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Research Paper

Antibacterial efficacy of Nisin, Pediocin 34 and Enterocin FH99 against *Listeria monocytogenes* and cross resistance of its bacteriocin resistant variants to common food preservatives

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Abstract

Antilisterial efficiency of three bacteriocins, viz, Nisin, Pediocin 34 and Enterocin FH99 was tested individually and in combination against *Listeria mononcytogenes* ATCC 53135. A greater antibacterial effect was observed when the bacteriocins were combined in pairs, indicating that the use of more than one LAB bacteriocin in combination have a higher antibacterial action than when used individually. Variants of *Listeria monocytogenes* ATCC 53135 resistant to Nisin, Pediocin 34 and Enterocin FH99 were developed. Bacteriocin cross-resistance of wild type and their corresponding resistant variants were assessed and results showed that resistance to a bacteriocin may extend to other bacteriocins within the same class. Resistance to Pediocin 34 conferred cross resistance to Enterocin FH 99 but not to Nisin. Similarly resistance to Enterocin FH99 conferred cross resistance to Pediocin 34 but not to Nisin. Also, the sensitivity of Nisin, Pediocin 34 and Enterocin FH99 resistant variants of *Listeria monocytogenes* to low pH, salt, sodium nitrite, and potassium sorbate was assayed in broth and compared to the parental wild-type strain. The Nisin, Pediocin 34 and Enterocin FH99 resistant variants did not have intrinsic resistance to low pH, sodium chloride, potassium sorbate, or sodium nitrite. In no case were the bacteriocin resistant *Listeria monocytogenes* variants examined were more resistant to inhibitors than the parental strains.

Key words: *Listeria monocytogenes*, Nisin, Pedioin 34, Enterocin FH99, cross resistance.

Introduction

Bacteriocins are ribosomally-synthesized antimicrobial peptides or proteins, produced mainly by lactic acid bacteria (LAB). Several LAB bacteriocins with broad spectra of inhibitory activity offer potential applications in food biopreservation (Galvez *et al.*, 2008). Several reports have described the emergence of nisin resistant mutants of *Listeria monocytogenes* on exposure of the nisin-sensitive cells to relatively high nisin concentrations (Ming and Daeschel, 1993, 1995; Davies *et al.*, 1996; Verheul *et al.*, 1997). Resistance has been correlated to an altered fatty acid composition (Ming and Daeschel, 1993; Mazotta and Montville, 1997) and altered phospholipids composition (Ming and Daeschel, 1995; Crandall and Montville, 1998). Studies have revealed the stability of bacteriocin resistance phenomenon (Rekhif *et al.*, 1994; Dykes and Hastings, 1998) and it has been reported to occur at either a low or a high level. In *Listeria monocytogenes* and *Enterococcus faecalis*, low-level resistance has been reported to be due to alterations in membrane lipid composition (Vadyvaloo *et al.*, 2002; Naghmouchi *et al.*, 2006). High-level resistance has been attributed to results from the inactivation of the *mptACD* operon and due to several changes in protein synthesis (Ming and Daeschel, 1993; Gravesen *et al.*, 2000; Ramanath *et al.*, 2000; Dalet *et al.*, 2001; Hechard *et al.*, 2001; Gravesen *et al.*, 2002; Calvez *et al.*, 2007).

The development of bacteriocin resistance might hinder application of bacteriocins in food preservation. The aim of the present study was to show that bacteriocins of lactic acid bacteria in combination have a higher antibacterial action against *Listeria* than when used individually. Further, it has been shown that resistance to a bacteriocin

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may extend to other bacteriocins within the same class. Also, in this study we examined the sensitivity of bacteriocin resistant variants of *Listeria monocytogenes* to sodium chloride, low pH, sodium nitrite, and potassium sorbate in comparison with the parental wild-type strains to determine if resistance to nisin confers cross resistance to these common food preservatives.

Materials and Methods

Bacterial strains and culture conditions

Enterococcus faecium FH99, bacteriocinogenic strain was a isolate from human faeces (Gupta *et al.*, 2010). *Pediococcus pentosaceous* 34, a bacteriocinogenic strain was an isolate from cheddar cheese. *Pediococcus acidilactici* LB 42 (a sensitive strain used for detection of bacteriocin producers), was obtained from Prof. Bibek Ray, Department of Animal Science, University of Wyoming, Laramie Wyoming, USA. *L. monocytogenes* ATCC 53135 was obtained from American Type Culture Collection (ATCC).

Bacteriocins

One hundred milliliter aliquots of MRS broth (De Man *et al.*, 1960) (pH 6.5) (HiMedia, Mumbai) were inoculated with active culture of *E. faecium* FH99 (Gupta *et al.*, 2010) and *P. pentosaceus* 34 (1%) and incubated at 37 °C for 24 h. Cell free culture supernatant (CFCS) were prepared by centrifugation of the cultures in refrigerated centrifuge at 10,000 rpm for 10 min. The supernatant was filter sterilized by passing through a 0.2 μm (Millipore), 45 mm diameter membrane filter and used for partial purification after neutralization. Crude enterocin FH99 and pediocin 34 were precipitated from broth media by 60% ammonium sulphate precipitation and the precipitates were dissolved in sterilized Milli Q water. Enterocin FH99 and Pediocin 34 were purified by the method earlier described by Gupta *et al.* (2010). Nisin A (Nisaplin ®) was obtained from Danisco (Gurgaon, India). Nisin stock solutions were prepared from pure nisin in 0.02 N HCl and autoclaved.

