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*Research Article*

# Efficient Inactivation of Multi-Antibiotics Resistant Nosocomial Enterococci by Purified Hiracin Bacteriocin

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**Abstract**

***Purpose:*** Because of the emergence of multi-antibiotic resistant bacteria, a number of infectious diseases have become a major concern to treat in health care services worldwide. This situation is worsened by the fact that very limited progress has been made in developing new and potent antibiotics in recent years. In this context antimicrobial peptides (AMPs) represent new potential therapeutic compounds with bactericidal or bacteriostatic activity against closely related bacterial strains.

***Methods:*** In this study, a collection of enterococci (n=170) from clinical sources were investigated for their potential to inhibit multiresistant nosocomial enterococci from Iranian hospitals.

***Results:*** Four isolates produced antimicrobial peptides that inhibited all the antibiotic resistant enterococci. This included three *Enterococcus faecium* isolates producing combinations of enterocin A, B and L50 AB. The most potent antagonism was produced by *E. faecalis* HO91. Purification and subsequent characterization by MALDI-TOF MS, Edman degradation and DNA-sequencing revealed that the antimicrobial compound was Hiracin. The purified Hiracin was evaluated for antibacterial activity against 12 multiresistant enterococcal isolates from clinical samples. The results demonstrated that Hiracin is highly effective towards enterococci which were resistant even to antibiotics from four distinct classes.

***Conclusion:*** The present research addresses Hiracin as a promising alternative to conventional antibiotics in treatment of multiresistant enterococcal infections.

## Introduction

The occurrence of multi-antibiotic resistant bacteria has become a serious clinical obstacle worldwide. Therefore, there is a need to much more research to develop new antimicrobial agents.[1,2] Bacteriocins which are produced by bacteria are a group of bactericidal compounds of peptide entity.[3] The widespread occurrence of bacterial species with bacteriocin production in microbial ecosystems such as the intestinal tract and epithelial surfaces has renewed interests in their bactericidal activities in recent years.[4-6]

Lactic acid bacteria (LAB) are perhaps the most bioprospected bacteriocin producers.[7-9] The LAB are gram-positive, facultative anaerobic, catalase-negative, non-spore-forming and non-motile cocci. Lactic acid-producing bacteria from the order *Lactobacilliales* containes several genera, including *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus.* These genera are of particular interest in terms of the widespread occurrence of them throughout the fermented meat, fruits, dairy products, and beverages, intestinal and genital tracts of both humans and animals. Most bacteriocins produced by LAB are exhibit activity only against LAB and other Gram-positive bacteria genetically closely related to the producer strain.[10] LAB has been widely studied for production of antimicrobial peptides. These contain several well-characterized bacteriocins.[7,11-13] Four classes of bacteriocins have been defined based on their molecular mass, structure, and thermo stability.[3] Class I bacteriocins includes lantibiotics, which are small post-translational modified peptides of 5 kDa characterizing by the presence of unusual amino acids lanthionine and methyl lanthionine. Nisin is a classic example of class I bacteriocins. Class II bacteriocins include a large and heterogeneous group of heat stable non-lanthionine-containing peptides of <10 kDa. Class III bacteriocins are large heat-labile proteins of >30 kDa. Class IV bacteriocins include complex glycoproteins and lipoproteins.[3]

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Bacteriocinogenic LAB and bacteriocins are now being considered for a variety of antimicrobial uses as therapeutic agents, food additives (preservatives) and starter cultures to control food borne pathogenic microorganisms.[1,14] This report describes the purification and characterization of a peptide from *E. faecalis* with antibacterial activity against clinically isolated multi-antibiotic resistant enterococci.

## Materials and Methods

### *Bacterial isolation and identification*

Blood samples obtained as part of regular diagnosis and treatment procedures of hospitalized patients from different hospitals of Orumiyeh were referred to our laboratory in school of pharmacy, Tabriz University of medical sciences (TUMS). Identification of enterococci was performed according to Manual of Clinical Microbiology.[15] Samples were suspended and incubated in Bile Esculin broth for 24 h at 37 ºC. In the next step, 20 µL of culture were surface plated on Bile Esculin sodium azide agar. The plates were then incubated at 37°C for 16 to 18 h and single black colonies were saved in 30% glycerol at -80 ºC as stocks.

