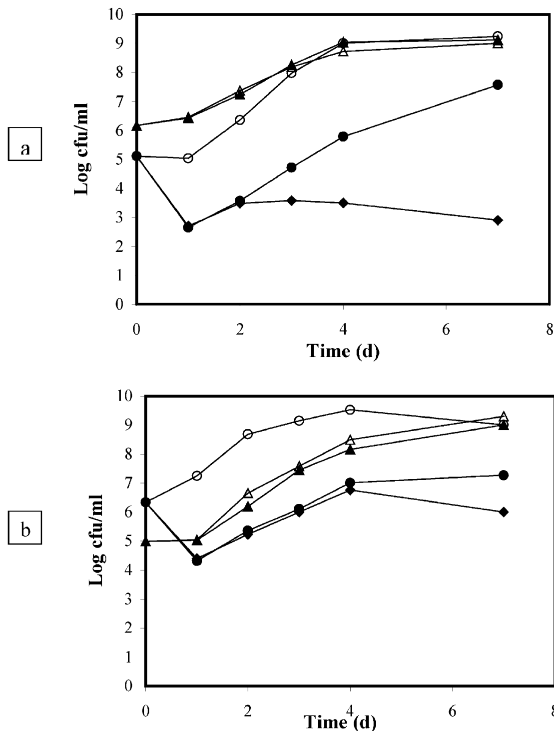

Fig. 1. Effect of nisin (100 IU/ml) and *E. faecium* BFE 900-6a inoculated at 1.5×10^6 (a) or 1×10^5 cfu/ml (b) on *L. monocytogenes* Scott A in Standard I Nutrient broth at 10 °C. (○) *L. monocytogenes* Scott A without nisin, (●) *L. monocytogenes* with nisin added, (♦) *L. monocytogenes* Scott A with nisin and *E. faecium* added, (△) *E. faecium* BFE 900-6a without nisin, (▲) *E. faecium* BFE 900-6a with nisin added.

samples was measured at each stage of microbiological analysis.

2.5.4. Inoculation experiments with homemade tofu

A 200 ml of a quarter-strength Ringers solution inoculated with *L. monocytogenes* Scott A or a combination of this strain with a protective culture was added to 110 g of tofu cubes. The inoculum of *L. monocytogenes* was prepared by adding 2 ml of a 10^{-3} or 10^{-4} dilution of an overnight culture to 200-ml quarter-strength Ringers solution. For the tofu samples to be inoculated with both *Listeria* and protective cultures, 20 ml of the undiluted overnight culture of *Lb. sakei* Lb 706-1a, *E. faecium* BFE 900-6a or *Lc. lactis* BFE 902 were added to 200 ml of the *Listeria* suspension. After 30 min at 10 °C, the bacterial suspension was decanted and the tofu kept for 7 days at 10 °C.

Bacteriological examination of tofu sampled after different time intervals was done as described under Section 2.5.3.

3. Results

3.1. Effect of nisin and protective cultures on L. monocytogenes Scott A in Standard I Nutrient broth at 10 °C

Lb. sakei Lb 706-1a and *E. faecium* BFE 900-6a were chosen to be used in combination with nisin to control growth of *L. monocytogenes* Scott A in Standard I Nutrient broth at 10 °C. These protective cultures had obtained resistance to nisin concentrations between 200 and 500 IU/ml by successive exposure to increasing nisin concentrations during a previous study (Schillinger et al., 1998). Addition of nisin at a concentration of 100 IU/ml to *L. monocytogenes* Scott A resulted in an initial reduction of viable numbers by about two log cycles (Fig. 1). An increase in *Listeria* viable counts, however, was observed after 48 h at 10 °C. During further incubation at the same temperature, *L. monocytogenes* multiplied at about the same growth rate as in the untreated control (Fig. 1). *E. faecium* BFE 900-6a was able to grow in the presence of 100 IU of nisin per ml and affected growth of surviving listerial cells. When 10^6 cfu of *E. faecium* was added to 1