Measurement of activity units (AU mL^{-1})

The antibacterial activity of nisin, pediocin 34 and enterocin FH99 was obtained using the spot on lawn assay as described by Ulhman *et al.* (1992), against *P. acidilactici* LB 42. Five microlitres of serial dilutions of the partially purified bacteriocin of *E. faecium* FH99 and *P. pentosaceus* 34 grown in MRS broth (De Man *et al.*, 1960) were spotted on the Tryptone Glucose Yeast Extract (TGE) agar plates (Biswas *et al.*, 1991) (1.5% agar). Before spotting, TGE agar plates were overlaid with TGE soft agar (0.75%) seeded with actively growing cells of the test organism. Plates were kept undisturbed for 3-4 h for diffusion of bacteriocin through agar and then incubated. The sensitivity of the strain in question was evaluated by checking for clear zones around the spots. Three independent replicates of experiment were done. The activity units of the culture broth were calculated using the following formula and expressed as activity units per mL:

Activity Units per mL (AU mL^{-1}) = 200 x Reciprocal of highest dilution that gave a clear zone

Bacteriocin susceptibility test and determination of Minimum Inhibitory Concentrations (MICs)

The inhibitory spectrum of activity was obtained using the spot on lawn assay as described by Ulhman *et al.* (1992) against *L. monocytogenes* ATCC 53135. Five microlitres of the partially purified bacteriocin of *E. faecium* FH 99 and *Pediococcus pentosaceus* 34 grown in MRS broth (De Man *et al.*, 1960) was spotted on the plates TGE agar plates (Biswas *et al.*, 1991) (1.5% agar). Before spotting, TGE agar plates were overlaid with TGE soft agar (0.75%) seeded with actively growing cells of the test organism. Plates were kept undisturbed for 3-4 h for diffusion of bacteriocin through agar and then incubated. The sensitivity of the strain in question was evaluated by checking for clear zones around the spots. For MIC determinations, 5 μL of a 1:2 dilution series of a bacteriocin solution was placed in wells. The Minimum Inhibitory Concentration (MIC) value was interpreted as the lowest concentration of bacteriocin that resulted in a clear inhibition halo after 18 h incubation at 37 °C. The MIC was defined as the lowest concentration of bacteriocin that induced an inhibition zone.

Kinetics of cell growth inhibition by bacteriocins

Overnight cultures of *L. monocytogenes* ATCC 53135 was inoculated into fresh BHI broth tubes (1%) containing either nisin, pediocin 34 or enterocin FH99. These bacteriocins were used individually or in combination; the mixture contained the calculated MICs of each bacteriocin. Additionally, the efficacy of nisin, pediocin 34 and enterocin FH99 in combination (half the concentration of MICs for each bacteriocin) was also evaluated. The concentrations of the bacteriocins used when tested against the target organism alone and in different combinations, respectively have been mentioned in footnote of Table 1. At different time intervals (1 h, 2 h, 4 h, 6 h and 24 h) the survivors were enumerated on Brain heart infusion (BHI) agar medium after appropriate dilutions in saline, and colonies were counted after 24-48 h of incubation at 37 °C. Three independent replicates of experiment were done.

Isolation of spontaneous bacteriocin resistant variants

Spontaneous resistant mutants of *L.monocytogenes* ATCC 53135 to nisin, pediocin 34 and enterocin FH99 were isolated after sequential exposure to a bacteriocin concentration 10-fold higher the MIC.

Table 1 - Viable cell count (log cfu mL^{-1}) of *Listeria monocytogenes* ATCC 53135 after treatment with nisin, pediocin 34 and enterocin FH 99 alone and in different combinations (mean ± standard deviation, n = 3).

Time	Control	Alone			Additive[a]				Synergistic[b]			
	WT	N	P	E	N+P	N+E	E+P	N+P+E	N+P	N+E	E+P	N+P+E
1 h	7.97 ± 0.03	4.20 ± 0.15	5.64 ± 0.34	5.68 ± 0.12	3.64 ± 0.03	3.07 ± 0.11	5.60 ± 0.20	2.30 ± 0.10	2.90 ± 0.09	1.60 ± 0.06	2.17 ± 0.091	1.14 ± 0.028
2 h	6.07 ± 0.09	4.20 ± 0.41	5.06 ± 0.44	6.20 ± 0.01	4.23 ± 0.04	2.60 ± 0.06	5.90 ± 0.03	2.60 ± 0.26	3.30 ± 0.03	1.30 ± 0.03	1.60 ± 0.06	1.77 ± 0.051
4 h	8.49 ± 0.02	2.55 ± 0.30	4.51 ± 0.32	5.07 ± 0.19	2.70 ± 0.07	2.20 ± 0.01	4.98 ± 0.03	1.44 ± 0.15	2.69 ± 0.075	2.00 ± 0.09	2.95 ± 0.04	1.47 ± 0.021
6 h	8.46 ± 0.05	6.73 ± 0.18	7.250.74	8.40 ± 0.14	5.76 ± 0.21	5.20 ± 0.02	8.33 ± 0.44	4.60 ± 0.02	3.50 ± 0.015	3.250.07	2.41 ± 0.07	2.77 ± 0.051
24 h	8.13 ± 0.03	5.20 ± 0.41	4.71 ± 0.34	8.18 ± 0.18	5.14 ± 0.61	6.77 ± 0.05	7.80 ± 0.61	6.07 ± 0.09	3.47 ± 0.021	5.00 ± 0.05	5.20 ± 0.01	3.17 ± 0.091

