ELSEVIER

International Journal of Food Microbiology 62 (2000) 65–75

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.nl/locate/ijfoodmicro

Nisin–curvaticin 13 combinations for avoiding the regrowth of bacteriocin resistant cells of *Listeria monocytogenes* ATCC 15313

Anne Bouttefroy[a], Jean-Bernard Millière[b,c,*]

[a]*ASEPT, Rue des Docteurs Calmette et Guerin, B.P. 2047, 53020 Laval Cedex 9, France*
[b]*Laboratoire de Fermentations et de Bioconversions Industrielles, ENSAIA-INPL, 2 Avenue de la Forêt de Haye, 54505 Vandoeuvre-Lès-Nancy, France*
[c]*IUT Nancy-Brabois, UHP Nancy 1, Villers-Lès-Nancy, France*

Received 13 November 1999; received in revised form 11 March 2000; accepted 5 June 2000

Abstract

Nisin (25–100 IU/ml) and curvaticin 13 (160 AU/ml), a bacteriocin produced by *Lactobacillus curvatus* SB13, were shown to have a bactericidal effect against *Listeria monocytogenes* ATCC 15313 in TSB-YE broth (pH 6.5), but it was only transitory. Regrowth was not due to the loss of bacteriocin activity. Cells surviving nisin or curvaticin 13 were more resistant to the respective bacteriocin than the parental strain. Survivors to curvaticin 13 were resistant to the class IIa bacteriocins (carnocin CP5, pediocin AcH) but remained sensitive to nisin. The frequencies of spontaneous nisin resistants decreased with increasing bacteriocin concentration and the presence of salts (NaCl, K_2HPO_4). The behaviour of nisin (1000 IU/ml) or curvaticin 13 (640 AU/ml) resistant variants (Nis^{1000}, $Curv^{640}$) was investigated in the presence of nisin (100 IU/ml) or curvaticin 13 (320 AU/ml) at 22 and 37°C, and compared with that of the parental strain. The effectiveness of nisin was the same at both temperatures, whereas curvaticin 13 displayed a faster bactericidal action at 37°C. Nis^{1000} cells were less sensitive to curvaticin 13 than the parental strain, whereas $Curv^{640}$ cells were more sensitive to nisin than the parental strain. Simultaneous or sequential additions of nisin (50 IU/ml) and curvaticin 13 (160 AU/ml) were performed at 22°C in broth inoculated with the parental strain. All combinations induced a greater inhibitory effect than the use of a single bacteriocin. Simultaneous addition of bacteriocins at t_0 led to the absence of viable cells in the broth after 48 h.

Keywords: *Listeria monocytogenes*; Nisin; Curvaticin 13; Bacteriocin resistance; Synergism

1. Introduction

Listeria monocytogenes is a psychrotrophic pathogen, ubiquitous in nature, which is characterized by its high tolerance for salt and its relative acid tolerance (Ralovich, 1992). These characteristics make this species difficult to control in food.

*Corresponding author. ENSAIA-INPL, Laboratoire de Fermentations et de Bioconversions Industrielles, 2 Avenue de la Forêt de Haye, 54505 Vandoeuvre-Lès-Nancy, France. Tel.: +33-3-8359-5882; fax: +33-3-8359-5915.

E-mail addresses: jean-bernard.milliere@iutnb.u-nancy.fr (J.-B. Millière), a.bouttefroy@asept.asso.fr (A. Bouttefroy).

0168-1605/00/$ – see front matter
PII: S0168-1605(00)00372-X

L. monocytogenes is often detected in a variety of foods and has caused major food-borne outbreaks worldwide (Farber and Peterkin, 1991).

Bacteriocins of lactic acid bacteria, natural antimicrobial peptides, have been proposed by many researchers for controlling *L. monocytogenes* in food products (Muriana, 1996). Nisin, produced by some *Lactococcus lactis* subsp. *lactis* strains, belongs to class I (lantibiotic peptides) (Klaenhammer, 1993). This bacteriocin has been approved by the World Health Organization as a preservative in the food industry (Ronk, 1988). The bacteriocins of class IIa, characterized by the presence of a consensus motif (YGNGV) in their N-terminal extremity and by their inhibitory activities towards *L. monocytogenes* (Klaenhammer, 1993), can be used as food preservatives (Schillinger et al., 1996). Curvaticin 13 is a listericidal bacteriocin produced by *Lactobacillus curvatus* SB13, a strain isolated from a Belgian sausage (Sudirman et al., 1993). This bacteriocin, with four amino acid residues 2–5 (YGNG) present in the consensus motif (unpublished data), may be a pediocin-like bacteriocin (class IIa).

It has been reported that bacteriocins induce a transitory bactericidal effect against *L. monocytogenes*, often followed by a regrowth of cells in food models and in laboratory media supplemented with nisin (Davies et al., 1997) or with class IIa bacteriocins (Rekhif et al., 1994; Song and Richard, 1997). This growth could be due to a too low concentration of bacteriocins to kill all cells, or to physico-chemical factors such as alkaline pH values (Liu and Hansen, 1990), NaCl (Bell and De Lacy, 1985; Bhunia et al., 1991) or fat (Jung et al., 1992; Blom et al., 1997), which decrease bacteriocin action, or due to the emergence of bacteriocin resistant cells. According to various authors, nisin resistant variants of *L. monocytogenes* appear at frequencies of 10^{-5} to 10^{-8} (Harris et al., 1991; Ming and Daeschel, 1993; Davies and Adams, 1994; Mazzotta and Montville, 1997). For the class IIa bacteriocins (leucocins A, B, E and sakacin A), Dykes and Hastings (1998) reported higher frequencies (10^{-4} to 10^{-6}). Spontaneous variants of *L. monocytogenes* ATCC 15313 resistant to mesenterocin 52 (class IIa), curvaticin 13 and plantaricin C19 were detected at frequencies of 10^{-3} to 10^{-4}, and the resistance phenotype was stable (Rekhif et al., 1994). Cross-resistance between pediocin-like bacteriocins has previously been observed by Rekhif et al. (1994), Wan et al. (1997), Dykes and Hastings (1998), and Rasch and Knøchel (1998).

