# Characterization of Bacteriocins Produced by Lactobacillus brevis NM 24 and L.fermentum NM 332 Isolated from Green Olives in Iran

N Mojgani, G Sabiri, M Ashtiani, M Torshizi

**Citation**

N Mojgani, G Sabiri, M Ashtiani, M Torshizi. *Characterization of Bacteriocins Produced by Lactobacillus brevis NM 24 and L.fermentum NM 332 Isolated from Green Olives in Iran*. The Internet Journal of Microbiology. 2008 Volume 6 Number 2.

**Abstract**

The aim of the study was to isolate and characterize bacteriocin producing Lactic Acid Bacteria (LAB) from local olive samples and to exploit their potential as biopreservative. The two lactobacillus strains namely L.brevis NM 24 and L.fermentum NM 332, exhibiting wide spectrum of activity against closely related strain were selected and screened for their bacteriocin producing ability. Cell free supernatant fluid collected from both the isolates was sensitive to the action to proteolytic enzymes, and complete loss of their antagonistic activity was seen. Several gram positive and gram negative pathogens such as Bacillus subtilis, Enterococcus faecalis,
Escherichia coli, Listeria monocytogenes, and Staphylococcus aureus were inhibited by the inhibitory action of bacteriocins in study. The bacteriocinogenic potential in these strains appeared non-inducible and no increase in their titer was observed after exposure to different concentrations of Mitomycin C and UV light.The concentrated crude bacteriocin samples subjected to ammonium sulphate precipitation resulted in an increased activity and high protein yield. During ultrafiltration studies, bacteriocin NM 332 produced by L.fermentum NM 332, was able to pass through the cellulose membranes with 10 KDa and 30KDa molecular weight cut off indicating the presence of a small protein molecule. By non-denaturing gel a band of approximately 8 KDa for L.fermentum NM 332 and band corresponding to 37 KDa in L.brevis NM 24 was seen. Physico-chemical characterization of the partially purified bacteriocin samples indicated heat (121°C for 60 min) and acidic pH stability (pH 2-6) of bacteriocin NM 24. Exposure to surfactants resulted in a significant increase in the bacteriocin titer of both the strains, except for Tween 20 which resulted in complete loss of activity. None of the strains showed presence of plasmids indicating chromosomal associated genes responsible for bacteriocin production. The bactericidal activity displayed by the bacteriocins in study was maintained during storage at -20 °C for more than three years.

## INTRODUCTION

Lactic acid bacteria (LAB) are a group of bacteria that can preserve dairy foods by producing a number of organic compounds that are antagonistic to other microorganisms (Lindgren and Dobrogosz, 1990; Brink, 1994). Among these compounds proteinaceous-bacteriocins have gained much attention especially regarding their role in the diary foods where they are known to strongly inhibit the growth of pathogens (Ruiz-Barbara et al., 1994; Benkerroum et al., 2007). Much research has focused on utilizing bacteriocins as novel food preservatives, but there is also interest in using them for the control of bacterial diseases in humans and animals. The probiotic potential of these bacteria is also vastly investigated. (Gilliand, 1990; Cleveland et al., 2001; Mojgani et al., 2006; Diez-Gonzalez, 2007).

In recent years food safety has become an important international concern. Great attention is being drawn towards application of the bacteriocins from lactic acid bacteria (LAB). Much interest have developed in the bacteriocins isolated from LAB as most of the bacteria in this group are reported as GRAS (generally regarded as safe) microorganisms and their bacteriocins are considered innocuous due to proteolytic degradation in the gastrointestinal (GI) tract. Only few reports are available which indicate bacteriocin production in LAB isolated from olives (Todorov and Dicks, 2005), and hence it appeared interesting to screen bacteriocin producing potential of LAB strains isolated from green olives in Iran. In this attempt, two bacteriocin producing lactobacillus strains were isolated from local green olive samples and characterized for exploiting their potential as suitable candidate for future application as a safe and efficacious biological preservative.

## MATERIALS AND METHODS

### I. BACTERIAL STRAINS AND CULTURE MEDIA

A number of green olive samples procured from local market were analyzed for the presence of Lactic Acid Bacteria. Initially, the seeds were removed from the olives and then crushed, homogenized with kitchen blender, and inoculated in the deMan Ragosa and Sharpe (MRS) broth for enrichment of resident LAB. From appropriate 10-fold dilutions, isolation of bacteria was carried out on MRS agar by incubation anaerobically at 37°C for 48 h. The cultures were purified by repeated streaking. Strains were identified to genus level by gram staining (morphological characteristics) and catalase test. The potentially interesting isolates were later identified to species level by biochemical tests, carbohydrate fermentation pattern using the AP1 50CH strips (AP1 Systems, Biomerieux Sa, France).