WT = Without Treatment.
N = Nisin, P = Pediocin 34, E = Enterocin FH99.
Units for concentrations: Nisin = IU mL^{-1}, Pediocin 34=AU mL^{-1}, Enterocin FH99 = AU mL^{-1}.
[a]In the additive assays the concentration of nisin (N), pediocin (P) and enterocin (E) were 13.2 IU mL^{-1}, 540 AU mL^{-1} and 120 AU mL^{-1}, respectively.
[b]In the synergistic assays the concentrations were 6.6 IU mL^{-1}, 270 AU mL^{-1} and 60 AU mL^{-1}, respectively.

Bacteriocin cross-resistance by Agar diffusion method

The sensitivity of *L. monocytogenes* ATCC 53135 and its resistant variants to nisin, pediocin 34 and enterocin FH99 were qualitatively determined by the agar well diffusion method. Briefly, 5 mL of molten TGE agar containing 0.75% (w/v) agar medium were cooled at 47 °C and seeded with 1% (v/v) overnight BHI culture of *L. monocytogenes* ATCC 53135 and its nisin, pediocin 34 or enterocin FH99 resistant variants. Seeded agar was then poured onto TGE agar plate and allowed to solidify at room temperature. Wells (8 mm) were cut in the solidified agar using a sterile metal cork borer and filled with 80 µL of sample. The plates were left at 5 °C for 2 h to allow diffusion of the tested aliquot and then incubated for 18 h at 37 °C. Absence or presence of inhibition zones was recorded.

Cross resistance to low pH, potassium sorbate, sodium chloride and sodium nitrite

Experiments were conducted to examine the sensitivity of bacteriocin resistant variants of *L. monocytogenes* ATCC 53135 to common food preservatives *i.e.* sodium chloride (NaCl), low pH, sodium nitrite and potassium sorbate in comparison with the parental sensitive-type strains and to determine if resistance to bacteriocins confers cross-resistance to these common food preservatives. BHI broth was supplemented with NaCl to final concentrations of 1%, 2%, 8%, 9%, 10%, 11%, 12%, 14% and 16% (wt/vol) (control, 0% additional NaCl); (ii) BHI broth was acidified with concentrated HCl to final pH values of 5.2, 5.0, 4.8, 4.6 and 4.4 (control, pH 7.4); BHI broth was supplemented with sodium nitrite to final concentrations of 20, 25, 30, 35, 40, 45, 50 and 55 µg/mL (control, no additional sodium nitrite) and potassium sorbate to final concentrations of 2, 2.5, 3.0, 3.5, 4, 4.5, 5, 5.5 and 6, mg/ml (control: no additional potassium sorbate). Three independent replicates of experiment were done.

Results

Kinetics of cell growth inhibition by bacteriocins

In the present study, the antibacterial efficacy of nisin, pediocin 34 and enterocin FH99 was evaluated alone as well as in different combinations against *L. monocytogenes* ATCC 53135 in BHI broth. The calculated MICs of the nisin, pediocin 34 and enterocin FH99 against *L. monocytogenes* were 13.2 IU mL^{-1}, 540 AU mL^{-1} and 120 AU mL^{-1}, respectively. These MICs were used to evaluate the antibacterial effect of bacteriocins alone. In order to evaluate additive and synergistic effect of bacteriocins the different combinations and the concentrations of bacteriocins were used against the target organisms. Nisin was also observed to be most effective in inhibiting the *L. monocytogenes* ATCC 53135, followed by pediocin 34 and

enterocin FH99. In case of *Listeria* , it was observed that even when nisin displayed the most rapid inhibitory activity at 1 h, the survivors resumed growth, reaching the highest cell counts at 24 h. The results of the present study indicate that combinations of different bacteriocins produce a more effective antibacterial effect against *L. monocytogenes* ATCC 53135 in comparison to the bacteriocins used alone. When the two non nisin bacteriocins were used together, a higher number of survivors were detected than with the pairs containing nisin. Also, synergistic action was observed between different combinations of bacteriocins when tested against *L. monocytogenes* ATCC 53135. A combination of nisin, pediocin 34 and enterocin FH99 was most effective against *L. monocytogenes* ATCC 53135 (Table 1).