### *Screening for bacteriocin production*

Isolated *Enterococcus* strains were evaluated for antagonistic activity against different indicator strains as S*taphylococcus aureus, Listeria monocytogenes*, *Li. innocua, Lactobacillus sakei, E. faecalis, E. faecium* and *E. hirae* which were obtained from PTCC (Persian Type Culture Collection) and LMGT (Norwegian University of Life Sciences) collections. The antagonistic activity of the bacteriocin was evaluated by a spot agar method of Zheng and Slavik.[16] Briefly, a single colony of each *Enterococcus* spp. strains was spotted onto a previously seeded agar plate. Plates were then incubated for 24 to 48 h at 30 °C. The antibacterial activity of the isolates was evaluated by measuring the diameters of visible zones of inhibition against indicator strains.

The amount of antimicrobial activity of bacteriocins was quantified with microtiter plate assay.[17,18] First, 100 µl media broth was added to each well in the microtiter plate before 100 µl of a each fraction to be tested for antimicrobial activity was added to the wells of column 1. In the next step, two-fold serial dilutions were made in each column. Subsequently, 100 µl of 20x diluted overnight indicator culture was added to each well. Lastly, the microtiter plate was incubated under conditions appropriate for the relevant indicator, and amount of growth was measured by reading optical density at 620 nm in a microtiter plate reader. One bacteriocin unit (BU) was defined as the reciprocal of the highest dilution exhibiting growth inhibition of indicator strain by 50% under the assay conditions (200-µl culture volume).

### *Genotypic differentiation of bacteriocin producing (Bac$^+$) isolates*

Total DNA was prepared by the method described previously[19] and stored as PCR template in the genotypic identification method at -20 °C. PCR amplifications was performed on a thermal cycler (Eppendorf AG, Hamburg, Germany) in a final volume of 25 µL containing 250 ng of DNA as template; 1 µL of each oligodeoxynucleotide primer set; 2 µL of 10 × PCR buffer (Cinnagen), 200 µmol (each) dATP, dCTP, dGTP, and dTTP (Cinnagen); 2.5 mmol $MgCl_2$; and 1 U of Taq DNA polymerase (Promega). The cycles used were 95 °C for 4 min for the first cycle; 95 °C for 45 s, 30 s at 54 °C (for *Enterococcus*-specific primers),[20] 56 °C (for species-specific primers)[21] and 72 °C for 1 min for the next 35 cycles; and 72 °C for 10 min for the last cycle. The three of PCR primers used are listed in Table 1. PCR products were resolved by electrophoresis on a 1.5% agarose gel electrophoresis (Hoefer scientific instruments).

### *PCR detection of bacteriocin structural genes*

PCR test was performed to investigate whether structural genes of well-known enterocins among enterococci were present in the bacteriocinogenic isolates. The primers used in this study are listed in Table 1. Plasmid or Total DNA was extracted as described before.[19] All the PCR reactions were carried out in a 25 µL reaction volume which consisted of 12.5 µL 2x PCR Master Mix (Cinnagen), 25 pmol of each primer, and 20-50 ng of template DNA. In the case of enterocin P (*entP*),[22] bacteriocin AS-48 (*bacAS-48*), bacteriocin 31 (*bac31*)[23] and enterocin L50A/B (*entL50A/B*),[24] the reaction conditions were 4 min denaturation at 94 ºC, followed by 40 cycles of 45 s at 95 ºC, 30 s at 56 ºC, and 35 s at 72 ºC; this was followed by 10 min at 72 ºC and a cool down to 4 ºC. For enterocin A (*entA*)[22] and enterocin Q (*entQ*),[25] 58 ºC and enterocin B (*entB*),[22] bacteriocin 1071 (*bac1071*), enterolysin A (*enlA*), and cytolysin (*cyl*),[24] the annealing temperature was 60 ºC. Amplified PCR fragments from these reactions were separated by 1% agarose gel electrophoresis, specific band were extracted from the agarose gel and purified. Enterocin sequences were determined by direct sequencing of PCR products.