All other bacteria used as indicator organisms for sensitivity tests were propagated in brain-heart-infusion broth (BHI, Difco) and trypticase soya broth (HiMedia, India) at 37°C for 24 hrs. For agar medium 1.6% w/v of granulated agar-agar was added to broth medium, while 0.7% semisolid medium was used. The isolate was maintained as frozen stock culture at -20°C in MRS broth with 5% glycerol and propagated twice before use in experiments.

### II. BACTERIOCIN SCREENING

All the isolated lactobacillus strains were screened for their antibacterial potential by the well diffusion and spot on lawn assay described earlier (Schillinger and Lucke, 1989; Harris et al., 1989; Aly and Abo-Amer, 2007). The two isolates identified as L.brevis NM 24 and L.fermentum NM 332, demonstrating maximum antibacterial activity were selected for further detailed investigations.

Preparation and Characterization of crude bacteriocin samples: Both the isolated strains were propagated in MRS broth (pH 7.0) for 48 h at 37°C anaerobically (Oxoid Gas Generating Kit). Cell-free solution from grown culture broth was obtained by centrifuging (10,000 rpm for 20 min at 4°C with Beckman L5050B) and adjusting the pH to 7.0 by means of 1M NaOH (fraction a). The involvement of hydrogen oxide for inhibitory action was ruled out by addition of 1 mg/ml catalase (C-100 bovine liver, Sigma) to the cell free supernatant fluid and studying the remaining activity (Daba et al., 1991). The supernatant was filtered through a 0.2 μm pore-size cellulose acetate filter and dialyzed for 24 h at 4°C (fraction b) and the remaining activity determined by the methods mentioned earlier.

To pertinacious nature of the antagonistic agent was evaluated by treating the crude bacteriocin samples to various enzymes. Enzymes (all obtained from Sigma) used were lipase, pronase E , pepsin, catalase, trypsin, lysozyme and proteinase K. 500 μl samples (fraction a) from both the isolates were incubated with 1 mg of each enzyme per ml at 37ºC except for samples containing trypsin and catalase, which were incubated at 25ºC. The remaining activity was determined after 1, 2, 4 and 12 hrs by well diffusion assay. Prior to being assayed for bacteriocin activity, preparations containing trypsin were treated with trypsin inhibitor (Sigma) according to the manufacturer's instructions (Wanda et al., 1991).

Determination of bacteriocin titer: The antibacterial titer of the bacteriocins in study was quantified by serial dilution assay (Mayr-Harting et al., 1972). Two fold serial dilutions of crude samples (fraction b) were made in saline solution and aliquots of 50 μl from each dilution were placed in wells in agar plates seeded with the indicator strain. A prediffusion at 4ºC for 2-4 hrs was allowed prior to incubation at 37ºC for 24hrs. The diameters of the inhibition zone were taken and recorded in millimeters (mm).

The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in arbitrary units per ml (AU ml-1)

Determination of bacteriocin activity and inhibitory spectrum: The antibacterial activity demonstrated by the two selected strains was studied against a number of gram positive and gram negative bacteria, according to well diffusion and spot-on lawn methods described earlier. The plates were incubated for 18-24 h, anaerobically at 37°C for lactobacillus indicators and aerobically at 37°C for non-lactic acid bacteria indicators. All plates were examined for the presence of clear zones of inhibition around the wells.

### III. PARTIAL PURIFICATION AND MOLECULAR SIZE ESTIMATION OF BACTERIOCIN SAMPLES

The filtered supernatant fluid (fraction b) collected from both producer strains was concentrated to one tenth of its original volume by PEG and Vacuum evaporation, followed by precipitation assays.

Poly ethylene glycol (PEG) 6000: The volume of the neutralized and filtered supernatant fluid (fraction b) was

reduced from 50ml to 5 ml by PEG (6000) dialysis. The mixture was dialyzed against 20% PEG in distilled water, in a cellulose membrane of 10-12 KDa cut-off (fraction c1).

Vacuum Evaporation: Fraction b from both the strains was frozen at -70°C in a chilling unit and later freeze dried till foam like material was achieved. The activity was assayed after dissolving the vacuum dried in 10mM sodium phosphate buffer pH 7.0 (fraction c2).

Ammonium Sulphate Precipitation: The bacteriocin samples obtained from previous step (fraction c2) were treated with solid ammonium sulphate (Mallinckrodth Chemical, Inc., Paris, KY, USA) to 0, 20, 40 and 60% saturation. The mixtures was stirred for 2 h at 4ºC and later centrifuged at 20,000 rpm for 1 h (4ºC). The precipitates were re-suspended in 25 ml of 0.05 M potassium phosphate buffer (pH 7.0) and passed through Sephadex G-25 (coarse) column (25 cm; Bio Rad) for desalting. The remaining activity was checked in both the precipitate and supernatant to determine which fraction contains the bacteriocin (Fraction d). The titer in AU/ml of fraction d was determined as described earlier. The protein concentration at each step was estimated with 1% BSA as standard.