The objectives of this study were to investigate the resistance of *L. monocytogenes* ATCC 15313 variants to bacteriocins from class I (nisin) or class IIa (curvaticin 13, carnocin CP5, pediocin AcH). The effect of incubation temperature (22 and 37°C) on the growth response was studied for two variants of *L. monocytogenes* (nisin and curvaticin 13 resistant strains) and the parental strain. The combined effects of nisin and curvaticin 13 were studied in order to obtain synergistic associations of bacteriocins which could prevent regrowth of bacteriocin resistant *L. monocytogenes* cells.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. monocytogenes ATCC 15313 was selected as the test organism and designated as the parental strain (PS). This strain was maintained at 4°C on trypcase-soy agar slants (TSA; code 51019, Bio Mérieux, Craponne, France) supplemented with 0.6% yeast extract (YE; code 112002, Biokar Diagnostics, Beauvais, France) and 1.2% agar (code 101001, Biokar Diagnostics) (TSA-YE). *Micrococcus luteus* A270 (Institut Pasteur Collection, Paris, France), the indicator strain for nisin assays, was maintained at 4°C on tryptose agar slants (code 0064-17-4, Fisher Scientific, Difco, Detroit, MI, USA). *Lactobacillus curvatus* SB13, the curvaticin 13 producer (Sudirman et al., 1993), was maintained at −20°C in MRS broth (code BK070, Biokar Diagnostics). Working cultures were obtained after two subcultures (2% v/v inoculum) for 24 h on TSB-YE (this broth has the same composition as TSA-YE without agar) at 37°C (*L. monocytogenes*), on MRS broth at 30°C (*Lb. curvatus*), or on brain heart infusion (code 0037-01-6, Difco) at 30°C under stirring at 250 rpm (*M. luteus*). The following bacteriocin-producing strains were used: *Lactococcus lactis* subsp. *lactis* ATCC 11454, the nisin producer, *Carnobacterium piscicola* CP5, which produces carnocin CP5 (Herbin et al., 1997), and *Pediococcus acidilactici* H, provided by B. Ray (Department of Animal Science, University of Wyoming, Laramie, WY, USA), which produces

pediocin AcH (Bhunia et al., 1988). *Lc. lactis* was grown in Elliker broth (code BK054, Biokar Diagnostics), whereas *C. piscicola* CP5 and *P. acidilactici* H were grown in TSB-YE or in MRS broth, respectively. All strains were incubated at 30°C for 24 h except for *L. monocytogenes* (37°C).

2.2. Preparation of nisin and curvaticin 13 solutions

Stock solution of nisin (code N5764, Sigma, Saint-Quentin Fallavier, France) containing 10^4 IU/ml consisted of 10 mg/ml in 0.02 mol/l HCl. Curvaticin 13 was partially purified by the modified adsorption–desorption method (Yang et al., 1992) from a culture of *Lb. curvatus* SB13 in 2 l MRS broth (30°C, 24 h). The desorption step was carried out without NaCl. Curvaticin 13 solution, in 100 ml demineralized water acidified to pH 2.0 with orthophosphoric acid (85%, code A4713501, Fisher Scientific, Labosi, Oulchy-le-Château, France), was concentrated 10-fold at 40°C (Rotavapor Büchni RE111, Bioblock, Illkirch, France). Acidic solutions of each bacteriocin were sterilized by heating (75°C, 10 min) and then adjusted to pH 6.5 with 10 mol/l NaOH.

2.3. Bacteriocins assay

The activity of curvaticin 13 solutions and the concentrations of bacteriocins in cultures of *L. monocytogenes* were determined from cell-free supernatants adjusted to pH 6.5 using a well diffusion assay. Two-fold dilutions of supernatants were then prepared in 50 mmol/l K_2HPO_4 (code 104873, Merck, Darmstadt, Germany)–Na_2HPO_4 (code 6579, Merck) buffer (pH 6.5). Aliquots (50 μl) were poured into wells (8 mm diameter) in IP (Institut Pasteur) agar plates (15 ml) previously seeded with a culture of the indicator strain. This strain was *M. luteus* A270 ($N_0 = 2 \times 10^6$ cfu/ml) for nisin assay or *L. monocytogenes* ATCC 15313 ($N_0 = 2 \times 10^7$ cfu/ml) for curvaticin 13 assay. The IP agar contained the following (g/l): bactopeptone (code 0118-17-0, Difco), 6; tryptone (code 104001, Biokar Diagnostics), 4; YE, 3; meat extract (code 117010, Biokar Diagnostics), 1.5; glucose (code A4815281, Labosi), 1; Tween 80 (code 822187, Merck), 2; agar, 9. Plates were first stored at 4°C for 16 h then incubated at 37°C for 24 h. The reciprocal of the highest dilution showing a distinct growth inhibition zone of the indicator strain gave the bacteriocin activity in units (AU) in 50 μl of sample (Mayr-Harting et al., 1972). The curvaticin 13 solution had a titre of 20 480 AU/ml.

2.4. Frequency of nisin resistant variants

In a previous study, we showed that nisin was more active in the absence of NaCl, hence more nisin-sensitive cells was killed (Bouttefroy et al., 2000). Therefore, the frequencies of resistant variants were determined with or without salts (NaCl, K_2HPO_4) in TSA-YE agar containing nisin (0, 100, 500 or 1000 IU/ml). The TSA-YE1 reconstituted agar, which had the same composition as TSA-YE but without NaCl (5 g/l) and K_2HPO_4 (2.5 g/l), contained the following (g/l): bio-trypcase (code 51091, BioMérieux), 17; bio-soyase (code 53401, BioMérieux), 3; glucose, 2.5; YE, 6; agar, 12. Three cultures of the PS in TSB-YE (37°C, 24 h) were 10-fold serial diluted in tryptone-salt broth (TS; code BK014, Biokar Diagnostics), and 0.1 ml of each dilution was streaked onto TSA-YE or TSA-YE1 plates with or without nisin (100, 500 or 1000 IU/ml). After incubation at 37°C for 48 h, the numbers of cfu on control and nisin-containing plates were determined. Experiments were conducted in duplicate. The frequency of spontaneous curvaticin 13 resistant variants of *L. monocytogenes* ATCC 15313 was previously determined in our laboratory; Rekhif et al. (1994) showed that the curvaticin 13 resistance of this strain was of stable character.

2.5. Behaviour of L. monocytogenes ATCC 15313 cells after a single exposure to nisin or curvaticin 13

The sensitivity of PS cells (1.5×10^4 cfu/ml) to nisin was determined in TSB-YE (pH 6.5) supplemented with different nisin concentrations (0, 25, 50, 100 or 125 IU/ml) at 22°C for 240 h. Similar experiments were performed with Nis^{100} cells obtained after a subculture of the PS strain in TSB-YE with 100 IU/ml nisin at 37°C for 24 h in order to obtain a population of 10^9 cfu/ml. The Nis^{100} cells (1.2×10^4 cfu/ml) were inoculated in TSB-YE with

or without 0, 50, 100, 150 or 200 IU/ml nisin, and cultures were incubated at 22°C for 96 h.