Bacteriocin cross resistance

The bacteriocin cross resistance profiles of wild type *L. monocytogenes* and its corresponding nisin, pediocin 34 and enterocin FH99 resistant variants is shown in Table 2. Wild type *L. monocytogenes* ATCC 53135 showed sensitivity to nisin, pediocin 34 and enterocin FH99. However, pediocin 34 resistant variant showed resistance to enterocin FH99 and not nisin, On the other hand nisin resistant variant retained the sensitivity to pediocin 34 as well as enterocin FH99. Resistance to enterocin FH99 conferred cross resistance to pediocin 34 but not to nisin (Table 2).

Table 2 - Comparison of susceptibility of wild type strains and resistant variants of *Listeria monocytogenes* ATCC 53135 to nisin, pediocin 34 (P) and enterocin FH99 (E) bacteriocins.

Pathogen	Nisin	P	E
WT	+	+	+
Nr	-	+	+
Pr	+	-	-
Er	+	-	-

WT = wild type, Nr = nisin resistant, Pr = Pedioicn 34 resistant, Er = Enterocin FH99 resistant. No inhibition = - , Inhibition = +.

Cross resistance of wild type and resistant strains to common food preservatives

Table 3 and Table 4 shows the viable cell count (log cfu mL^{-1}) of wild type *L. monocytogenes* ATCC 53135 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium with pH 4.4, 4.8 and 5.0 and sodium chloride at concentration of 1, 2, 8, 9, 10 and 11% (w/v), respectively. Table 5 and Table 6 shows the viable cell count (log cfu mL^{-1}) of wild type *L. monocytogenes* ATCC 53135 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/mL and sodium nitrite at concentration of 20,

Table 3 - Viable cell count (log cfu mL^{-1}) of wild type *Listeria monocytogenes* ATCC 53135 (WT) and its nisin (Nr) resistant, pediocin 34 resistant and enterocin FH99 resistant variant at different time intervals after growth in medium with pH 4.4, 4.8 and 5.0 (mean ± standard error, n = 3).

pH	Culture	Time (h)					
		1	2	4	6	8	24
Control	WT	7.97 ± 0.03	7.74 ± 0.03	8.49 ± 0.13	7.86 ± 0.03	8.95 ± 0.02	8.11 ± 0.09
	Nr	7.95 ± 0.04	7.71 ± 0.03	8.47 ± 0.01	7.83 ± 0.09	8.93 ± 0.08	8.04 ± 0.03
	Pr	7.99 ± 0.05	7.72 ± 0.06	8.86 ± 0.09	7.83 ± 0.09	8.94 ± 0.03	8.00 ± 0.13
	Er	7.94 ± 0.09	7.71 ± 0.03	8.47 ± 0.01	7.85 ± 0.02	8.93 ± 0.09	8.04 ± 0.03
4.4	WT	3.51 ± 0.85	4.81 ± 0.29	3.60 ± 0.20	3.39 ± 0.79	2.250.35	3.51 ± 0.13
	Nr	4.20 ± 0.04	2.47 ± 0.12	2.62 ± 0.32	2.11 ± 0.81	2.14 ± 0.61	2.60 ± 0.20
	Pr	2.11 ± 0.39	1.60 ± 0.20	2.27 ± 0.60	1.77 ± 0.15	1.11 ± 0.39	2.00 ± 0.83
	Er	3.34 ± 0.24	2.34 ± 0.81	2.46 ± 0.23	2.32 ± 0.22	2.90 ± 0.30	3.17 ± 0.60
4.8	WT	6.17 ± 0.12	5.73 ± 0.23	6.04 ± 0.13	6.68 ± 0.12	5.88 ± 0.08	5.90 ± 0.30
	Nr	4.44 ± 0.31	4.49 ± 0.13	3.830.25	4.250.05	3.81 ± 0.13	4.04 ± 0.26
	Pr	4.77 ± 0.51	3.90 ± 0.30	3.77 ± 0.15	3.62 ± 0.23	3.49 ± 0.13	3.44 ± 0.31
	Er	3.60 ± 0.20	3.51 ± 0.13	3.50 ± 0.51	3.69 ± 0.04	3.39 ± 0.79	3.07 ± 0.91
5.0	WT	5.38 ± 0.61	5.70 ± 0.01	5.50 ± 0.14	6.55 ± 0.30	6.63 ± 0.34	7.27 ± 0.60
	Nr	4.81 ± 0.16	5.00 ± 0.85	5.41 ± 0.49	5.250.27	4.770.25	4.00 ± 0.49
	Pr	4.77 ± 0.12	4.69 ± 0.04	4.60 ± 0.20	4.54 ± 0.40	4.41 ± 0.49	4.14 ± 0.61
	Er	4.47 ± 0.71	4.17 ± 0.60	3.84 ± 0.50	4.00 ± 0.82	3.84 ± 0.50	3.54 ± 0.44

WT = Wild Type, Nr = Nisin resistant variant, Pr = Pediocin 34 resistant variant, Er = Enterocin FH99 resistant variant.

Table 4 - Viable cell count (log cfu mL^{-1}) of wild type *Listeria monocytogenes* ATCC 53135 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in supplemented with Sodium Chloride at concentration of 1, 2, 8, 9, 10 and 11% (w/v) (mean ± standard error, n = 3).