Estimation of the size of the bacteriocin molecule: The apparent molecular weight of the bacteriocins in subject was determined by ultrafilteration studies. A 2ml sample from each strain (fraction a) was ultrafiltered through cellulose membranes with 5, 10 and 30 KDa exclusion units (Centricon, Micro concentrations, USA). Assay of the bacteriocin activity was carried out in both the precipitate and supernatant to determine which fraction contains the bacteriocin.

SDS-PAGE Analysis: The partially purified fractions (fraction d) were analyzed by SDS-PAGE as described by Laemmli (1970). The gel was run at constant current 20-25 mA until the tracking dye (bromophenol blue) had migrated to the end.

Non-Denaturing Gel: The non-denaturing gel (10 and 15%) of the native protein was performed by the method described by Bhunia and his colleagues (1991). After electrophoresis the gel was divided into two parts, one part was stained and the other part was overlaid with semisolid agar embedded with indicator strain and incubated at 37 C for 24 hrs. The protein responsible for activity was located by the appearance of inhibition zone around the respected band.

## IV. PHYSICO-CHEMICAL CHARACTERIZATION OF PARTIALLY PURIFIED BACTERIOCIN SAMPLES

The partially purified bacteriocin samples (fraction d) were characterized with respect to thermal and pH stability, susceptibility to salt and surfactants, stability during storage, treatment with dissociating agents and mitomycin C and UV light induction.

Heat Resistance: 400 µl of fraction d was exposed to various heat treatments: 60, 80, 100 and 121ºC. Samples were collected after every 10 min for a maximum of 3 hrs and the remaining titer determined as described previously.

pH Sensitivity: 400 µl of fraction d was adjusted to pH 2, 4, 6, 8, 10, and 12 with 4M hydrochloric acid (HCl) and or 3M sodium hydroxide (NaOH), incubated for 4 h at 37ºC and similarly assayed.

Salt Tolerance: The purified bacteriocin fractions (400 µl of fraction d) were incubated at 37ºC with varying concentrations of NaCl (10-40%) (Merck, Germany). The activity was assessed after different time intervals by well diffusion assay.

Effect of surfactant on bacteriocin activity: This study was carried out by incorporating non-ionic (triton X100, tween 20, tween 80) and anionic (sodium dodecyl sulphate, deoxycholic acid) surfactants. The surfactants were obtained from Sigma Chemical Co. and were added to purified bacteriocin fractions (d) at a final concentration of 1%. The preparations were incubated at 37ºC and assayed for bacteriocin activity against indicator organisms after 4, 6, 12 and 24 hrs.

Induction of Bacteriocin: Effect of Mitomycin C and UV light was studied on the bacteriocinogenic potential of the strains. Mitomycin C was added at a final concentration of 0.5, 1.0 and 2.0 µg/ml to freshly grown culture broth of the producer strains. Incubation was carried out at 30ºC, and samples were analyzed for bacteriocin activity at 30 and 60 min of time interval.

To study the effect of UV light, a 10 ml aliquot of cultured broth was placed in a sterile petri dish and exposed to short – wave UV light (254nm) from a Electric germicidal bulb at a distance of 20 cm. Times of exposure ranged from 0 to 2 min. (Wanda and Bonita, 1991). After each time interval, bacteriocin activity was analyzed.

Plasmid Isolation: Trials were made to isolate Plasmid DNA from both the producer strain by following the method described earlier Burger and Dicks, (1994).

## V. STABILITY OF BACTERIOCIN DURING STORAGE

Purified bacteriocin fractions (d) from both lactobacillus strains was stored at three different temperatures (–20, 4 and 37ºC). After every month of interval the bacteriocin activity was determined by previously described method, till the activity existed.

## RESULTS

Initially, two lactobacillus strains demonstrating maximum inhibitory action against other closely related strains, was isolated from green olive samples. The isolates were later identified to species level and studied in detail for their bacteriocin producing ability. The two isolates identified as L. brevis NM 24 and L. fermentum 332 (data not shown) were able to inhibit the growth of a number of bacteria against which tested. The cell free culture supernatant fluid of both the strains was subjected to different treatments. In contrast to the action of catalase and lysozyme, the antibacterial activity demonstrated by both the strains in study was lost after treatment with proteolytic enzymes (Table- 1). Lipase treatment decreased the antibacterial activity in L.brevis NM 24, while had no effect on the bacteriocin of L.fermentum NM 332.