Cultures of PS cells (10^4 cfu/ml) were performed in TSB-YE (pH 6.5) with or without curvaticin 13 (160 AU/ml) at 22°C for 24 h. Cells (PS or $Curv^{160}$) were then harvested by centrifugation for 10 min at 10 000×*g*, resuspended and washed in TSB-YE. Their sensitivity towards nisin, curvaticin 13, and two class IIa bacteriocins (carnocin CP5 and pediocin AcH) was evaluated using the well diffusion method. The culture supernatant of each bacteriocin-producing strain was used as the bacteriocin solution. These supernatants were adjusted to pH 6.5, and bacteriocin titres were determined with *L. monocytogenes* ATCC 15313 as the indicator strain.

2.6. Generation of bacteriocin resistant variants and stability of the nisin resistance phenotype

In order to obtain the nisin (1000 IU/ml) resistant variant (Nis^{1000}), *L. monocytogenes* PS (1×10^7 cfu/ml) was first grown in TSB-YE with 100 IU/ml nisin at 37°C for 24 h. This culture was then serially transferred into TSB-YE broths containing increasing concentrations of nisin in 100 IU/ml steps until 1000 IU/ml. The curvaticin 13 (640 AU/ml) resistant variant ($Curv^{640}$) was obtained by successive subcultures of PS cells in TSB-YE supplemented with 160, 320 and 640 AU/ml curvaticin 13 at 37°C. The nisin or curvaticin 13 resistance phenotype was checked using the well diffusion method with the parental strain as the control.

In order to determine the stability of the nisin-resistance phenotype, Nis^{1000} cells (10^4 or 10^7 cfu/ml) were subcultured 15 times in TSB-YE with or without nisin (1000 IU/ml) at 37°C for 24 h. For *L. monocytogenes* ATCC 15313, Rekhif et al. (1994) reported that the resistance phenotype to curvaticin 13 was stable.

2.7. Effect of nisin and curvaticin 13 on PS, Nis^{1000} and $Curv^{640}$ cells

PS (1.2×10^4 cfu/ml), Nis^{1000} (4.6×10^3 cfu/ml) or $Curv^{640}$ (3.3×10^4 cfu/ml) cells were inoculated into 100 ml TSB-YE (pH 6.5) supplemented with nisin (100 IU/ml) or curvaticin 13 (320 AU/ml). Cultures were incubated at 22 and 37°C. Experiments were carried out in triplicate.

2.8. Effect of simultaneous or delayed addition of nisin and curvaticin 13 on L. monocytogenes PS

The effectiveness of nisin (50 IU/ml) and curvaticin 13 (160 AU/ml), alone or in combination, was analyzed according to the behaviour of *L. monocytogenes* PS cells (1.5×10^4 cfu/ml) in TSB-YE (pH 6.5) at 22°C. Both bacteriocins were added at 0 h (t_0) and/or at 4 h of incubation.

2.9. Microbial analysis

For *L. monocytogenes* enumeration, 1 mg/ml of protease (type XIV from *Streptomyces griseus*, code P5147, Sigma) was added to culture samples in order to inactivate bacteriocins. Samples were incubated at 37°C for 20 min. Under our experimental conditions, this protease had no inhibitory effect on *L. monocytogenes*. For determination of the viable population (cfu/ml), 1 ml of a given 10-fold dilution of the sample in TS broth was plated in duplicate into TSA-YE and incubated at 37°C for 48 h. The absence of *L. monocytogenes* in 1, 5 or 10 ml of sample was checked by an enrichment procedure in 9, 45 or 90 ml UVM broth, respectively (UVM base code 52331, UVM1 supplement code 52352 and UVM2 supplement code 52362, Oxoid, Hampshire, UK), following the AFNOR norm (NF V08-055, AFNOR, 1993).

3. Results

3.1. Frequency of nisin resistant variants

The frequency of spontaneous nisin resistant variants decreased with increasing nisin concentration (Table 1). Thus, the variants were isolated on agar plates at a frequency of 10^{-2} with 100 IU/ml or 10^{-5} with 500 IU/ml nisin. With 1000 IU/ml nisin, the frequency was estimated below 10^{-9}, because no survivor was obtained. The frequency was salt-dependent (NaCl, K_2HPO_4) because, in the presence of 500 IU/ml nisin without salt, variants were isolated at a lower frequency (10^{-8}).

Table 1
Influence of nisin concentration and salt addition (NaCl, K_2HPO_4) on the frequency of spontaneous nisin resistant variants[a]

Nisin (IU/ml)	TSA-YE	TSA-YE1
100	5.9×10^{-2}*	2.6×10^{-3}
	2.9×10^{-2}**	5.8×10^{-4}
500	3.5×10^{-5}*	1×10^{-8}
	1.5×10^{-5}**	2.4×10^{-8}
1000	$<10^{-9}$***	$<10^{-9}$

[a] TSA-YE1 had the same composition as TSA-YE but without NaCl or K_2HPO_4. For each inoculum of *Listeria monocytogenes* ATCC 15313 (*2.8×10^8, **5×10^8, ***9.8×10^8 cfu/ml), experiments were carried out in duplicate.

3.2. Behaviour of Nis^{100} cells in the presence of nisin

The behaviour of PS and Nis^{100} cells was determined in TSB-YE with nisin during incubation at 22°C (Fig. 1). For the PS strain, the addition of nisin resulted in an immediate bactericidal effect, with cell counts decreasing with increasing bacteriocin concentration between 25 and 125 IU/ml (Fig. 1A). The population level was 20 cfu/ml with 25 IU/ml nisin and 1 or 0 cfu/ml with concentrations between 50 and 125 IU/ml after 4 h. Nevertheless, a transitory bactericidal effect was observed because regrowth occurred after 6, 12 or 24 h in the presence of 25, 50 or 100 IU/ml, respectively. Total inhibition was only observed with 125 IU/ml and no *L. monocytogenes* were detected during 240 h in 10 ml pre-enriched culture. No variation in nisin titres was noted, indicating that regrowth was not associated with the loss of bacteriocin activity.