### *Partial purification of bacteriocins from culture supernatants*

Cell free supernatants of the bacterial strain producing the largest inhibition zones were prepared by removing the bacteria by centrifuging the 16 h cultures for 10 min at 10,000 g. In the first step, the supernatant containing bacteriocins were concentrated by ammonium sulphate (40%) precipitation. The protein pellets resulted from centrifugation at 15,000 g for 30 min (4 ºC)[26] were dissolved in a small portion of potassium phosphate buffer (pH 7.0) and filter sterilized.

### *Enzyme, pH and heat stability of partially purified bacteriocin*

The influence of enzymes, pH and heat on bacteriocin activity was determined by agar spot method against *Li. innocua* as indicator strain. Untreated bacteriocin served

as the control. The stability of bacteriocin to the following enzymes was evaluated by using spot test at 37 ºC for 24 h: proteinase K (900 U/ mL), trypsin (5 mg/ mL) and catalase (2 mg/ mL).

To evaluate the thermal sensitivity of the antimicrobial substances, the bacteriocin solution was checked to exposure for 10 min at 100 ºC. Furthermore, the stability of bacteriocin in different pH value was evaluated by adjusting the pH of partially purified bacteriocin to 2.0, 4.0, 6.0, 8.0 and 10.0 using 5 M HCl and 8 M NaOH. After incubation for 1 h and 6 h at room temperature and readjusting the pH of treated partially purified bacteriocin to acidic pH, the residual activity was assayed.

**Table 1.** List of primer pairs used in PCR reactions and expected product size

| Primer | Sequence (5′-3′) | Fragment size (bp) | Primer Reference |
|---|---|---|---|
| *Enterococcus* specific | f: TACTGACAAACCATTCATGATG<br>r: AACTTCGTCACCAACGCGAAC | 112 | (Ke et al., 1999) |
| *Enterococcus faecalis* | f:ATCAAGTACAGTTAGTCTTTATTAG<br>r: ACGATTCAAAGCTAACTGAATCAGT | 941 | (Kariyama et al., 2000) |
| *Enterococcus faecium* | f: TTGAGGCAGACCAGATTGACG<br>r: TATGACAGCGACTCCGATTCC | 658 | (Kariyama et al., 2000) |
| Enterocin A | f: AAATATTATGGAAATGGAGTGTAT<br>r: GCACTTCCCTGGAATTGCTC | 155 | (du Toit et al., 2000) |
| Enterocin B | f: GAAAATGATCACAGAATGCCTA<br>r: GTTGCATTTAGAGTATACATTTG | 201 | (du Toit et al., 2000) |
| Enterocin P | f: TATGGTAATGGTGTTTATTGTAA<br>r: ATGTCCCATACCTGCCAAAC | 121 | (du Toit et al., 2000) |
| Enterocin Q | f: ATGAATTTTCTTAAAAATGGTATCGCAAAA<br>r: TTAACAAGAAATTTTTTCCCATGGCAAG | 105 | (Brandao et al., 2010) |
| Bacteriocin 31 | f: CCTACGTATTACGGAAATGGT<br>r: GCCATGTTGTACCCAACCATT | 248 | (De Vuyst et al., 2003) |
| Enterocin L50A/B | f: TTGGGTGGCCTATTGTTAAA<br>r: TCTATTGTCCATCCTTGTCCA | 224 | (Solheim et al., 2009) |
| Bacteriocin 1071 | f: ATGCTGTAGGTCCAGCTGC<br>r: TTTCCAGGTCCTCCACCAGT | 219 | (Solheim et al., 2009) |
| Bacteriocin AS-48 | f: GAGGAGTATCATGGTTAAAGA<br>r: ATATTGTTAAATTACCAA | 339 | (De Vuyst et al., 2003) |
| Enterolysin A | f: CGCAGCTTCTAATGAGTGGT<br>r: CATACACACTGCCATTTCCA | 161 | (Solheim et al., 2009) |
| Cytolysin | f: TGGCGGTATTTTTACTGGAG<br>r: TGAATCGCTTCCATTTCTTC | 250 | (Solheim et al., 2009) |
| Hiracin | f: TGTCTAGCTGGCATCGGTACAG<br>r: TACCTTCTAGGTGCCCATGGAC | 170 | This study |

***Purification of the E. faecalis HO91 antimicrobial agent***

One L cell free supernatant of an overnight culture of strain *E. faecalis* HO91 was precipitated with ammonium sulphate and dissolved in 100 mL of $dH_2O$. The sample was then filtering sterilized through 0.22 µm membrane filter and pH adjusted to about 3.0 using 1 M HCl. Comparison of minimum inhibitory concentration (MIC) towards the pediocin sensitive *E. faecalis* LMGT 2708 and its resistant descendant LMGT 3348 was used to check whether *E. faecalis* HO91 produced a ClassIIa bacteriocin.