### Figure 1

Table 1. Effect of enzymes on the activity (AU/ml) of bacteriocin produced by the isolated strains

| Treatments | *L. brevis* NM 24 | *L. fermentum* NM 332 |
|---|---|---|
| Control | 6400 | 12800 |
| Lipase | 3200 | 12800 |
| Pronase E | 0 | 0 |
| Pepsin | 0 | 0 |
| Catalase | 6400 | 12800 |
| Trypsin | 0 | 0 |
| Lysozyme | 6400 | 12800 |
| Proteinase K | 0 | 0 |

Table. 2 depicts the antagonistic effect of the bacteriocins on the growth of other Gram-positive and Gram-negative bacteria used as indicator. The bacteriocins in study were able to inhibit the growth of B. subtilis, E. coli, E.faecalis L.monocytogenes, and S. aureus while Candida albicans and Pseudomonas aeruginosa were resistant to their action. The spectrum of inhibition by L. fermentum NM 332 was significantly wider than L.brevis NM 24, as it further inhibited the growth of L innocua and S.typhimurium. Both the producer strains were immune to the inhibitory effect of their own bacteriocin as no inhibition was observed when tested against themselves.

A significant increase in yield and purification fold of the bacteriocin in study was observed during different purification stages (Table- 3). The crude supernatant fluid of L. brevis NM 24 and L. fermentum NM 332 were concentrated before subjecting them to four rounds (0-20, 20-40, 40-60 and 60-80%) of ammonium sulphate precipitations. All the activity was recovered in the pellet at 80% saturation. Finally, these fractions were subjected to ultra filtration using various filtron membranes. The eluted and retained fractions were collected and assayed for bacteriocin activity. At this stage, when filtered through 5, 10 and 30 KDa molecular weight cut off, L.fermentum NM 332 bacteriocin was able to pass through 10 and 30 KDa cellulose membranes, as antagonistic activity was observed in the filtrate. Filtration was not achieved for L.brevis NM 24 bacteriocin under these conditions, and the activity was recovered only in the retentate fraction.

### Figure 2

Table 2. Inhibition of various indicator organisms by bacteriocin produced by the selected strains

| Indicator organisms | Strain No | *L. brevis* NM 24 | *L. fermentum* NM 332 |
|---|---|---|---|
| *Bacillus subtilis* | ATCC 25293 | +(5mm) | +(8mm) |
| *Escherichia coli* | ATCC 8739 | +(5mm) | +(5mm) |
| *Enterococcus faecalis* | Local isolate | + (12mm) | + (14mm) |
| *Candida albicans* | ATCC 10231 | - | - |
| *Listeria monocytogenes* | ATCC 7973 | +(8mm) | +(14mm) |
| *Listeria monocytogenes* | ATCC 5214 | + (4mm) | +(12mm) |
| *Listeria innocua* | Local isolate | + (4mm) | - |
| *Lactobacillus acidophilus* | DSMZ 20079 | - | +(5mm) |
| *Lactobacillus brevis* | DSMZ 20054 | - | +(7mm) |
| *Lactobacillus fermentum* | DSMZ 20174 | +(6mm) | - |
| *Lactobacillus reuteri* | DSMZ 20016 | +(5mm) | (8mm) |
| *Lactobacillus delbrueckii* | DSMZ 20081 | +(10mm) | +(12mm) |
| *Lactobacillus helveticus* | DSMZ 20075 | - | - |
| *Lactobacillus fermentum* | DSMZ 20052 | +(14mm) | +(14mm) |
| *Pseudomonas aeruogenosa* | ATCC 9027 | - | - |
| *Salmonella typhimurium* | ATCC 13311 | + ( 8mm) | - |
| *Staphylococcus aureus* | ATCC 6538 | +( 6mm) | +( 6mm) |
| *Streptococcus pyogenes* | ATCC 194 | - | - |

**Figure 3**

Table 3: Recovery of Bacteriocins produced by test isolates during partial purification

| Bacteriocin Producing strain | Purification stages | Volume (ml) | Total Activity AU | Total Protein mg | Specific Activity (AU/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| *L.brevis* NM 24 | a.Culture supernatant | 1000 | 640000 | 21600 | 30 | 100 | 1 |
| | b. Conc. Supernatant | 100 | 286000 | 1142 | 250 | 44 | 171 |
| | c. 80 % Ammonium sulphate ppt | 7 | 15360 | 30 | 5120 | 2.4 | 176 |
| *L.fermentum* NM 332 | a.Culture supernatant | 1000 | 1280000 | 38350 | 33 | 100 | 1 |
| | b. Conc. Supernatant | 100 | 1024000 | 1235 | 829 | 80 | 25 |
| | c. 80 % Ammonium sulphate ppt | 10 | 100000 | 24 | 4149 | 8 | 126 |

Specific Activity (AU/mg) = Total activity of the subsequent purification step/ Total protein of the same step Yield (%) =Total activity of the subsequent purification step/ Total activity in the crude culture supernatant Purification (fold) = Specific activity of the subsequent purification step/ specific activity of the crude culture supernatant

Attempts to size the bacteriocin under denaturing conditions were obscured due to diffuse banding. However under non-denaturing conditions the exact location of the protein giving activity was detected. The bacteriocin NM 332 was resolved as a single band of approximately 8 KDa while that of NM 24 appeared to be 37 KDa. These bands showed zone of clearance when overlaid with the indicator lawn and thus were confirmed to be bacteriocin related.