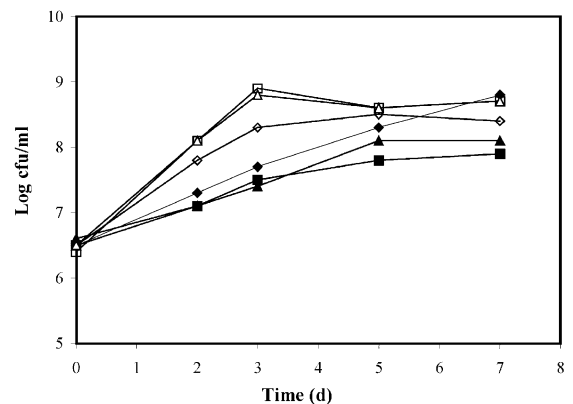

Fig. 2. Growth of *Lb. sakei* Lb 706-1a (♦), *E. faecium* BFE 900-6a (▲) and *Lc. lactis* BFE 902 (■) in soymilk (open symbols) and MRS broth (closed symbols) at 10 °C.

Table 1
Bacteriocin production of *Lb. sakei* Lb706-1a, *E. faecium* BFE 900-6a and *Lc. lactis* BFE 920 in soymilk (pH 6.3) and MRS broth (pH 6.1) at 10 °C

Strain	Inoculum level (log cfu/ml)	Medium	Detection of bacteriocin activity[a]			Maximum bacteriocin activity (AU/ml)
			48 h	72 h	144 h	
Lb. sakei Lb 706-1a	6.5	MRS broth	+	+	+	1600
Lb. sakei Lb 706-1a	6.5	Soymilk	−	+	+	400
E. faecium BFE 900-6a	6.6	MRS broth	−	−	+	100
E. faecium BFE 900-6a	6.5	Soymilk	−	+	+	200
Lc. lactis BFE 902	6.5	MRS broth	−	−	−	−[b]
Lc. lactis BFE 902	6.4	Soymilk	−	+	+	400

[a]Bacteriocin activity determined in the culture supernatant using the agar spot assay.
[b]No bacteriocin activity detected after 7 days of incubation at 10 °C.

ml of the *Listeria* culture exposed to 100 IU of nisin, survivors to nisin were prevented from growing at an early stage (Fig. 1a). There was only a slight increase in viable counts by 0.8 log units after 48 h and then *Listeria* cell numbers remained at a low level during further incubation at 10 °C. A 10-fold lower inoculum of *E. faecium* (10^5 cfu/ml) was much less effective in controlling growth of listerial cells that had survived nisin action (Fig. 1b). An increase of the viable counts to 5×10^6 cfu/ml was not prevented after 4 days. Comparable results were obtained with *Lb. sakei* Lb 706-1a (data not shown).

3.2. Growth and bacteriocin production of protective cultures in soymilk at 10 °C

Protective cultures selected for use in combination with nisin in a food product should be able to grow and to produce bacteriocins in this product at a relevant temperature. Soymilk was chosen in order to investigate the growth behaviour of the three protective cultures *Lb. sakei* Lb 706-1a, *E. faecium* BFE 900-6a and *Lc. lactis* BFE 902 in a food-related medium. For comparison, growth and bacteriocin production were also studied in MRS broth at 10 °C.

All three protective cultures grew well in soymilk at pH 6.3 and even more rapidly than in MRS broth at 10 °C (Fig. 2). However, differences in the growth behaviour were observed among the three strains. *Lc. lactis* BFE 902 and *E. faecium* BFE 900-6a

Table 2
Growth and bacteriocin production of protective cultures on tofu stored at 10 °C

Strain	Storage time (days)	Viable numbers (log cfu/g)	pH	Bacteriocin detected
Lb. sakei	0	6.15	6.3	−
Lb 706-1a	2	7.78	6.3	+
	5	8.27	6.1	+
E. faecium	0	6.33	6.3	−
BFE 900-6a	2	7.28	6.3	+
	5	8.61	6.3	−
Lc. lactis	0	6.26	6.3	−
BFE 902	2	7.75	6.3	+
	5	8.45	6.3	+