Concentration (% w/v)	Culture	Time (h)					
		1	2	4	6	8	24
Control	WT	7.97 ± 0.03	7.74 ± 0.03	8.49 ± 0.13	7.86 ± 0.03	8.95 ± 0.02	8.11 ± 0.09
	Nr	7.95 ± 0.04	7.71 ± 0.03	8.47 ± 0.01	7.83 ± 0.09	8.93 ± 0.08	8.04 ± 0.03
	Pr	7.99 ± 0.05	7.72 ± 0.06	8.86 ± 0.09	7.83 ± 0.09	8.94 ± 0.03	8.00 ± 0.13
	Er	7.94 ± 0.09	7.71 ± 0.03	8.47 ± 0.01	7.85 ± 0.02	8.93 ± 0.09	8.04 ± 0.03
1	WT	5.34 ± 0.13	6.07 ± 0.08	6.30 ± 0.10	6.78 ± 0.05	7.22 ± 0.05	7.17 ± 0.09
	Nr	4.95 ± 0.03	4.90 ± 0.09	5.11 ± 0.03	7.32 ± 0.14	6.97 ± 0.08	6.39 ± 0.04
	Pr	4.69 ± 0.07	4.77 ± 0.06	4.90 ± 0.09	6.74 ± 0.08	7.11 ± 0.06	6.62 ± 0.09
	Er	4.63 ± 0.08	4.59 ± 0.15	5.66 ± 0.08	6.43 ± 0.04	5.68 ± 0.07	5.36 ± 0.17
2	WT	5.39 ± 0.03	6.08 ± 0.06	6.14 ± 0.10	6.30 ± 0.03	7.11 ± 0.12	6.77 ± 0.08
	Nr	4.84 ± 0.08	4.84 ± 0.09	4.77 ± 0.08	5.50 ± 0.05	6.50 ± 0.10	5.95 ± 0.09
	Pr	4.68 ± 0.04	4.39 ± 0.07	4.98 ± 0.01	5.96 ± 0.10	5.97 ± 0.04	6.30 ± 0.03
	Er	4.46 ± 0.02	4.65 ± 0.03	5.82 ± 0.05	5.50 ± 0.15	5.44 ± 0.08	5.00 ± 0.09
8	WT	4.20 ± 0.12	4.23 ± 0.09	4.20 ± 0.13	4.00 ± 0.06	4.00 ± 0.19	3.60 ± 0.06
	Nr	3.69 ± 0.07	3.60 ± 0.06	3.30 ± 0.03	3.60 ± 0.14	3.07 ± 0.06	3.04 ± 0.03
	Pr	3.68 ± 0.04	3.39 ± 0.07	3.98 ± 0.07	3.96 ± 0.08	3.97 ± 0.04	3.30 ± 0.04
	Er	3.94 ± 0.09	3.71 ± 0.11	3.47 ± 0.08	3.85 ± 0.06	3.93 ± 0.09	3.04 ± 0.07
9	WT	3.95 ± 0.03	3.47 ± 0.02	4.00 ± 0.11	4.69 ± 0.07	3.60 ± 0.06	3.69 ± 0.08
	Nr	3.60 ± 0.06	3.47 ± 0.07	3.60 ± 0.01	3.00 ± 0.09	3.00 ± 0.10	3.14 ± 0.06
	Pr	3.47 ± 0.07	3.34 ± 0.03	3.55 ± 0.06	3.11 ± 0.04	3.17 ± 0.09	3.250.03
	Er	3.47 ± 0.11	3.40 ± 0.06	3.69 ± 0.08	3.83 ± 0.10	3.45 ± 0.02	3.00 ± 0.10
10	WT	3.39 ± 0.04	3.46 ± 0.08	3.71 ± 0.03	3.69 ± 0.07	3.07 ± 0.08	3.07 ± 0.10
	Nr	3.30 ± 0.03	3.36 ± 0.11	3.30 ± 0.06	2.78 ± 0.05	2.90 ± 0.09	2.95 ± 0.03
	Pr	3.27 ± 0.04	3.250.03	3.20 ± 0.02	2.95 ± 0.03	2.99 ± 0.05	2.07 ± 0.08
	Er	3.14 ± 0.08	3.17 ± 0.09	3.49 ± 0.09	3.36 ± 0.08	2.95 ± 0.03	2.81 ± 0.04
11	WT	2.82 ± 0.05	2.60 ± 0.06	3.14 ± 0.08	3.30 ± 0.06	3.250.05	3.04 ± 0.09
	Nr	2.69 ± 0.07	2.47 ± 0.04	2.47 ± 0.02	2.50 ± 0.09	2.77 ± 0.11	2.84 ± 0.08
	Pr	2.47 ± 0.10	2.62 ± 0.03	2.55 ± 0.07	2.51 ± 0.04	2.99 ± 0.05	2.91 ± 0.04
	Er	2.97 ± 0.08	2.54 ± 0.06	2.49 ± 0.02	2.86 ± 0.03	2.95 ± 0.03	2.79 ± 0.08

WT = Wild Type, Nr = Nisin resistant variant, Pr = Pediocin 34 resistant variant, Er = Enterocin FH99 resistant variant, ND = Not Detected.