The effects of heat, pH, organic solvents, salt, and surfactants on bacteriocin activity were determined. The bacteriocin produced by L. brevis NM 24 was considered to be heat stable as it retained its activity (6400 AU/ml) after heating at 121ºC for 60 min compared to the bacteriocin produced by L. fermentum NM 332 which completely lost its activity at the same temperature within 5min only (Table-4). However, the pH stability (Table- 5) and salt tolerance of bacteriocin NM 332 appeared greater than that of NM 24. The mentioned bacteriocin was stable at pH 2 to 10 and in salt concentrations of 10-40%, compared to the bacteriocin produced by L. brevis NM 24 which was stable between pH 2 to 6 and resisted 10% salt, only.

**Figure 4**

Table 4: Effect of heat on the bacteriocin titers of the selected strains

| Heat Treatment | *L. brevis* NM 24 | *L. fermentum* NM 332 |
|---|---|---|
| 60 °C for 150 min | 6400 | 12800 |
| 80 °C for 120min | 6400 | 1600 |
| 100 °C for 40min | 6400 | 0 |
| 121 °C for 60 min | 6400 | 0 |

**Figure 5**

Table 5. Effect of pH treatment on bacteriocin activity (AU/ml) produced by the selected strains during 4 hrs of incubation

| pH | *L. brevis* NM 24 | *L. fermentum* NM 332 |
|---|---|---|
| 2 | 1200 | 6400 |
| 4 | 6400 | 6400 |
| 6 | 6400 | 6400 |
| 8 | 400 | 3200 |
| 10 | 400 | 3200 |
| 12 | 400 | 3200 |

Table- 6 shows the effect of dissociating agents on bacteriocin activity. Exposure to most of surfactants tested resulted in an increase in the bacteriocin titer (by at least one to two fold dilutions). However, Tween 20 had adverse effect on these bacteriocins and their activity was completely demolished after subjection to this surfactant within only 2 hrs of incubation.

**Figure 6**

Table 6. Effect of surfactants on activity of bacteriocin (AU/ml) produced by the selected strains.

| Surfactants | *L. brevis* NM 24 | *L. fermentum NM 332* |
|---|---|---|
| Control | 6400 | 12800 |
| EDTA | 25600 | 51200 |
| Sodium dodeyl sulphate | 12800 | 12800 |
| Tween 20 | 0 | 0 |
| Tween 80 | 12800 | 51200 |
| Tritox X – 100 | 12800 | 25600 |
| Deoxycholic acid | 6400 | 12800 |

Both the physical and chemical inducing agents adopted for induction of the bacteriocinogenic strains, failed to induce the activity and no increase in titer was seen when the

producer cells were subjected to Mitomycin C or UV light. Moreover, the possibility of plasmid encoded bacteriocin production in both the isolates was ruled out as no plasmid was observed in these strains.

Effect of storage on bacteriocin activity indicated full stability of the bacteriocins in study at –20ºC during three years of storage; partial stability for 120 days at 4ºC, while no activity was detected after storage for 30 days at 37ºC.

## DISCUSSION

In the last few decades, tremendous interest has swelled in the potential use of bacteriocins from Lactic Acid Bacteria (LAB). The bacteriocins produced by this group of bacteria are considered potent bio-preservative agents and their application in food is currently the subject of extensive research.

The present investigation highlights the isolation and characterization of bacteriocin producing LAB strains from Iranian green olives. To date, only few bacteriocin producing LAB has been reported in olives (Frantz et al., 1996; Lean et al., 1998; Todorov and Dicks, 2005). We here report the isolation and characterization of two wide spectrum bacteriocin producing LAB strains namely, L.brevis NM 24 and L.fermentum NM 332 isolated for the first time from local green olives in Iran.

According to Fricourt and his co-workers, lactic acid bacteria synthesize bactericidal agents that vary in their spectra of activity (Fricourt et al., 1994). Many of these agents are bacteriocins with a proteinaceous active moiety, while others are non-protein agents (Piard and Desmazeaud, 1991; 1992; Atrih et al., 1993; Lash et al., 1995). During our investigations we recorded the proteinaceous status of the bacteriocins in study, and the antagonistic activity demonstrated by these strains was completely lost when exposed to proteases. The antimicrobial activity of the bacteriocins appeared unrelated to hydrogen peroxide or acidity as their activity was not lost after treatment with catalase or adjustment of pH to 7.0. However, the activity of bacteriocin NM 332 was affected by lipase treatment which might indicates its linkage to a non-protein moiety such as lipids.