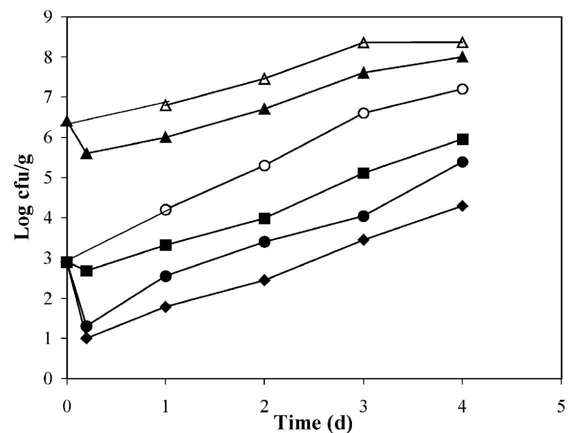

Fig. 3. Effect of nisin (3000 IU/ml) and *E. faecium* BFE 900-6a on *L. monocytogenes* Scott A in commercial tofu stored at 10 °C. (○) *L. monocytogenes* Scott A without nisin, (●) *L. monocytogenes* Scott A with nisin added, (■) *L. monocytogenes* Scott A with *E. faecium* BFE 900-6a added, (◆) *L. monocytogenes* Scott A with both nisin and *E. faecium* added, (△) *E. faecium* BFE 900-6a without nisin, (▲) *E. faecium* BFE 900-6a with nisin added.

grew to higher cell densities (3 to 8×10^8 cfu/ml) in soymilk than in MRS broth (7×10^7 to 1.5×10^8 cfu/ml) and maximum cell yield was achieved earlier (day 3). *Lb. sakei* Lb 706-1a showed a somewhat faster growth in soymilk as compared to MRS broth. It, however, did not achieve higher cell densities in soymilk than in MRS broth. Bacteriocins were produced by all three protective cultures in soymilk at 10 °C (Table 1). In soymilk, *E. faecium* and *Lc. lactis* were able to produce their bacteriocins earlier and a higher bacteriocin activity was detected as compared to MRS broth where bacteriocin production of these strains was rather poor (*E. faecium*) or not detected at all (*Lc. lactis*) even after 7 days due to the slow growth at 10 °C (Table 1). In contrast to this, *Lb. sakei* Lb 706-1a produced high bacteriocin amounts in MRS broth and bacteriocin activity was also detected earlier in MRS broth than in soymilk (Table 1).

3.3. Inoculation experiments with tofu

3.3.1. Growth and bacteriocin production of the protective cultures on commercial tofu at 10 °C

Commercially available tofu was inoculated with the three protective cultures to investigate their ability to grow and to produce bacteriocins on tofu at 10 °C. All strains grew to high cell densities $> 10^8$ cfu/g within 5 days without affecting pH of the product (Table 2). In situ bacteriocin production was