30, 40 and 50 µg/mL, respectively. The results clearly show that resistance to nisin, pediocin 34 and enterocin FH99 did not confer intrinsic resistance to any of the preservatives tested. In no case were the bacteriocin resistant *L. monocytogenes* ATCC 53135 variants examined, resistant to inhibitors than the parental strains.

Discussion

Kinetics of cell growth inhibition by bacteriocins

Nisin was observed to be most effective in inhibiting the *L. monocytogenes* ATCC 53135. It was observed that even when the bacteriocins displayed the most rapid inhibitory activity at 1 h, the survivors resumed growth, reaching the highest cell counts at 24 h. Similar observations were also made by Schillinger *et al.* (1998), who reported a regrowth of survivors of *L. monocytogenes* Scott A after exposure to nisin concentrations between 10 and 500 IU mL^{-1} as well as with those of Song and Richard (1997), who observed that survivors of *L. innocua* resumed growth after the addition of nisin, pediocin AcH, and enterococcin EFS2 into TSBYE broth. According to Muriana (1996), several studies indicated the immediate decrease of target cells by one to three log cycles cfu/mL when a bacteriocin was added, with none or little effect on future inoculations.

Table 5 - Viable cell count (log cfu mL^{-1}) of wild type *Listeria monocytogenes* ATCC 53135 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/mL (mean ± standard error, n = 3).

Concentration (mg/mL)	Culture	Time (h)					
		1	2	4	6	8	24
Control	WT	7.97 ± 0.03	7.74 ± 0.03	8.49 ± 0.13	7.86 ± 0.03	8.95 ± 0.02	8.11 ± 0.09
	Nr	7.95 ± 0.04	7.71 ± 0.03	8.47 ± 0.01	7.83 ± 0.09	8.93 ± 0.08	8.04 ± 0.03
	Pr	7.99 ± 0.05	7.72 ± 0.06	8.86 ± 0.09	7.83 ± 0.09	8.94 ± 0.03	8.00 ± 0.13
	Er	7.94 ± 0.09	7.71 ± 0.03	8.47 ± 0.01	7.85 ± 0.02	8.93 ± 0.09	8.04 ± 0.03
2.0	WT	5.23 ± 0.04	5.23 ± 0.09	5.55 ± 0.03	6.57 ± 0.04	6.69 ± 0.06	7.95 ± 0.04
	Nr	4.59 ± 0.05	4.83 ± 0.09	5.44 ± 0.08	6.53 ± 0.09	6.32 ± 0.09	7.77 ± 0.01
	Pr	5.250.03	5.47 ± 0.02	5.50 ± 0.05	6.57 ± 0.04	6.71 ± 0.03	7.63 ± 0.08
	Er	4.90 ± 0.09	5.07 ± 0.08	4.95 ± 0.03	5.53 ± 0.09	5.57 ± 0.04	6.78 ± 0.05
3.0	WT	4.20 ± 0.02	4.95 ± 0.03	5.11 ± 0.03	5.90 ± 0.09	6.30 ± 0.03	6.00 ± 0.13
	Nr	3.51 ± 0.04	3.30 ± 0.03	4.30 ± 0.03	4.00 ± 0.12	5.30 ± 0.11	5.17 ± 0.01
	Pr	3.30 ± 0.10	3.38 ± 0.11	3.47 ± 0.01	3.50 ± 0.05	3.30 ± 0.03	3.90 ± 0.90
	Er	3.56 ± 0.02	3.41 ± 0.03	3.67 ± 0.08	3.75 ± 0.01	3.77 ± 0.01	3.81 ± 0.13
4.0	WT	3.44 ± 0.08	3.60 ± 0.06	4.00 ± 0.12	4.20 ± 0.02	4.250.03	4.47 ± 0.01
	Nr	2.90 ± 0.09	2.69 ± 0.02	3.30 ± 0.03	3.59 ± 0.05	3.77 ± 0.01	3.250.03
	Pr	3.20 ± 0.02	3.27 ± 0.04	3.11 ± 0.02	2.71 ± 0.03	2.43 ± 0.04	2.87 ± 0.06
	Er	2.36 ± 0.08	2.00 ± 0.09	ND	ND	ND	ND
5.0	WT	2.39 ± 0.04	2.53 ± 0.09	2.60 ± 0.06	2.65 ± 0.03	2.69 ± 0.07	2.77 ± 0.01
	Nr	1.90 ± 0.09	1.95 ± 0.03	2.30 ± 0.03	2.39 ± 0.04	2.30 ± 0.03	1.95 ± 0.03
	Pr	2.00 ± 0.16	2.07 ± 0.01	2.30 ± 0.03	2.54 ± 0.08	2.18 ± 0.04	2.65 ± 0.06
	Er	2.250.03	1.74 ± 0.05	ND	ND	ND	ND
6.0	WT	1.87 ± 0.05	1.60 ± 0.02	1.47 ± 0.01	1.54 ± 0.08	1.60 ± 0.06	1.47 ± 0.01
	Nr	1.60 ± 0.06	1.47 ± 0.01	1.60 ± 0.06	1.47 ± 0.01	ND	ND
	Pr	1.90 ± 0.09	1.47 ± 0.01	ND	ND	ND	ND
	Er	1.95 ± 0.01	1.60 ± 0.06	ND	ND	ND	ND

WT = Wild Type, Nr = Nisin resistant variant, Pr = Pediocin 34 resistant variant, Er = Enterocin FH99 resistant variant, ND = Not Detected.