The nisin sensitivity of Nis^{100} cells was determined in TSB-YE with increasing concentrations of nisin between 0 and 200 IU/ml. Without nisin, Nis^{100} cells grew from 1.8×10^4 to 4.2×10^8 cfu/ml in 30 h (Fig. 1B) and, after a short stationary phase (18 h), the population level decreased to 6×10^7 cfu/ml at 96 h, while the level of PS cells was stable at 3.1×10^8 cfu/ml. With Nis^{100} cells, nisin always induced a transitory bactericidal effect which was enhanced with increasing nisin concentrations. However, the reduction in Nis^{100} cell counts was lower than that with PS cells, and cells quickly resumed growth. Thus, 200 IU/ml nisin reduced Nis^{100} counts by 2.5 $\log_{(10)}$ in 6 h, but regrowth occurred after 8 h, while PS cells were totally inhibited by 125 IU/ml nisin.

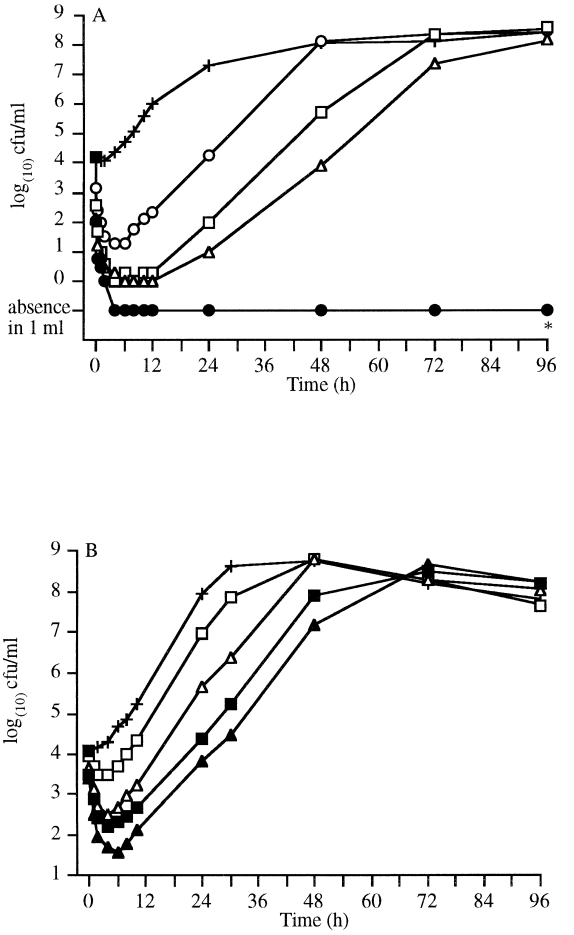

Fig. 1. Effect of nisin against *Listeria monocytogenes* ATCC 15313 (parental strain) PS (A) and a spontaneous nisin resistant variant (Nis^{100}) (B) in TSB-YE broth (pH 6.5) at 22°C. Nisin concentrations (IU/ml): (+) 0; (○) 25; (□) 50; (△) 100; (●) 125; (■) 150; (▲) 200. Absence in 1 ml: no cells were present in 1 ml sample culture after an enrichment procedure. *Until 240 h regrowth was not observed and no cells were present in 10 ml of sample.

3.3. Sensitivity of $Curv^{160}$ cells to bacteriocins

The sensitivity of PS and $Curv^{160}$ cells to bacteriocins was determined by the diameter of the inhibition zone produced by curvaticin 13 (1280 AU/ml), carnocin CP5 (640 AU/ml), pediocin AcH (10 240 AU/ml) and nisin (640 AU/ml) (Table 2).

Table 2
Sensitivity to bacteriocins of *Listeria monocytogenes* ATCC 15313 cells obtained from cultures in TSB-YE with ($Curv^{160}$) or without (PS) 160 AU/ml curvaticin 13 at 22°C

Cell	Culture supernatant of the bacteriocin-producing strain (titre in AU/ml)			
	Curvaticin 13 (1280)	Carnocin CP5 (640)	Pediocin AcH (10 240)	Nisin (640)
PS[a]	13.16±0.28[c]	7.16±0.28	8.83±0.76	9.33±0.28
$Curv^{160}$ [b]	1.52±0.51 (bl)[d]	0	0	10.97±0.55

[a] PS, parental strain of *L. monocytogenes* ATCC 15313.
[b] $Curv^{160}$, cultivar of PS grown in TSB-YE with curvaticin 13 (160 AU/ml).
[c] Mean diameter of inhibition zones (diameter of the well was subtracted) and standard deviation ($n = 3$).
[d] bl, blurred zone of inhibition.

$Curv^{160}$ cells remained slightly sensitive to curvaticin 13; the inhibition zones were blurred with a diameter of inhibition of 1.5 mm, a value significantly lower than that obtained with the PS cells (13.1 mm). $Curv^{160}$ cells were resistant to carnocin CP5 and pediocin AcH, two bacteriocins of class IIa, while PS cells were sensitive to both bacteriocins. $Curv^{160}$ cells were sensitive to nisin as well as the PS strain.

3.4. Effect of nisin and curvaticin 13 on PS, Nis^{1000} and $Curv^{640}$ cells

The behaviour of PS, Nis^{1000} and $Curv^{640}$ cells in TSB-YE with or without nisin (100 IU/ml) or curvaticin 13 (320 AU/ml) was investigated at 22 and 37°C for 72 h. The PS strain was sensitive to both bacteriocins at 22°C (Fig. 2A) and at 37°C (Fig. 2B). The effectiveness of nisin, expressed as the maximum viability loss ($\log_{(10)}$), was the same at both temperatures. Curvaticin 13 displayed a quicker bactericidal action at 37°C, with a maximal reduction in 2 h (1.3 $\log_{(10)}$); at 22°C, the bactericidal effect was only obtained in 8 h (1 $\log_{(10)}$). With both bacteriocins, the regrowth of surviving cells was faster at 37°C than that at 22°C.

The Nis^{1000} cells were unaffected by 100 IU/ml nisin at 22°C (Fig. 3A) and 37°C (Fig. 3B). However, they were sensitive to curvaticin 13; growth occurred after a bacteriostatic phase of 8 or 6 h at 22 or 37°C, respectively. PS cells were more sensitive to curvaticin 13 than Nis^{1000} cells. The resistance phenotype to nisin (Nis^{1000}) was stable after 15 subcultures with or without nisin.