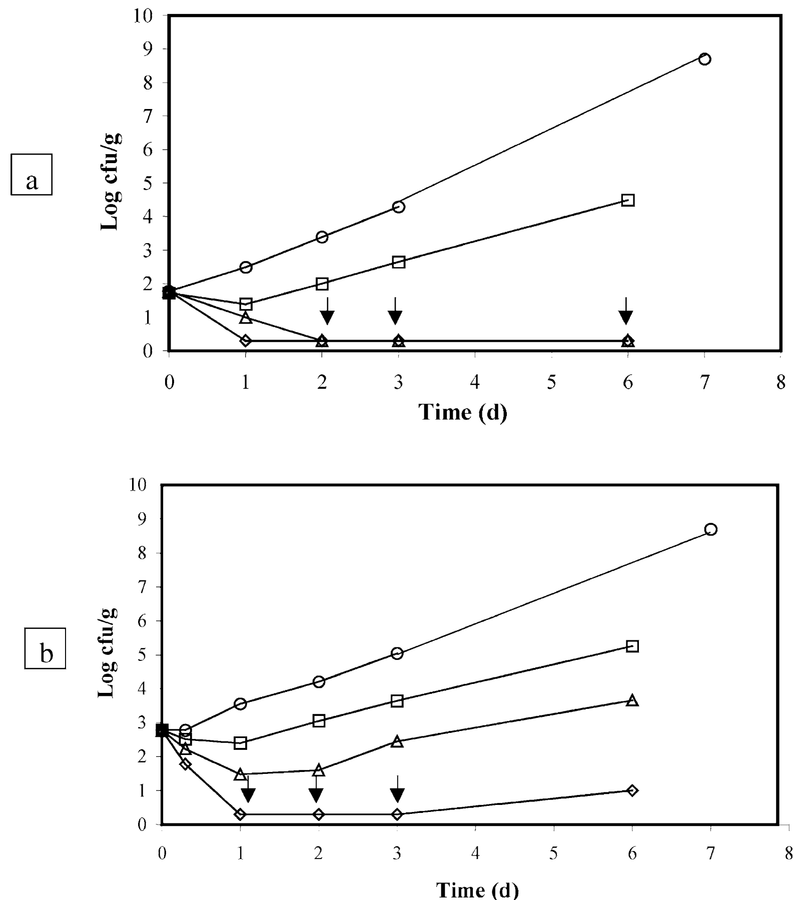

Fig. 4. Effect of various concentrations of nisin on *L. monocytogenes* Scott A inoculated at 60 cfu/g (a) and 6×10^2 cfu/g (b) in homemade tofu at 10 °C. (○) 0, (□) 500, (△) 1000, (◇) 2000 IU/ml soymilk added. Arrows indicate that *Listeria* counts fell below the detection limit.

demonstrated with all protective cultures after 48 h of incubation (Table 2).

3.3.2. Effect of nisin in combination with protective cultures on commercially available tofu

In our first experiments, tofu purchased from a local supermarket was artificially inoculated with *L. monocytogenes* Scott A (10^3 cfu/g) and then exposed to a nisin solution of 3000 IU/ml for 30 min. The application of nisin in this concentration resulted in a reduction of initial *Listeria* numbers by 1.6 log units (Fig. 3). Nisin, however, did not prevent a rapid regrowth of *L. monocytogenes* during storage of tofu at 10 °C. An increase of viable *Listeria* numbers by more than one log cycle was observed within 24 h, and after 4 days, *Listeria* viable counts had increased by four log units to a cell density of 2.5×10^5 cfu/g (Fig. 3). When a protective culture was used in addition to nisin, a retardation of *Listeria* regrowth was observed. With *E. faecium* BFE 900-6a inoculated at 2.5×10^6 cfu/g, growth of *Listeria* cells surviving nisin action was delayed and *Listeria* viable counts were lower than in tofu samples without protective culture added (Fig. 3). The other two protective cultures, *Lb. sakei* Lb 706-1a and *Lc. lactis* BFE 902, behaved very similarly (data not shown) also showing some inhibitory effect on the *Listeria* cells not killed by nisin.

3.3.3. Effect of nisin on L. monocytogenes in homemade tofu at 10 °C

Homemade tofu was prepared from soy beans, and various amounts of nisin were added during the precipitation step of the heated soy milk. The resulting nisin-containing tofu was contaminated with *L. monocytogenes* Scott A at two inoculum levels and viability of *Listeria* was studied during storage at 10 °C. When nisin was used at a high concentration of 2000 IU/ml nisin effectively suppressed *Listeria* growth in tofu stored at 10 °C (Fig. 4a + b). *Listeria* viable numbers rapidly declined to around the detection level of 10 cfu/g where they remained. A concentration of 500 IU of nisin per ml caused a smaller reduction in *Listeria* viable numbers and did not prevent an increase in viable counts after 2 days at both inoculum levels. When nisin was used at a concentration of 1000 IU/ml, regrowth was prevented in tofu inoculated with a low number of *Listeria* (60 cfu/g) (Fig. 4a) but not at a 10-fold higher inoculum (Fig. 4b).

3.3.4. Effect of nisin in combination with protective cultures on L. monocytogenes in homemade tofu at 10 °C

Homemade tofu with nisin added during manufacture was used to study the effect of nisin in combination with a protective culture on *L. monocytogenes* Scott A. A nisin concentration of 700 IU per ml soymilk was chosen and tofu was inoculated with *L. monocytogenes* at a level of 1×10^2 cfu/g. When *E. faecium* BFE 900-6a was added at an inoculum level of 2×10^6 cfu/g to tofu prepared without nisin, an inhibitory effect against *L. monocytogenes* was observed after several days of storage at 10 °C (Fig. 5). At the end of the storage period, viable counts of *Listeria* were three log cycles lower than in the control tofu samples without protective culture added. Bacteriocin activity was detected in tofu inoculated with *E. faecium* BFE 900-6a. In the tofu samples with 700 IU of nisin added to 1-ml soymilk during manufacture, *Listeria* numbers were initially reduced to a low level below the detection limit and increased again to 2.3×10^2 cfu/g after 5 days (Fig. 5). This regrowth of *Listeria* was prevented in tofu with both nisin and *E. faecium* BFE 900-6a added.