The results of the present study indicate that combinations of different bacteriocins produce a more effective antibacterial effect against *L. monocytogenes* ATCC 53135 in comparison to the bacteriocins used alone. Similar observations were reported by Hanlin *et al.* (1993) that a mixture containing more than one bacteriocins would have greater bactericidal effect to a sensitive population, since cells resistant to one bacteriocin might be killed by the other bacteriocin. Moreover synergistic effects were reported when the interactions between pairs of bacteriocins from lactic acid bacteria were tested which are in accordance with the results obtained by Mullet-Powell *et al.* (1998). The effectiveness of different bacteriocin pairs could be be explained by the fact that the bacteriocins used in this study belonged to different classes, which might vary considerably in the nature and sequence of amino acid residues as also earlier suggested in astudy conducted by Moll *et al.* (1999). The synergistic action of combinations of two different bacteriocins with different structures produced by the same strain has also been reported in agar medium by Limonet *et al.* (2004). Similar results have been reported by Jamuna *et al.* (2005) who showed that the bacteriocins from *L. acidophilus* and *L. casei* have a better antibacterial activity in combination with Nisin than when used alone against food spoilage and pathogenic organisms in liquid and food systems. Vignolo *et al.* (2000) also reported that the combined effect of lactocin 705, enterocin CRL35, and nisin against *L. monocytogenes* FBUNT in meat slurry showed no viable counts after incubation for 3 h. Jamuna and Jeevaratnam (2009) have also reported the synergistic effect of Nisin and bacteriocin from *Pediococcus acidilactici* to be more effective in inhibiting the growth of *L. monocytogenes* and *S. aureus* in sealed pouches of vegetable pulav.

Table 6 - Viable cell count (log cfu ml^{-1}) of wild type *Listeria monocytogenes* ATCC 53135 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in supplemented with Sodium Nitrite at concentration of 20, 30, 40, and 50 μg/mL (mean ± standard error, n = 3).

Concentration (μg/mL)	Culture	Time (h)					
		1	2	4	6	8	24
Control	WT	7.97 ± 0.03	7.74 ± 0.03	8.49 ± 0.13	7.86 ± 0.03	8.95 ± 0.02	8.11 ± 0.09
	Nr	7.95 ± 0.04	7.71 ± 0.03	8.47 ± 0.01	7.83 ± 0.09	8.93 ± 0.08	8.04 ± 0.03
	Pr	7.99 ± 0.05	7.72 ± 0.06	8.86 ± 0.09	7.83 ± 0.09	8.94 ± 0.03	8.00 ± 0.13
	Er	7.94 ± 0.09	7.71 ± 0.03	8.47 ± 0.01	7.85 ± 0.02	8.93 ± 0.09	8.04 ± 0.03
20	WT	6.38 ± 0.11	6.19 ± 0.13	6.28 ± 0.07	6.00 ± 0.16	7.00 ± 0.15	6.63 ± 0.08
	Nr	4.99 ± 0.06	5.17 ± 0.09	5.04 ± 0.09	5.34 ± 0.03	5.65 ± 0.13	6.250.07
	Pr	5.39 ± 0.04	6.250.03	6.30 ± 0.03	6.15 ± 0.06	6.01 ± 0.07	6.09 ± 0.02
	Er	5.11 ± 0.03	5.90 ± 0.09	6.17 ± 0.01	5.32 ± 0.09	6.29 ± 0.03	6.00 ± 0.06
30	WT	4.60 ± 0.16	4.84 ± 0.05	4.14 ± 0.12	4.14 ± 0.18	4.60 ± 0.20	4.95 ± 0.04
	Nr	4.07 ± 0.09	4.30 ± 0.10	4.92 ± 0.09	4.39 ± 0.04	4.53 ± 0.10	4.38 ± 0.11
	Pr	4.00 ± 0.12	4.41 ± 0.03	4.87 ± 0.06	3.90 ± 0.09	4.44 ± 0.05	4.39 ± 0.09
	Er	4.30 ± 0.03	4.36 ± 0.08	4.54 ± 0.18	4.47 ± 0.03	4.36 ± 0.06	4.11 ± 0.08
40	WT	3.39 ± 0.04	3.47 ± 0.07	3.90 ± 0.09	4.27 ± 0.04	4.17 ± 0.04	3.90 ± 0.09
	Nr	3.07 ± 0.08	3.00 ± 0.06	3.04 ± 0.04	3.44 ± 0.07	3.60 ± 0.06	3.69 ± 0.07
	Pr	3.04 ± 0.03	3.08 ± 0.08	3.55 ± 0.03	3.47 ± 0.01	3.65 ± 0.03	3.69 ± 0.06
	Er	3.00 ± 0.10	3.19 ± 0.05	3.65 ± 0.07	3.250.03	3.94 ± 0.08	3.90 ± 0.09
50	WT	3.27 ± 0.02	3.11 ± 0.03	3.23 ± 0.09	3.89 ± 0.05	3.81 ± 0.03	3.44 ± 0.08
	Nr	2.69 ± 0.07	2.50 ± 0.05	2.60 ± 0.06	2.73 ± 0.04	2.50 ± 0.05	2.59 ± 0.06
	Pr	2.90 ± 0.09	2.97 ± 0.04	3.00 ± 0.07	3.07 ± 0.01	3.27 ± 0.08	3.01 ± 0.03
	Er	2.80 ± 0.08	2.89 ± 0.05	3.14 ± 0.05	2.95 ± 0.03	3.03 ± 0.06	2.91 ± 0.08