The bacteriocin was then more purified by cation-exchange chromatography. Five mL SP sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) was applied in filtration bed columns, to bind to the positive net charge proteins/peptide molecules such as class IIa bacteriocins. The SP sepharose column was washed with 100 mL $dH_2O$ then equilibrated to a pH of 3 using 10 mmol acetic acid. Then, the sample was applied to the column by the rate of approximately 1 mL/min, in the following stage, washing by 20 mmol phosphate buffer pH 6.8. Next, the sample was eluted a gradient concentration of 0.1 M, 0.3 M, 1.0 M and 5.0 M NaCl. The activity of all samples was checked and they kept at 4 °C for further analysis.

The bacteriocin was further purified by applying the sample to the Äkta purifier FPLC system. The most active fraction (observed on the microtiter assay) was first diluted to a total volume of 50 mL in $dH_2O$ and applied to the reverse phase column (Resource$^{TM}$ RPC 3 mL) with the rate of 2 mL/min. Then, the gradient proportion 20-60% was generated by 0.1% TFA (Trifluroacetic acid) in water and 0.1% TFA with isopropanol at flow rate of 1 mL/min to elute the purified

bacteriocin. Fractions showing candidate peaks on the chromatogram were assayed for antimicrobial activity against indicator LMGT 2708, 2785 and 3348 on a microtiter plate. The protein concentrations of pooled fractions used for subsequent purification steps were measured at 280 nm by NanoDrop spectrophotometer.

*Mass Spectrometry*

To determine the precise molecular weight of purified peptide (bacteriocin), the fraction with high antimicrobial activity from reverse phase chromatography was concentrated about 20 times by speed vacuum and subjected to Matrix Assisted Laser Desorption /Ionization- Time Of Flight mass spectrometry (MALDI-TOF). The peptide mass homology search was done through the http://au.expasy/org proteomics server and the MultiIdent tool, where molecular weight of all published peptides and proteins are available.

*Edman degradation (N-terminal amino acid sequencing)*

The amino acid sequence of 50 fold concentrated purified bacteriocin was determined by Edman degradation (Alphalyse A/S, Denmark). The primary amino acid sequence was entered into BLAST[27] to search for peptides with similar sequences.

*DNA-sequence analysis of Hiracin structureal gene in E. faecalis HO91*

Nucleotide sequence of *E. faecium* T8 which contained Hiracin (bacteriocin T8) structural gene was reported in GeneBank database Accno DQ402539.1. Forward and Reverse primers TGTCTAGCTGGCATCGGTACAG and TACCTTCTAGGTGCCCATGGAC respectively (Table 1) were designed to amplify a 170 bp fragment containing the hiracin structural gene *hirA*. DNA-sequencing was performed of gel purified PCR-amplicon.

***Determination of antibiotic resistance among clinical enterococci***

The resistance patterns of 38 of 170 clinical enterococcal blood isolates which randomly selected were determined by soft agar overlay disc diffusion assay after 24 h incubation of plates at 37 ºC as recommended by National Committee for Clinical Laboratory Standards.[28] The antibiotic-containing disks (Himedia) used were ampicillin (Amp) 10 µg, chloramphenicol (Chl) 30 µg, ciprofloxacin (Cip) 5 µg, erythromycin (Ery) 15 µg, gentamicin (Gen) 10 µg, streptomycin (Stp) 10 µg and vancomycin (Van) 30 µg.