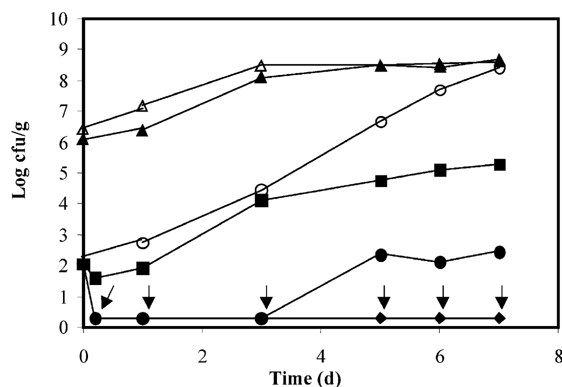

Fig. 5. Effect of nisin and *E. faecium* BFE 900-6a on *L. monocytogenes* Scott A in homemade tofu stored at 10 °C. (○) *L. monocytogenes* Scott A without nisin, (●) *L. monocytogenes* Scott A with nisin added, (■) *L. monocytogenes* Scott A with *E. faecium* BFE 900-6a added, (◆) *L. monocytogenes* Scott A with both nisin and *E. faecium* added, (△) *E. faecium* BFE 900-6a without nisin, (▲) *E. faecium* BFE 900-6a with nisin added. Arrows indicate that *Listeria* counts fell below the detection limit.

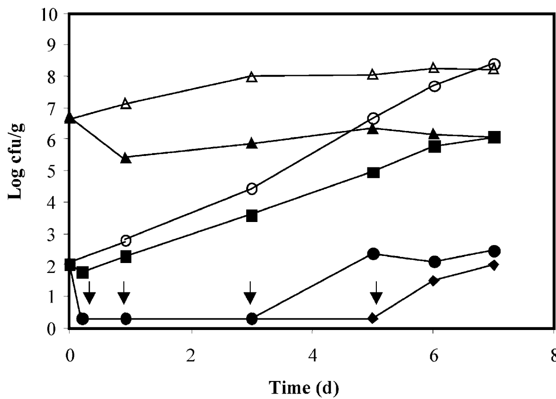

Fig. 6. Effect of nisin and *Lb. sakei* Lb 706-1a on *L. monocytogenes* Scott A in homemade tofu stored at 10 °C. (○) *L. monocytogenes* Scott A without nisin, (●) *L. monocytogenes* Scott A with nisin added, (■) *L. monocytogenes* Scott A with *Lb. sakei* Lb 706-1a added, (♦) *L. monocytogenes* Scott A with both nisin and *Lb. sakei* Lb 706-1a added, (△) *Lb. sakei* Lb 706-1a without nisin, (▲) *Lb. sakei* Lb 706-1a with nisin added. Arrows indicate that *Listeria* counts fell below the detection limit.

Identical results were obtained with *Lc. lactis* BFE 902 (data not shown). Only with a combination of nisin with one of these two protective cultures, *Listeria* viable counts remained below the detection limit during 7 days at 10 °C. The combination of nisin with *Lb. sakei* Lb 706-1a was less effective in suppressing regrowth of *L. monocytogenes* (Fig. 6). This combination did not prevent an increase of *Listeria* viable counts after 6 days at 10 °C. This is probably due to a lack of growth of *Lb. sakei* Lb 706-1a in tofu when 700 IU of nisin was added during manufacture.