The $Curv^{640}$ cells were unaffected by 320 AU/ml

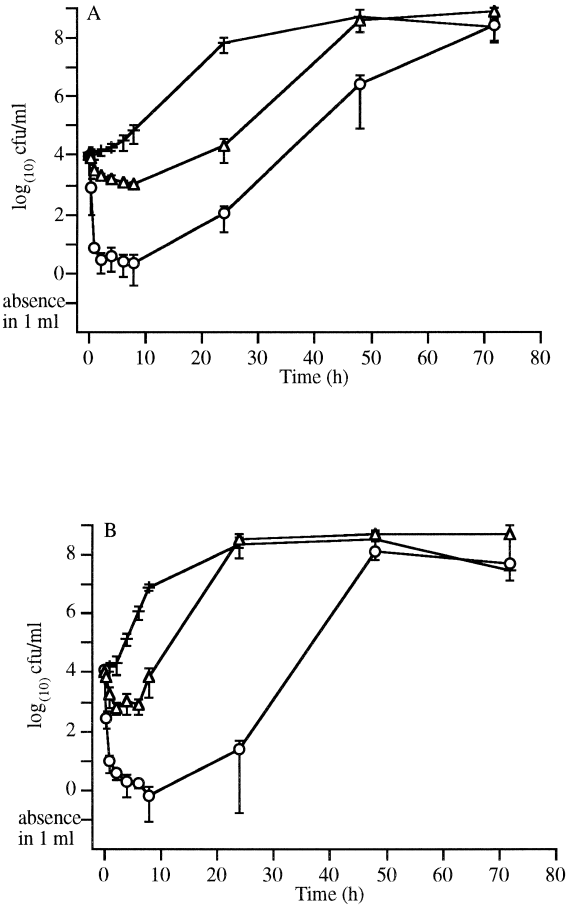

Fig. 2. Behaviour of *Listeria monocytogenes* ATCC 15313 PS in TSB-YE broth (pH 6.5) in the presence of nisin or curvaticin 13 at 22°C (A) and 37°C (B). Without bateriocins (+), cultures with 100 IU/ml nisin (○) or 320 AU/ml curvaticin 13 (△).

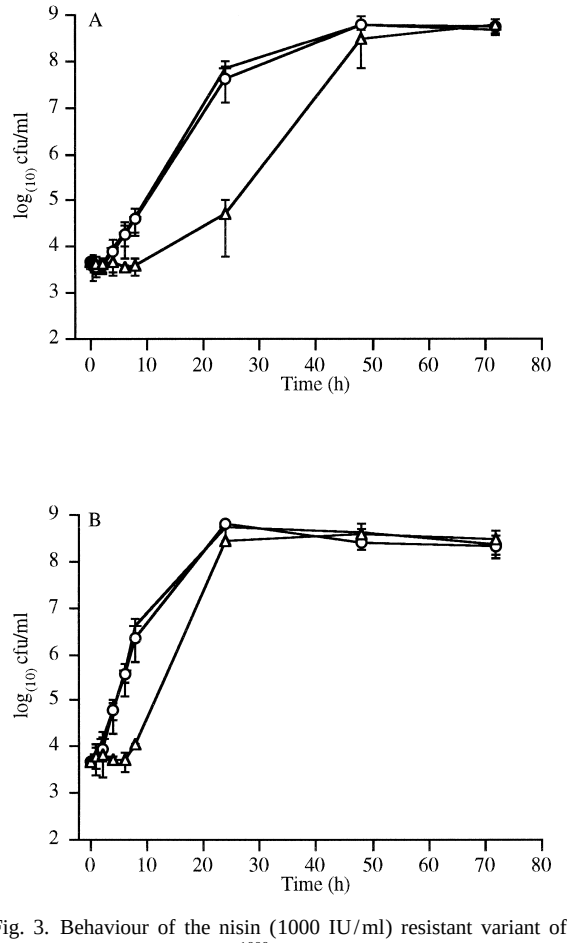

Fig. 3. Behaviour of the nisin (1000 IU/ml) resistant variant of *Listeria monocytogenes* (Nis^{1000}) in TSB-YE broth (pH 6.5) in the presence of nisin or curvaticin 13 at 22°C (A) and 37°C (B). Without bateriocins (+), cultures with 100 IU/ml nisin (○) or 320 AU/ml curvaticin 13 (△).

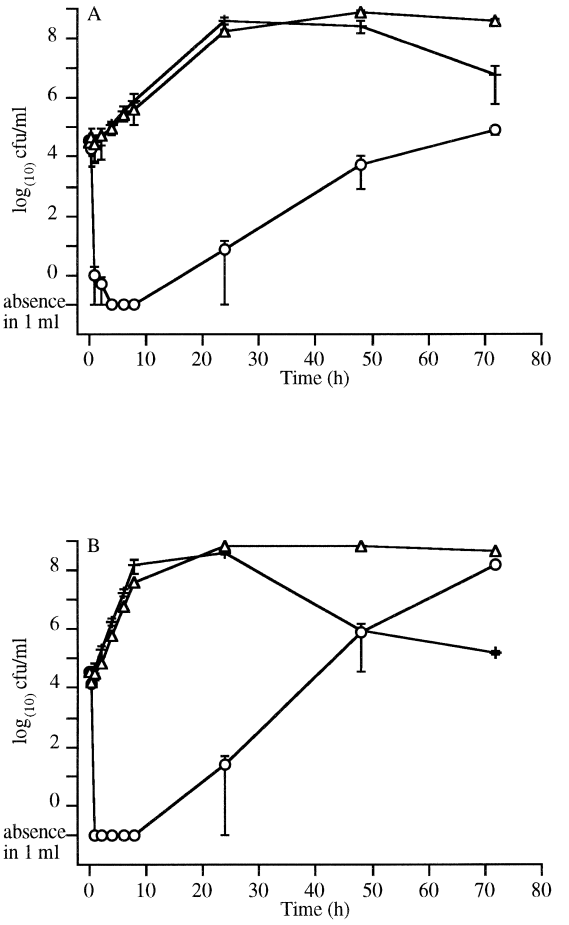

Fig. 4. Behaviour of the curvaticin 13 (640 AU/ml) resistant variant of *Listeria monocytogenes* ($Curv^{640}$) in TSB-YE broth (pH 6.5) in the presence of nisin or curvaticin 13 at 22°C (A) and 37°C (B). Without bateriocins (+), cultures with 100 IU/ml nisin (○) or 320 AU/ml curvaticin 13 (△).

curvaticin 13 at both temperatures, with the same growth rate in culture with or without curvaticin 13 (Fig. 4). However, without bacteriocin, the stationary phase was shorter, and cell counts decreased faster at 37°C (Fig. 4B) than at 22°C (Fig. 4A). At 37°C, the population level was 1.6×10^5 cfu/ml in broth without bacteriocin after 72 h and 4.2×10^8 cfu/ml with curvaticin 13. In the presence of 100 IU/ml nisin, the maximal bactericidal effect ($4.5\ \log_{(10)}$) was observed in 1 h at both temperatures, whereas with PS cells a lower inhibition was obtained after 8 h. The $Curv^{640}$ cells were more sensitive to nisin than the PS cells.