***Evaluation of sensitivity of multi-antibiotic resistant enterococci to Hiracin***

The Minimum inhibitory concentration (MIC) of purified Hiracin was determined towards 12 antibiotic resistant enterococcal strains using the microtiter assay as described previously. The MIC is the concentration of bacteriocin needed to obtain 50% inhibition of growth.

## Results and Disscussion

***Bacteriocin production is prevalent among clinical enterococci in Iran***

A total of 170 clinical blood isolates phenotypically classified as *Enterococcus* spp. were investigated for their ability to produce bacteriocins by agar spot method. A total of 32 (18.8 %) of the evaluated isolates exhibited inhibitory activity towards *E. faecalis, E. faecium, E. hirae, Li. monocytogenes*, and *Li. innocua.* Surprisingly, *E. faecium* were the predominant strain, 88% (28 of 32) and the remaining isolates were *E. faecalis.* This encouraged us to investigate these isolates further for efficient antimicrobials.

Amongst these, 4 isolates (HS8, HO9, HO87 and HO91) displayed very large inhibition zones on agar and the highest antimicrobial activity in liquid broth media against the previously mentioned indicators. As the strong antilisterial and antienterococcal activity is consistent with the expression of known bacteriocins including class IIa, regularly found among enterococci,[29,30] the isolates were investigated for presence of known bacteriocin structural genes.

***Genotyping of Bacteriocin producing isolates and PCR detection of known enterocin genes***

Four $Bac^+$ isolates with highest antimicrobial activity were further identified by PCR as *E. faecium* (HS8, HO9 and HO87) and *E. faecalis* (HO91) using *Enterococcus* genus and species specific primers (results not shown).

The purified DNAs of the 4 bacteriocin producer strains (HS8, HO9, HO87 and HO91) were investigated for the presence of ten known structural genes encoding bacteriocins (Table 1) by PCR. The results showed that all three *E. faecium* strains (HS8, HO9 and HO87) harbored the enterocin A, B and L50A/B genes, while presence of enterolysin A and cytolysin genes were also detected in HO9 and HO87. *E. faecalis* HO91 carried only the enterolysin A structural gene. None of the $Bac^+$ strains harbored enterocin Q, P, AS-48, 1071A/B or bacteriocin 31 structural genes. This is in line with previous studies showing that *entA; enlA* and *entL50A/B* are mostly detected among enterococcal isolates originated from blood and feces.[31,32]

***Biochemical stability of partially purified bacteriocin***

Acid production is probably the major mechanism of antimicrobial action among LAB; however enterococci are not as efficient acid producers as other members of LAB. On the other hand, they are potential bacteriocin producers and can antagonize competing bacteria by their potent antimicrobial peptides.[33,34]

As shown in Table 2, the partially purified (concentrated) antimicrobial substances were sensitive to proteinase K and trypsin; in addition the activity retained after treatment with catalase which these two features confirmed the proteinaceous structure of antimicrobial agents. LAB such as enterococci, lactobacilli and bifidobacteria can kill or inhibit the growth of pathogenic

bacteria by producing antimicrobial agents like lactic acid, hydrogen peroxide and bacteriocins.

**Table 2.** Effect of various treatments on the activity of the bacteriocins produced by selected enterococcal strains

| | Treatment [a] | Residual activity of antimicrobial agents after treatments [c] | | | |
|---|---|---|---|---|---|
| | | HS8 | HO9 | HO87 | HO91 |
| Enzyme | Catalase | + | + | + | + |
| | Trypsin | - | - | - | - |
| | Proteinase K | - | - | - | - |
| pH | 2.0 (1 h at RT)[b] | + | + | + | + |
| | 2.0 (6 h at RT) | + | + | + | + |
| | 4.0 (1 h at RT) | + | + | + | + |
| | 4.0 (6 h at RT) | + | + | + | + |
| | 6.0 (1 h at RT) | + | + | + | + |
| | 6.0 (6 h at RT) | + | + | + | + |
| | 8.0 (1 h at RT) | + | ↓ | ↓ | + |
| | 8.0 (6 h at RT) | ↓↓ | ↓↓ | ↓↓ | ↓↓ |
| | 10.0 (1 h at RT) | + | ↓ | ↓ | + |
| | 10.0 (6h at RT) | ↓↓ | ↓↓ | ↓↓ | ↓↓ |
| Heat | 60 °C (30 min) | + | + | + | + |
| | 100 °C (10 min) | + | + | + | + |