4. Discussion

Nisin shows a strong antilisterial activity and may therefore be used as a biopreservative to control *L. monocytogenes* in various types of minimally processed foods. Several studies have been performed on the use of nisin to inhibit *Listeria* in dairy and fish and meat products (Davies et al., 1997; Dean and Zottola, 1996; Ferreira and Lund, 1996; Scannell et al., 1997; Nilsson et al., 1997). The application of nisin in soybean derived products has not been studied so far. Tofu is a non-fermented soybean product with a relatively high pH, which may be contaminated during processing with spoilage or pathogenic bacteria including *L. monocytogenes*. These bacteria may proliferate even at low temperatures. This may especially be the case in absence of unfavourable factors such as competing microflora. *L. monocytogenes* Scott A inoculated in a commercial tofu or in a homemade fresh tofu was able to grow rapidly to a high cell density during storage at 10 °C.

When nisin was applied in tofu by exposing the food product to a nisin solution, relatively large amounts of the bacteriocin (3000 IU/ml) were required to cause a significant reduction in *Listeria* viable counts. This type of application of nisin resulted in a short-term inhibitory effect. The decrease in viable counts was followed by rapid regrowth of survivors to nisin during storage of tofu at 10 °C. After 24 h, *Listeria* had already achieved a cell density which nearly compensated the initial reduction caused by nisin.

A stronger inhibitory effect on *L. monocytogenes* can be achieved when nisin is added during the manufacture of tofu. Tofu is prepared from soybeans by a relatively simple procedure. It is made from soybean milk, which is the liquid extracted from soybeans after the beans have been soaked in water and blended. The soymilk is then heated to boiling and with the addition of a precipitant, a curd forms that is pressed into cakes of tofu. As nisin is heat stable, it can be added during the precipitation step of heated soymilk. When nisin was applied in a concentration of 2000 IU per ml soymilk, the resulting homemade tofu did not allow growth of *L. monocytogenes* Scott A at 10 °C. This relatively high nisin concentration resulted in a complete loss of viability of the *Listeria* population. *Listeria* initial numbers decreased from 6×10^2 cfu/ml to a level below the detection limit and remained very close to this level for at least 6 days. When lower concentrations of nisin were used in homemade tofu, proliferation of surviving *Listeria* cells was not prevented in most cases during storage at refrigeration temperature. A strong inhibition, however, can also be achieved using nisin amounts below 1000 IU per ml soymilk provided the biopreservative is applied in combination with an appropriate protective culture.

The enhancement of the antilisterial efficacy of nisin by combination with a bacteriocinogenic pro-

tective culture, had already been shown in a laboratory medium at 30 °C in a previous study (Schillinger et al., 1998). Similar observations were made at 10 °C. The combination of nisin with nisin-resistant mutants of *Lb. sakei* Lb 706 or *E. faecium* BFE 900 was more effective in suppressing growth of *L. monocytogenes* Scott A than nisin alone.

The protective cultures *Lb. sakei* Lb 706-1a, *E. faecium* BFE 900-6a and *Lc. lactis* BFE 902 were found to be able to grow very well and to produce bacteriocins in soymilk at 10 °C. It is interesting to note that growth and bacteriocin production of the *Enterococcus* and the *Lactococcus* were even better in soymilk than in MRS broth commonly used for cultivation of these bacteria. Bacteriocins of these strains were also detected during growth in home-made tofu at 10 °C. When the protective cultures were used in addition to nisin in tofu, the antilisterial effect of nisin was enhanced. The effectiveness of this approach depended on the strain used as protective culture. *Lb. sakei* Lb 706-1a was less effective in inhibiting *Listeria* than *E. faecium* BFE 900-6a and *Lc. lactis* BFE 902 which both were able to completely suppress regrowth of survivors to nisin.

Enterocins produced by *E. faecium* BFE 900-6a during growth in tofu may have been responsible for the inhibition of *Listeria* cells not killed by nisin. The bacteriocin produced by *Lc. lactis* is nisin and therefore the strong inhibitory effect of this strain on listerial cells that survived nisin action was surprising. Survivors to nisin, however, are not necessarily resistant to this bacteriocin. They may also have escaped the action of nisin due to insufficient bacteriocin available to completely kill the *Listeria* population. Indeed, examination of survivors isolated from tofu samples prepared with nisin revealed that they were still sensitive to nisin (data not shown). Consequently, they could be inhibited by the nisin producing *Lc. lactis*. Organic acids produced by the protective culture probably also affected the viability of *Listeria* and contributed to the inhibition observed during storage of tofu at 10 °C.