In all experiments with bacteriocins, no variation in titres of bacteriocins was observed, indicating that the bacteriocin resistant variants did not produce proteases. The nisin or curvaticin 13 resistant phenotype was verified in IP agar using the well diffusion method. No inhibition zone was observed for Nis^{1000} cells with 1000 IU/ml nisin or for $Curv^{640}$ cells with 5120 AU/ml curvaticin 13.

3.5. Effect of simultaneous or delayed addition of nisin and curvaticin 13 on L. monocytogenes

The behaviour of PS cells was investigated in TSB-YE (pH 6.5) at 22°C, with addition of nisin (50 IU/ml) and/or curvaticin 13 (160 AU/ml) at sub-

lethal concentration at t_0 and/or after 4 h of incubation.

The addition of nisin or curvaticin 13, at both incubation times, always induced an immediate transitory bactericidal effect (Fig. 5A). However, the action of nisin was dependent on the physiological state of the cells. Therefore, the bactericidal effect of nisin added at t_0 was greater ($3\log_{(10)}$) than that observed with nisin added at 4 h ($0.8\log_{(10)}$); in both cases, growth was resumed after 6 h of incubation. The addition of curvaticin 13 at t_0 or after 4 h reduced counts by $1\log_{(10)}$ unit, and regrowth occurred after 12 h.

Simultaneous addition of both bacteriocins at t_0 induced an immediate bactericidal phase which led, in 48 h, to reduction of the population to a non-detectable level; the absence of cells in 5 ml culture sample was verified after an enrichment procedure up to 96 h (Fig. 5B). When nisin and curvaticin 13 were added after 4 h, the reduction in cell counts was $2.5\log_{(10)}$ after 12 h of incubation, but cells resumed growth after 24 h and reached the control culture level (4×10^8 cfu/ml) after 72 h. Addition of nisin at t_0 followed by curvaticin 13 at 4 h induced a strong bactericidal effect ($4.1\log_{(10)}$), with only 1 cfu/ml between 6 and 12 h. However, regrowth occurred between 12 and 24 h and the population reached the control culture level after 96 h (data not shown). When curvaticin 13 was added at t_0 and nisin at 4 h, the bactericidal effect was lower ($2.7\log_{(10)}$), the cells resumed growth between 12 and 24 h and the population level was 4.5×10^8 cfu/ml after 72 h. All combinations of bacteriocins induced greater inhibitory effects than obtained with only a single bacteriocin.

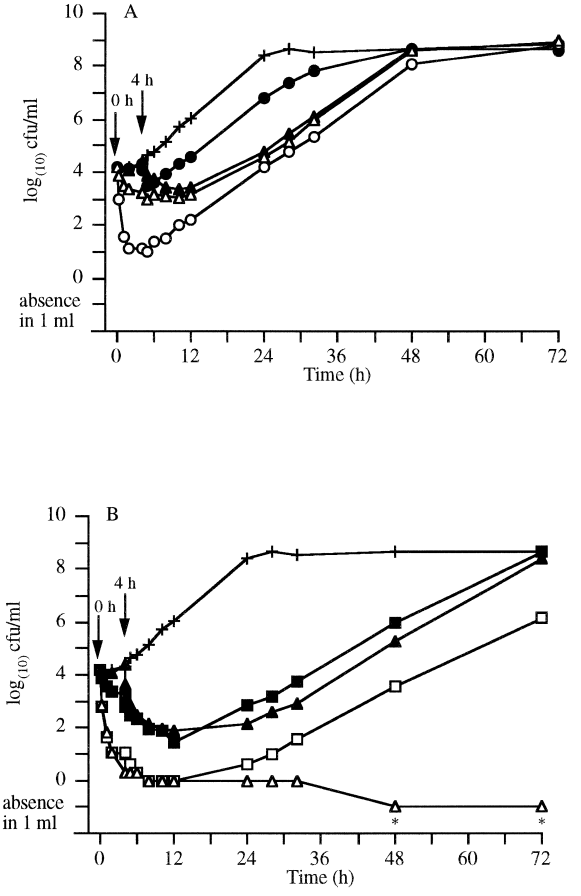

Fig. 5. Behaviour of *Listeria monocytogenes* ATCC 15313 PS in TSB-YE broth (pH 6.5) in the presence of nisin (50 IU/ml) and/or curvaticin 13 (160 AU/ml) added at t_0 and/or after 4 h of incubation at 22°C. No bacteriocin was added to the control culture (+). (A) nisin added at t_0 (○) or at $t_{4\,h}$ (●); curvaticin 13 added at t_0 (△) or at $t_{4\,h}$ (▲). (B) Both bacteriocins added at t_0 (△) or at $t_{4\,h}$ (▲); nisin added at t_0 and curvaticin 13 at $t_{4\,h}$ (□); curvaticin 13 added at t_0 and nisin at $t_{4\,h}$ (■). *Viable cells not detectable.

4. Discussion

Curvaticin 13 and nisin induced a bactericidal effect on *L. monocytogenes* followed by regrowth of the target cells. This transitory inhibitory effect of nisin (lantibiotic) or class IIa bacteriocins has been reported previously (Mathieu et al., 1994; Rekhif et al., 1994; Song and Richard, 1997). Cells resumed growth because of an insufficient amount of bacteriocins, and/or the emergence of more resistant cells. However, exposure of 1.5×10^4 cells of *L. monocytogenes* ATCC 15313 to 125 IU of nisin led to total inhibition. In this species, the resistance to nisin (Ming and Daeschel, 1993; Mazzotta and Montville, 1997) or to class IIa bacteriocins (Rekhif et al., 1994; Song and Richard, 1997) was not attributed to the production of a protease such as a nisinase previously described for *Bacillus* species (Jarvis, 1967). Nisin resistance of *Listeria* was attributed to changes in cytoplasmic membrane fatty acid and phospholipid compositions in the cell wall (Ming and Daeschel, 1995; Davies et al., 1996; Maisnier-Patin and Richard, 1996; Verheul et al.,

1997; Crandall and Montville, 1998). Pore formation in the cytoplasmic membrane by nisin was decreased in relation to the decrease in the net negative charge of the membrane and in membrane fluidity. Crandall and Montville (1998) reported that divalent cations were required by the variant to resist nisin. Little is known about the mechanism of resistance to non-nisin bacteriocins. Robichon et al. (1997) showed that when a σ^{54} transcriptional factor is not expressed in *L. monocytogenes*, cells become resistant to mesentericin Y105.