[a] Determined by spot-on-lawn method using *Li. Innocua* LMGT 2785 as an indicator microorganism;
[b] RT= room temperature;
[c] + = Presence of activity; - = Absence of activity;
↓ = Reduction in activity; ↓↓ = Serious reduction in activity

It is seen from Table 2 that the stability of pH treated bacteriocin was detected up to pH 8.0. In pH 8.0 and 10.0, losses in the inhibitory activity started to be seen for two strains at room temperature incubation for 1 h. When the treated bacteriocins (pH 8.0 and 10.0) were incubated for 6 h at room temperature, bacteriocins activity were dramatically reduced. This feature can be attributed to the solubility of the bacteriocins produced by $Bac^+$ strains under their Isoelectric points (8.0-10.0).[35] Interestingly, the activity was not significantly affected by heating at 60 ºC and 100 ºC for 30 and 10 min, respectively. Heat stability is one of the main characteristic features of many bacteriocins and stable cross-linkages, presence of strong hydrophobic regions as well as high glycine content are factors contributing to their heat resistance.[35] Residual antimicrobial activity was determined against *Li. Innocua* LMGT 2785 by the agar spot method.

### *Purification of the antimicrobial substance produced by E. faecalis HO91*

*E. faecalis* HO91 showed noticeable zones of inhibition on the spot on the lawn assay against applied indicator strains. The substance with antimicrobial activity were protease sensitive, as well as stable at pH 2.0 and 100 °C for 10 minutes. Furthermore, the results of PCR screening showed that HO91 only possessed bacteriocin structural gene of enterolysin A. As enterolysin A belongs to the class III, the large and heat-labile bacteriocins;[36] hence substantial reduction in antimicrobial activity after boiling at 100 ºC was inevitable. However, no reduction in the inhibition activity of partially purified bacteriocin was observed, so it could be concluded that there was no expression for enterolysin A. Then, the antimicrobial activity of the ammonium sulphate precipitate of HO91 culture supernatant was tested against indicators LMGT 2708 and its pediocin-resistant mutant strain LMGT 3348 (2708 RS), the latter is resistant to all class IIa bacteriocin. This was performed to determine whether the produced substance was from pediocin family (class IIa bacteriocin). The results indicated that the precipitate of HO91 only could inhibit the growth of 2708 but not 2708 RA. Hence; based on the above described results, the antimicrobial substance was considered a member of pediocin-like bacteriocin. Briefly, the bacteriocin was purified using SP-sepharose ion exchange chromatography, followed by reverse phase chromatography (RPC 3). Table 3 briefly describes the result of each purification stage of the bacteriocin. Interestingly, reverse phase chromatography (RPC) gave a pure and distinct peak with absorbance at both $A_{214}$ and $A_{280}$ (Figure 1).

**Table 3.** Purification of the *E. faecalis* HO91 bacteriocin

| Fraction | Vol [a] (ml) | Activity (BU/ml) | Protein (mg pr/ ml) | Sp.Act.[b] (BU/ mg pr) | Total Activity (BU) | Yeild (%) |
|---|---|---|---|---|---|---|
| Culture Supernatant | 1000 | 800 | 20.3 | 39.4 | 800000 | 100 |
| ASP[c] | 100 | 25600 | 14.5 | 1765.5 | 2560000 | 320 |
| Cation Exchange Ch[d]. | 20 | 1600 | 0.73 | 2191.8 | 32000 | 4 |
| RPC[e]-3-1 | 1 | 51200 | ND[f] | - | 51200 | 6.4 |
| RPC-3-2 | 0.5 | 51200 | 0.18 | 284444 | 25600 | 3.2 |

[a] Vol= volume
[b] Sp. Act. = specific activity
[c] ASP= ammonium sulphate precipitate
[d] Ch= chromatography
[e] RPC= reverse phase chromatography
[f] ND= Not determined

### *MALDI-TOF molecular weight determination and peptide mass homology search*

A fraction containing the purified antimicrobial compound from the 2nd RPC was used for further biochemical characterization. The monoisotopic molecular weight was determined by MALDI-TOF MS to 5088.9 Da (in Figure 2). Performing a peptide mass homology search (MultiIdent tool Expasy) produced one

significant hit, the bacteriocin Hiracin (bacteriocin T8) with the average molecular weight of 5094 Da. Hiracin is a class IIa bacteriocin which was isolated from both *E. hirea* and *E. faecium* previously.[37,38] The purified peptide was sent for sequencing because of two reasons; firstly, the difference in peptide mass (about 6 Da) and secondly, the fact that determined producer strains were different from ours (*E. faecalis* HO91).