On the other hand, the application of the protective cultures alone did not result in a successful suppression of *Listeria* growth in tofu at low temperature. Thus, an initial increase of *Listeria* viable numbers cannot be prevented by a protective culture on its own, as it needs time to grow to a high cell density in order to produce bacteriocins and other metabolic products such as organic acids. When the protective culture is used in combination with nisin, the *Listeria* population is kept at a low level by nisin for several days and during this time the protective culture can reach sufficiently high numbers to suppress growth of survivors to nisin.

Acknowledgements

The authors gratefully acknowledge the skillful technical assistance of Andrea Springer. The research was financially supported by commission of the European Union through contract FAIR CT 96-1148. We are indebted to J. Delves-Broughton from Aplin and Barrettt who kindly provided nisin.

References

Davies, E.A., Bevis, H.E., Delves-Broughton, J., 1997. The use of the bacteriocin, nisin, as a preservative in ricotta-like cheeses to control the food-borne pathogen *Listeria monocytogenes*. Lett. Appl. Microbiol. 24, 343–346.

Dean, J.P., Zottola, E.A., 1996. Use of nisin in ice cream and effect on the survival of *Listeria monocytogenes*. J. Food Prot. 59, 476–480.

Delves-Broughton, J., 1990. Nisin and its uses as a food preservative. Food Technol. 44, 100–117.

Delves-Broughton, J., Blackburn, P., Evans, R.J., Hugenholtz, J., 1996. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek 69, 193–202.

Ferreira, M.A.S.S., Lund, B.M., 1996. The effect of nisin on *Listeria monocytogenes* in culture medium and long-life cottage cheese. Lett. Appl. Microbiol. 22, 433–438.

Food and Drug Administration, 1998. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Fed. Regist. 53, 11247–11251.

Franz, C.M.A.P., Du Toit, M., von Holy, A., Schillinger, U., Holzapfel, W.H., 1997. Production of nisin-like bacteriocins produced by *Lactococcus lactis* strains isolated from vegetables. J. Basic Microbiol. 37, 187–196.

Harris, L.J., Fleming, H.P., Klaenhammer, T.R., 1991. Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A and UAL500 to nisin. J. Food Prot. 54, 836–840.

Mohamed, G.G.E., Seaman, A., Woodbine, M., 1984. Food antibiotic nisin: comparative effects on *Erysipelothrix* and *Listeria*. In: Woodbine, M. (Ed.), Antimicrobials and Agriculture; The Preceedings of the 4th International Symposium on Antibiotics in Agriculture: Benefits and Malefits. Butterworth, London, pp. 435–442.

Nilsson, L., Huss, H.H., Gram, L., 1997. Inhibition of *Listeria monocytogenes* on cold-smoked salmon by nisin and carbon dioxide atmosphere. Int. J. Food Microbiol. 38, 217–227.

Rasch, M., Knochel, S., 1998. Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA-1 and bavaricin A. Lett. Appl. Microbiol. 27, 275–278.

Richard, J.A., 1996. Use of bacteriocin producing starters advantageously in dairy industry. In: Bozoglu, T.F., Ray, B. (Eds.), Lactic Acid Bacteria: Current Advances in Metabolism, Genetics and Applications. NATO ASI, Ser. H, vol. 98, Springer, Berlin, pp. 137–154.

Scannell, A.G.M., Hill, C., Buckley, D.J., Arendt, E.K., 1997. Determination of the influence of organic acids and nisin on shelf-life and microbiological safety aspects on fresh pork sausage. J. Appl. Microbiol. 83, 407–412.

Schillinger, U., Stiles, M.E., Holzapfel, W.H., 1993. Bacteriocin production by *Carnobacterium piscicola* LV 61. Int. J. Food Microbiol. 20, 131–147.

Schillinger, U., Chung, H.-S., Keppler, K., Holzapfel, W.H., 1998. Use of bacteriocinogenic lactic acid bacteria to inhibit spontaneous nisin-resistant mutants of *Listeria monocytogenes* Scott A. J. Appl. Microbiol. 85, 657–663.

Ukuku, D.O., Shelef, L.A., 1997. Sensitivity of six strains of *Listeria monocytogenes* to nisin. J. Food Prot. 60, 867–869.