The bacteriocin produced by L. fermentum NM 332 exhibited a wider spectrum of inhibition compared to the bacteriocin produced by L.brevis NM 24, as it further inhibited the growth of L.innocua and S.aureus. The potential of these bacteriocins to inhibit the food pathogens such as E.coli, S.aureus, S.typhi, Listeria and bacillus spp makes it of crucial interest especially in processed foods where there is risk of food pathogens. Due to the phenomenon of immunity the bacteriocin from the producer organism were resistant to the organism producing it.

During the purification procedures, each step resulted in considerable loss of protein concentration while specific activity increased. At 80% saturation with ammonium sulphate highest increase in activity was observed. This agreed with the findings of Ivanova et al. (2000). The Increase in activity could be due to release of active monomers from bacteriocin complexes. During salt precipitation various amount of the protein was fractionated as a surface pellicle, this might be due to the association of bacteriocin molecules with the hydrophobic globular micelle like structure in the supernatant fluid. Similar observations have also been recorded for lactocin S and lactacin F (Muriana and Klaenhammer, 1991).

During ultrafiltration experiments, both the bacteriocins were unable to pass through 5 KDa molecular weight cut-off membranes. A tendency to aggregate with other proteins has been reported in bacteriocins produced by other lactic acid bacteria (Bhunia et al., 1991; Toba et al., 1991), and might have contributed to the reason why the bacteriocins could not pass through the membrane with low molecular weight cut-off. While, slight activity of bacteriocin NM 24 recovered in the filtrate of 30 KDa units, could be attributed to the monomeric form of this bacteriocin. The bounded dimeric form of this bacteriocin was present in the retentate of the respective exclusion units. Moreover, as reported by other researchers the possible involvement of two peptides could not be ruled out (Yang et al., 1992). We could not confirm the presence of two peptides, as single bands giving activity were observed for both the bacteriocins during 15% non-denaturing gel experiments. However, the possibility of smaller proteins not being resolved on this percentage of gel exists. A considerable loss of the bacteriocin activity was observed during ultrafiltration which might be due to absorption of the bacteriocin on the membrane.

The phenomenon of heat stability of LAB bacteriocins have been reported earlier for plantaricin A (Daeschel et al., 1990), plantaricin C19 (Audisio, 1999), plantaricin S (Jimenez-Diaz et al., 1990), plantaricin 149 (Kato et al., 1994), plantaricin SA6 (Ralph et al., 1995), plantaricin 423 (Van-Reenen, 1998), pentocin TV35b (Okkers et al., 1999),

lactocin RN 78 (Mojgani and Amirinia, 2007) and a bacteriocin produced by L.brevis 0G1 (Ogunbanwo et al., 2003). Our findings are also in agreement with the above mentioned reports as we observed heat stability of L. brevis NM 24 bacteriocin. The retention of activity by this bacteriocin after heating at 121ºC for 60 min, place it within heat stable low molecular weight group of bacteriocins. This quality of the bacteriocin makes it superior in processed food stuffs where high heat is applied.

Like most LAB bacteriocins reported to date (Daeschel et al., 1990; Lade et al., 2006), the activity of the bacteriocin in study appeared pH dependent. The bacteriocin NM 24 exhibited highest activity in acidic pH range of 2 to 6, while almost lost its activity in alkaline pH range. Similar phenomenon of acid stability has also been demonstrated previously in plantaricin, bulgarican, and lactobulgarican (Reddy et al., 1994; Lade et al., 2006). In contradiction to the bacteriocin NM 24 and similar to lactocin RN 78 (Mojgani and Amirinia, 2007), the bacteriocin produced by L.fermentum 332 was stable at alkaline pH values and resisted high salt concentrations which makes them an attractive applicant in food supplies.

Exposure of the bacteriocin samples to surfactants resulted in an increase in the bacteriocin titers. This increase might be due to the effect of surfactant on the permeability of the cell membrane (Graciela et al., 1995). It has also been suggested that the dispersion of the bacteriocin complex into active subunits ultimately results in more lethal hits and consequently enhanced activity is witnessed (Muriana and Klaenhammer, 1991).

The high stability of the bacteriocins in study during prolong storage makes them superior to a number of other reported natural and synthetic bacteriocins. Both the bacteriocins remained fully stable after storage for three years at -20°C, but became non-detectable within 30 days of storage at 37°C, indicating that cold temperature may be the most appropriate preservation technique.

## CONCLUSION

The peculiar antimicrobial characteristics and technological properties and especially heat and storage stability of L.brevis NM 24 and L.fermentum NM 332, can positively have impact on their use as biopreservative, with a view to improving the hygiene and safety of the food products especially processed foods. However, more efforts are required to analyze the amino acid sequence of these bacteriocins which could lead us a step ahead in future utility of these bacteriocins for health benefits.