Spontaneous nisin resistants were obtained at a frequency ranging from 10^{-2} to 10^{-5} with nisin between 100 and 500 IU/ml at 37°C. These frequencies are 1000-fold higher than those for *L. monocytogenes* Scott A as reported by Mazzotta and Montville (1997) and De Martinis et al. (1997). In *L. monocytogenes*, nisin resistance was strain-dependent. The frequency of nisin resistance was drastically reduced in the absence of salts (0.5% NaCl and 0.25% K_2HPO_4). Without salts, cells formed colonies at a frequency of 10^{-8} with 500 IU/ml at 37°C. Bell and De Lacy (1985) have reported similar results with *B. licheniformis*; NaCl appears to antagonize the sporicidal action of nisin by interfering with its adsorption onto the spores. De Martinis et al. (1997) have previously studied the effect of NaCl, between 0.5 and 3.5%, on the frequency of nisin resistance in *L. monocytogenes* Scott A. A protective effect of NaCl was observed at concentrations around 2% at low temperature (10°C), but not at higher temperatures (20–30°C). This effect could be due to monovalent cations, which may be able to bind to phospholipids and prevent nisin adsorption.

L. monocytogenes cells surviving nisin or curvaticin 13 were more resistant to the bacteriocin than cells of the parental strain. The nisin resistance was a stable phenotype, as reported by Ming and Daeschel (1993), Mazzotta and Montville (1997), and Schillinger et al. (1998). The phenomenon of cross-resistance between curvaticin 13 and two bacteriocins of class IIa (carnocin CP5, pediocin AcH) was observed, but not with nisin. These results indicate that the mechanism of resistance of *L. monocytogenes* to curvaticin 13 is probably the same as for the class IIa bacteriocins, but should be different to the mechanism of nisin resistance. Previously, Rekhif et al. (1994) reported that curvaticin 13 resistant cells displayed cross-resistance towards mesenterocin 52, another pediocin-like bacteriocin (Revol-Junelles et al., 1996). Cross-resistance between class IIa bacteriocins has been reported for pediocin AcH/bavaricin A (Rasch and Knøchel, 1998), pediocin AcH/piscicolin 126 (Wan et al., 1997) and leucocins A, B, E/sakacin A (Dykes and Hastings, 1998).

Without bacteriocin, Nis^{1000} and $Curv^{640}$ cells have the same growth rates as the PS cells at 22 or 37°C. The growth kinetics of nisin and curvaticin 13 resistant cells were similar with or without the respective bacteriocin. However, without bacteriocin the population level of curvaticin 13 resistant cells quickly decreased after a short stationary phase. No previous study has reported this instability for a bacteriocin resistant variant. Drastic acidification (pH 6.5 to 4.6) of TSB-YE was always observed in relation to the growth of the parental strain and both variants (data not shown). Curvaticin 13 resistant cells would be less acid-tolerant than the parental strain due to membrane modifications. Dykes and Hastings (1998) reported that the variants resistant to class IIa had a slower growth rate than the sensitive parental strain and, in the absence of bacteriocin, variants were unable to invade populations of the sensitive strain when grown in co-culture. These authors concluded that the resistance phenotype was unstable, in opposition to the results of Rekhif et al. (1994). The variant resistant to curvaticin 13 did not display cross-resistance to nisin, and was slightly more sensitive to nisin than the parental strain. The nisin resistant cells were sensitive to curvaticin 13, but were more tolerant than the parental strain. These results agree with those reported by Schillinger et al. (1998) and Rasch and Knøchel (1998), who reported that a nisin resistant cell did not display cross-resistance to sakacin A, enterocin A, pediocin AcH or bavaricin A.

As no nisin/curvaticin 13 cross-resistance was found, combinations of these bacteriocins were investigated in broth at 22°C. After 4 h of culture without bacteriocin, cells were more resistant to nisin than those in the lag phase (bacteriocin added at t_0). Zapico et al. (1998) described similar results with *L. monocytogenes* Scott A. The effectiveness of curvaticin 13 was identical on cells in the lag phase or on growing cells (bacteriocin added at 4 h). Curvaticin 13 and nisin had a synergistic bactericidal effect on *L. monocytogenes*. When both bacteriocins were added at t_0, their combined inhibitory effect

resulted in the death of all cells (1.5×10^4 cfu/ml), whereas regrowth was observed with a single bacteriocin. Synergistic combinations of two bacteriocins have already been reported for pediocin AcH/nisin (Hanlin et al., 1993), lactacin B or lactacin F with nisin or pediocin AcH, and lactacin 481/pediocin AcH (Mulet-Powell et al., 1998). After 4 h, addition of nisin and curvaticin 13 reduced their bactericidal effect because cells were more resistant to nisin. When bacteriocins were applied sequentially, they produced a lower inhibitory effect than that obtained with simultaneous additions at t_0.

Based on the above data, the transitory bactericidal effect of bacteriocins was due to the growth of spontaneous resistant variants. No cross-resistance was found between nisin and curvaticin 13, a bacteriocin probably of class IIa. Curvaticin 13 and nisin had a synergistic listericidal effect without regrowth when the bacteriocins were added simultaneously at t_0. This combination may prevent the emergence of spontaneous variants resistant to nisin or curvaticin 13. Experiments are currently being conducted in order to test this association of bacteriocins on *L. monocytogenes* in dairy products at refrigeration temperatures. Furthermore, the sequence of curvaticin 13 will be determined.

References

AFNOR, 1993. Association française de normalisation: recherche de *Listeria monocytogenes*. Norme française NF V08-055, pp. 1–23.

Bell, R.G., De Lacy, K.M., 1985. The effect of nisin–sodium chloride interactions on the outgrowth of *Bacillus licheniformis* spores. J. Appl. Bacteriol. 59, 127–132.

Bhunia, A.K., Johnson, M.C., Ray, B., 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. 65, 261–268.

Bhunia, A.K., Johnson, M.C., Ray, B., Kalchayanand, N., 1991. Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. J. Appl. Bacteriol. 70, 25–30.

