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 monocytogenes* 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 FH99 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, Pediocin 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...
Materials and Methods

Bacterial strains and culture conditions

Enterococcus faecium FH99, bacteriocinogenic strain was a isolate from human faeces (Gupta et al., 2010). Pediococcus pentosaceus 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 microlites 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 microliteres 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 FH99 alone and in different combinations (mean ± standard deviation, n = 3).

| Time   | Control | N | P | E | N-P | N-E | N-P-E | N-P+E |
|--------|---------|---|---|---|-----|-----|-------|-------|
| 1h     | 7.97±0.03 | 4.20±0.15 | 5.64±0.34 | 5.06±0.12 | 3.64±0.03 | 3.07±0.11 | 5.60±0.20 | 2.30±0.10 |
| 2h     | 6.07±0.09 | 4.20±0.31 | 5.06±0.44 | 6.20±0.01 | 4.22±0.04 | 2.60±0.06 | 5.90±0.03 | 3.30±0.33 |
| 4h     | 8.49±0.02 | 2.50±0.30 | 4.51±0.12 | 5.07±0.19 | 2.70±0.20 | 1.20±0.11 | 4.98±0.03 | 3.44±0.15 |
| 6h     | 8.46±0.05 | 6.73±0.18 | 7.29±0.74 | 8.40±0.14 | 5.76±0.21 | 5.20±0.02 | 8.33±0.04 | 4.60±0.02 |
| 24h    | 8.13±0.03 | 5.20±0.34 | 4.71±0.34 | 8.18±0.18 | 5.14±0.61 | 6.77±0.05 | 7.69±0.61 | 6.07±0.09 |

WT = Without Treatment. N = Nisin, P = Pediocin 34, E = Enterocin FH99.

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 nitrate to final concentrations of 20, 25, 30, 35, 40, 45, 50 and 55 μg/mL (control, no additional sodium nitrate) 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 = pediocin 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 ± 0.35 | 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 | 2.22 ± 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.
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.

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### 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  | 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.
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          | 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 ± 0.09|
|                       | 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 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 | 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 (mesenterocin 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 nitrates, 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-conferring 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 \textit{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 \textit{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 \textit{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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