WT = Wild Type, Nr = Nisin resistant variant, Pr = Pediocin 34 resistant variant, Er = Enterocin FH99 resistant variant.

Bacteriocin cross resistance

Several reports suggest that resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes. The nisin resistant strain of *L. monocytogenes* has been reported to show cross resistance to the class IIa bacteriocin pediocin PA-1 and the class IV leuconocin S (Crandall and Montville, 1998). *L. monocytogenes* mutants resistant to mesenterocin 52, curvaticin 13, and plantaricin were also reported to be cross-resistant to the other bacteriocins (Rekhif *et al.*, 1994). In addition, piscicolin 126-resistant mutants of *L. monocytogenes* which emerged in cheese made from milk containing the bacteriocin were also resistant to pediocin P02 (Wan *et al.*, 1997). These reports of cross-resistance indicate that the use of multiple bacteriocins to achieve greater antibacterial efficacy (Hanlin *et al.*, 1993) might not be feasible. Cross-resistance between bacteriocins has also been observed when the sensitivity of *Listeria* variants to lactocin 705, enterocin CRL35, and nisin was tested. Similar results were obtained by Rekhif *et al.* (1994) who reported that mutants of *L. monocytogenes* ATCC 15313 resistant to one of three bacteriocins tested (mesentericin 52, curvaticin 13, and plantaricin C19), displayed more resistance to the other two, but not to nisin. Insensitivity of a variant to lactocin 705 and enterocin CRL35 while retaining sensitivity to nisin, and vice versa, was associated with the mechanism by which a bacteriocin enters the cell following binding to the cell surface, as well as with the ability to form pores in bacterial membranes.

Cross Resistance of Wild Type and Resistant Strains to Common Food Preservatives

The application of bacteriocins as part of hurdle technology has received great attention in recent years (Chen and Hoover, 2003; Ross *et al.*, 2003; Deegan *et al.*, 2006). Bacteriocins can be used purposely in combination with selected hurdles in order to increase microbial inactivation. Many reports have reported the ultimate failure of bacteriocin based preservation systems due to the eventual growth of resistant strains (Motlagh *et al.*, 1992; Ming and Daeschel, 1993; Jamuna *et al.*, 2005). Several studies focused in the application of potential synergists of nisin's activity. These included the use of nitrites, low pH, pasteurization, controlled atmosphere, and food ingredients (Rayman *et*

al., 1981; Somers and Taylor, 1981; Taylor *et al.*, 1981; Taylor *et al.*, 1985; Motlagh *et al.*, 1992; Rogers and Montville, 1994; Scott and Taylor, 1998a, 1998b) but these reports lacked the data regarding the possible emergence of nisin resistant strains. There are only few reports on the development of intrinsic resistance to other preservation factors. Our results clearly show that resistance to nisin, pediocin 34 and enterocin FH99 did not confer intrinsic resistance to low pH, sodium chloride, potassium sorbate, or sodium nitrite. In contrast, resistant variants were more or equally sensitive than wild-type strain. The resistance mechanism(s) of the nisin, pediocin 34 and enterocin FH99 resistant variants is specific to bacteriocin resistance and do not confer general resistance. Bacteriocins act on sensitive cells by a common mechanism that dissipates the chemical and energy gradient across the cytoplasmic membrane (Okereke and Montville, 1991; Montville and Bruno, 1994). Studies have shown that resistance to nisin-conferred cross-resistance to other antimicrobial peptides but did not confer intrinsic resistance to heat (Mazotta and Montville, 1997; Moll *et al.*, 1999) or the other preservative tested in this study, which act on the cell by different mechanisms.

Conclusion

In conclusion, results presented here indicate that nisin, pediocin 34 and enterocin FH99 in combination have a higher antibacterial action against *L. monocytogenes* ATCC 53135 *than* when used individually Also the results suggest that resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes that might lower the efficacy of bacteriocins when used in combinations. This study also demonstrates that the bacteriocin resistant *L. monocytogenes* variants tested did not become resistant to other preservation factors. The results showed that the nisin, pediocin 34 and enterocin resistant strains of *L. monocytogenes* ATCC 53135 were generally more preservative-sensitive, therefore, design of hurdle preservation systems containing bacteriocins can improve food safety without being undermined by resistance-related phenomena and also the synergy between different antimicrobial factors might allow the use of lower doses compared to their individual application. Since bacteriocins are considered as potential tools for biopreservation, more study is needed to determine the distribution of bacteriocin-resistance phenomena among food borne pathogens.

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