Blom, H., Katla, T., Hagen, B.F., Axelsson, L., 1997. A model assay to demonstrate how intrinsic factors affect diffusion of bacteriocins. Int. J. Food Microbiol. 38, 103–109.

Bouttefroy, A., Mansour, M., Linder, M., 2000. Millière, J.B., 2000. Inhibitory combinations of nisin, sodium chloride, and pH on *Listeria monocytogenes* ATCC 15313 in broth by an experimental design approach. Int. J. Food Microbiol. 54, 109–115.

Crandall, A.D., Montville, T.J., 1998. Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl. Environ. Microbiol. 64, 231–237.

Davies, E.A., Adams, M.R., 1994. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. Int. J. Food Microbiol. 21, 341–347.

Davies, E.A., Falahee, M.B., Adams, M.R., 1996. Involvement of the cell envelope of *Listeria monocytogenes* in the acquisition of nisin resistance. J. Appl. Bacteriol. 81, 139–146.

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

De Martinis, E.C.P., Crandall, A.D., Mazzotta, A.S., Montville, T.J., 1997. Influence of pH, salt, and temperature on nisin resistance in *Listeria monocytogenes*. J. Food Prot. 60, 420–423.

Dykes, G.A., Hastings, J.W., 1998. Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. Lett. Appl. Microbiol. 26, 5–8.

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food borne pathogen. Microbiol. Rev. 55, 476–511.

Hanlin, M.B., Kalchayanand, N., Ray, P., Ray, B., 1993. Bacteriocins of lactic acid bacteria in combination have greater antibacterial activity. J. Food Prot. 56, 252–255.

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.

Herbin, S., Mathieu, F., Brulé, F., Branlant, C., Lefebvre, G., Lebrihi, A., 1997. Characteristics and genetic determinants of bacteriocin activities produced by *Carnobacterium piscicola* CP5 isolated from cheese. Curr. Microbiol. 35, 319–326.

Jarvis, B., 1967. Resistance to nisin and production of nisin-inactivating enzymes by several *Bacillus* species. J. Gen. Microbiol. 47, 33–48.

Jung, D.S., Bodyfelt, F.W., Daeschel, M.A., 1992. Influence of fat and emulsifiers on the efficacy of nisin in inhibiting *Listeria monocytogenes* in fluid milk. J. Dairy Sci. 75, 387–393.

Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39–86.

Liu, W., Hansen, J.N., 1990. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. Appl. Environ. Microbiol. 56, 2551–2558.

Maisnier-Patin, S., Richard, J., 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in presence of high nisin concentrations. FEMS Microbiol. Lett. 140, 29–35.

Mathieu, F., Michel, M., Lebrihi, A., Lefebvre, G., 1994. Effect of the bacteriocin carnocin CP5 and of the producing strain *Carnobacterium piscicola* CP5 on the viability of *Listeria monocytogenes* ATCC 15313 in salt solution, broth and skimmed milk, at various incubation temperatures. Int. J. Food Microbiol. 22, 155–172.

Mayr-Harting, A., Hedges, A.J., Berkeley, R.C.W., 1972. Methods for studying bacteriocins. In: Norris, J.R., Ribbons, D.W.P. (Eds.). Methods in Microbiology, Vol. 7A. Academic Press, New York, pp. 315–422.

Mazzotta, A.S., Montville, T.J., 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10°C and 30°C. J. Appl. Microbiol. 82, 32–38.

Ming, X., Daeschel, M.A., 1993. Nisin resistance of foodborne

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

Ming, X., Daeschel, M.A., 1995. Correlation of cellular phospholipid content with nisin resistance of *Listeria monocytogenes* Scott A. J. Food Prot. 58, 416–420.

Mulet-Powell, N., Lacoste-Armynot, A.M., Vinas, M., Simeon De Buochberg, M., 1998. Interactions between pairs of bacteriocins from lactic bacteria. J. Food Prot. 61, 1210–1212.

Muriana, P.M., 1996. Bacteriocins for control of *Listeria* spp. in food. J. Food Prot. (Suppl.), 54–63.

Ralovich, B., 1992. Data for the properties of *Listeria* strains (a review). Acta Microbiol. Hung. 39, 105–132.

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

Rekhif, N., Atrih, A., Lefebvre, G., 1994. Selection and properties of spontaneous variants of *Listeria monocytogenes* ATCC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. Curr. Microbiol. 28, 237–241.

Revol-Junelles, A.M., Mathis, R., Krier, F., Fleury, Y., Delfour, A., Lefebvre, G., 1996. *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 synthesise two distinct bacteriocins. Lett. Appl. Microbiol. 23, 120–124.

Robichon, D., Gouin, E., Débarbouillé, M., Cossart, P., Cenatiempo, Y., Héchard, Y., 1997. The *rpoN* (σ^{54}) gene from *Listeria monocytogenes* is involved in resistance to mesentericin Y105, an antibacterial peptide from *Leuconostoc mesenteroides*. J. Bacteriol. 179, 7591–7594.

Ronk, R., 1988. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Fed. Reg. 53, 11247–11251.

Schillinger, U., Geisen, R., Holzapfel, W.H., 1996. Potential of antagonistic microorganism and bacteriocins for the biological preservation of foods. Trends Food Sci. Technol. 7, 158–164.

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

Song, H.J., Richard, J., 1997. Antilisterial activity of three bacteriocins used at sub minimal inhibitory concentrations and cross-resistance of the survivors. Int. J. Food Microbiol. 36, 155–161.

Sudirman, I., Mathieu, F., Michel, M., Lefebvre, G., 1993. Detection and properties of curvaticin 13, a bacteriocin produced by *Lactobacillus curvatus* SB13. Curr. Microbiol. 27, 35–40.

Verheul, A., Russell, N.J., Van't Hof, R., Rombouts, F.M., Abee, T., 1997. Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. Appl. Environ. Microbiol. 63, 3451–3457.

Wan, J., Harmark, K., Davidson, B.E., Hillier, A.J., Gordon, J.B., Wilcock, A., Hickey, M.W., Coventry, M.J., 1997. Inhibition of *Listeria monocytogenes* by piscicolin 126 in milk and Camembert cheese manufactures with a thermophilic starter. J. Appl. Microbiol. 82, 273–280.

Yang, R., Johnson, M.C., Ray, B., 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. 58, 3355–3359.

Zapico, P., Medina, M., Gaya, P., Nunez, M., 1998. Synergistic effect of nisin and the lactoperoxidase system on *Listeria monocytogenes* in skim milk. Int. J. Food Microbiol. 40, 35–42.