## References

1. Atrih, A., Rekhif N., Milliere, J.B. and Lefebvre, G.1993. Detection and characterization of a bacteriocin produced by Lactobacillus plantarum C19, Canadian J Microbial 39: 1173–1179.
2. Audisio, M.C., Oliver, G. and Paella, M.C.1999. Antagonistic effect of E.faecium J96 against human and poultry pathogenic Salmonellae species. J. food Prot 62: 751-755.
3. Aly, E. and Abo-Amer.2007. Characterization of a bacteriocin like inhibitory substance produced by L.plnatarum isolated from Egyptian Home made yogurt. Science Asia 33: 313-319.
4. Benkerroum, N., Ghouati, Y. and Ghalfi, H. 2007. Screening for bacteriocin producing LAB from various Moraccan food products and partial characterization of putative bacteriocins. Biotechnol. 4:481-488.
5. Bhunia, A.K., Johnson, M.C., Ray, B. and Kalchayanand, N. 1991. Mode of action of pediocin AcH from P.acidilactis H on sensitive bacterial strains. J. Appl. Bacteriol. 70: 25-33.
6. Brink ten, B., Minekns, M., Vander Vossen, J.M.B.M., Leer, R.J. and Huis in't Veld, J.H.J. 1994. Antimcrobial activity of lactobacilli. J. Appl. Bacteriol. 77: 140 – 148.
7. Burger, J.H. and Dicks, L.M.T. 1994. Technique for isolating plasmids from exopolysaccharide producing Lactobacillus spp. Biotechnol. Tech. 8:769–772.
8. Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L. 2001.Bacteriocins: safe, natural antimicrobial for food preservation. Int. J. Food Microbiol. 71:1-20.
9. Daba, H., Pandian, S., Gosselin, J.F., Simard, R.E., Huang, J. and Lacroix, C. 1991. Detection and activity of bacteriocin produced by Leuconostoc mesenteriodes. Appl. Environ. Microbiol. 57: 3450-3455.
10. Daeschel, M. A., McKeney, M. C. and McDonald, L. C. 1990. Bacteriocidal activity of L. plantarum C-11. Food Microbiol 7: 91–98.
11. Diez-Gonzalez, F. 2007. Applications of bacteriocins in livestock. Curr Issues Intestinal Microbiol 8, 15–24.
12. Frantz, C. M. A. P., Schillinger, U. and Holzapfel, W. H. 1996. Production and characterization of enterocin 900, a bacteriocin produced by Enterococcus faecium BFE 900 from black olives. Int. J. Food Microbiol. , 29, 255-270.
13. Fricourt, B.V., Barefoot, S.F., Testin, R.F. and Hayasaka, S.S. 1994. Detection and activity of plantaricin F, an antibacterial substance from L. plantarum BF001 isolated from processed channel catfish. J. Food Prot. 57:698-702.
14. Gilliand, S.E. 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. 87: 175-178.
15. Graciela, M., Vignolo, M., de Kairuz, M., Aida, A.P., de Ruiz, H. and Oilver, G. 1995. Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by L. casei CRL 705. J. Appl. Bacteriol. 78: 5 – 10.
16. Harris, L.J., Daeschel, Stiles M.E. and klaenhammer, T. R. 1989. Antimicrobial activity of lactic acid bacteria against L.monocytogenes. J. Food Prot 52: 384-387.
17. Holo, H., Nilssen, O. and Nes, I.F. 1991. Lactococcin A, a new bacteriocin from Lactococcus lactis subsp. cremoris: isolation and characterization of the protein and its gene. J. Bacteriol. 173: 3879-3887.
18. Invanova, I., Kabadjova, P., Panter, A., Danova, S. and Dousset, X. 2000. Detection, purification and partial characterization of a novel, Bacteriocin subsp. Lactis B14 isolated from Boza-Bulgarian Traditional cereal Beverage:

Biocatalysis, Fundamentals and Applications 6: 47-53.
19. Jimenez-Diaz, R., Piard, J. C., Ruiz-Barba, J. L. and Desmazeaud, M. J. 1990. Isolation of a bacteriocin-producing L.plantarum strain from green olive fermentation. Third symposium on lactic acid bacteria. FEMS Microbiol. Rev., 87, 91.
20. Kato, T., Matsuda, T., Ogawa, E., Ogawa, H., Kato, H., Doi, U. and Nakamura, R. 1994. Plantaricin-149, a bacteriocin produced by L.plantarum NRIC 149. J. Ferment. Bioeng 77, 277–282.
21. Klaenhammer, T.R.1988. Bacteriocins of lactic acid bacteria. Biochime 70:337-349.
22. Lade, H. S., Chitanand, M. P., Gyananath, G. and Kadam, T. A. 2006. Studies on some properties of bacteriocins produced by Lactobacillus species isolated from agro-based waste. The Internet J. Microbiol., 2(1).
23. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227: 680–685.
24. Lash, B.W., Mysliwiec, T.H. and Gourama, H. 2005. Detection and partial characterization of a broad-range bacteriocin produced by L. plantarum. J Appl Microbiol 99: 77-84.
25. Lean, M. V., Baras, M., Ruiz-Barba, J. L., Floriano, B. and Jiménez-Díaz, R. 1998. Bacteriocin production and competitiveess of L.plantarum LPCO10 in olive juice broth, a culture medium obtained from olive. Int. J. Food Microbiol. , 43: 129-134.
26. Lindgren, S.W. and Dobrogosz, W.J. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87: 149-164.
27. Mayr-Harting, A., Hedges, A.J. and Berkeley, R.C.W. 1972. Methods for studying bacteriocins. Methods Microbiol. 7A : 315–422 .
28. Mojgani, N. and Ashtiani, M.P. 2006. In vitro inhibition of mastitis pathogens by bacteriocin RN 86 produced by an indigenous strain of L.casei RN 86. J Appl Sciences 6: 2629-2634.
29. Mojgani, N. and Amirinia, C. 2007. Kinetics of growth and bacteriocin production in L.casei RN 78 isolated from a dairy sample in Iran. Int J Dairy Sci.2: 1-12.
30. Muriana, P.M. and Klaenhammer, T.R. 1991. Purification and partial characterization of lactacin F, a bacteriocin produced by L.acidophilus 11088. Appl Environ Microbiol. 57:114–121.
31. Nissen-Meyer, J., Holo, H., Håvarstein, L.S., Sletten, K., Nes, I.F. 1992. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J. Bacteriol. 174:5686-5692.
32. Ogunbanwo, S., Sanni, A and Onilude A. 2003. Influence of cultural conditions on the production of bacteriocin by Lactobacillus brevis OG1. African Journal of Biotechnology 7:179-184.
33. Okkers, D.J., Dicks, L.M.T., Silvester, M., Joubert, J.J. and Odendaal, H.J. 1999. Characterization of pediocin TV35b, a bacteriocin-like peptide isolated from L pentosus with a fungistatic effect on C. albicans, J App Microbiol 87: 726–734.
34. Piard, J.C and Desmazeaud, M. 1991. Inhibiting factors produced by LAB part L. oxygen metabolites and catabolism end-products. Lait 71: 525-541.
35. Piard, J.C. and Desmazeaud, M. 1992. Inhibiting factors produced by lactic acid bacteria. Part Z. bacteriocins and other antibacterial substances. Lait 72: 113-142.
36. Ralph, W.J., Tagg, J.R. and Ray, B. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. 59: 249–252.
37. Reddy, G.C., Shahani, K.M., Friend, B.A. and Chandan, R.C. 1984. Natural antibiotic activity of L.acidophilus and bulgaricus, production and partial purification of Bulgaricus cultured. J. Dairy Products 8: 15 – 19.
38. Ruiz-Barbara, J.L., Cathcart, D.P., Warner, P.J. and Jimmenez-Diaz, R. 1994. Use of L.plantarum LPCO10, a bacteriocin producer as a starter culture in Spanish style green olive fermentations. Appl Environ Microbiol 2059-2064.
39. Schillinger, U. and Lucke, F.K. 1989. Antimicrobial activity of L.sake isolated from meat. Appl. Environ. Microbiol. 55:1901-1906.
40. Takahiro, T., Emiko, Y. and Takatoshi, I. 1991. Lacticin, a bacteriocin produced by L.delbrueckii sub sp. lactis. Lett. Appl. Microbiol. 12: 43-45.
41. Toba, T., Samant, S.K., Yoshioka, E. and Itoh, T. 1991. Reutericin 6, a new bacteriocin produced by L. reuteri LA6. Lett. Appl. Microbiol. 13:281-286.
42. Todorov, S. and Dicks, L.M.T. 2005. Optimiztion of bacteriocin ST311 production by E.faecium, isolated from spoiled black olives. J Microbiol 370-374.
43. Van-Reenen, C.A., Dicks, L.M.T. and Chikindas, L.M. 1998. Isolation 423, a bacteriocin produced by L.plantarum, J Appl Bacteriol 84:1131–1137.
44. Wanda, J. L. and Bonita Glatz, A. 1991. Partial Purification and Characterization of a Bacteriocin Produced by Propionibacterium thoenii. Appl Environ Microbiol.3: 701-706.
45. Yang, R., Johnson, M.C. and Ray, B. 1992. Novel method to extract large amounts of bacteriocin from LAB. Appl. Environ. Micrbiol. 58: 3355-3359.

## Author Information

**Naheed Mojgani, M.Sc., Ph.D**
Biotechnology Department, Razi vaccine and serum research Institute

**Ghulamhossein Sabiri, MD**
Health Clinic, Razi vaccine and serum research Institute

**Mehdi P. Ashtiani, DVM., Ph.D**
Biotechnology Department, Razi vaccine and serum research Institute

**Mohd A K Torshizi, M.Sc., Ph.D**
Department of Poultry Science, College of Agriculture, Tarbiat Modares University