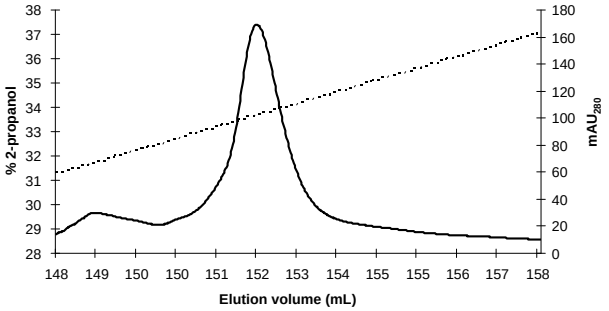


**Figure 1.** Chromatogram of the final reversed phase purification of Hiracin. Solid line: $mAU_{280}$ nanometers; Dotted line: % 2-propanol

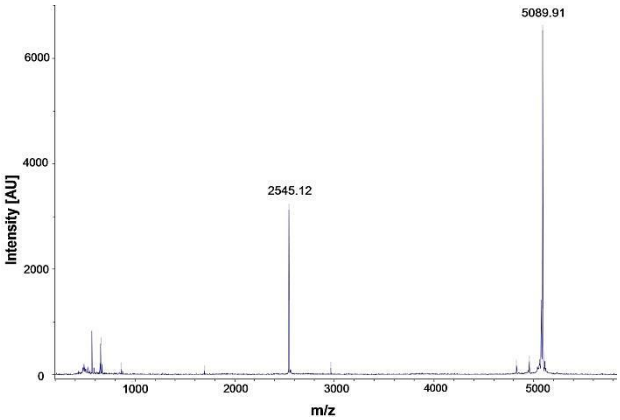


**Figure 2.** MALDI-TOF analysis of purified bacteriocin, corresponding to monoisotopic molecular weight of Hiracin (5089.9 m+1/z) and doubly charged ion (2545.1 m+2/z).

### *N-terminal amino acid sequencing*

N-terminal sequencing for 48 cycles presented the following amino acids and nucleotides:

A T Y Y G N G L Y X N K E K X W V D W N Q A
K G E I G K I I V N G W V N H G P W A (A/P) R R

This sequence was identical in 41 of 44 amino acids of Hiracin, where the 2 'X' residues corresponded to cysteine residues. Hence it was obvious that the purified bacteriocin was Hiracin by the different producer strain (*E. faecalis*).

### *Confirmation that E. faecalis HO91 is a bacteriocin T8 (Hiracin)- producer strain*

To confirm that the bacteriocin produced by *E. faecalis* HO91 was Hiracin, a PCR analysis was performed by designing primers for published sequence of bacteriocin T8 structural gene of *E. faecium* T8. A 170 bp fragment was amplified from purified DNA of *E. faecalis* HO91 and sequenced, confirming 100% identity to the *hirA* structural gene (data not shown).

### *Assessment of antibiotic resistance patterns*

The antibiotic resistance phenotype of 38 $Bac^+$ enterococcal strains was tested. In overall, 97.4% of the enterococcal isolates were resistant to at least one of the seven antibiotics applied in this survey. Interestingly, UDS1 was the only strain with sensitivity profile to all tested antimicrobial agents.

Regarding the individual antibiotics, the enterococcal resistance was detected for all antimicrobial agents tested to different degrees (Figure 3). Majority of isolates (> 50%) showed resistance for gentamicin (92%), ciprofloxacin (84%), erythromycin (76%), streptomycin (74%) and chloramphenicol (53%). The prevalence of resistance to ampicillin and vancomycin was about (34%) and (13%), respectively.

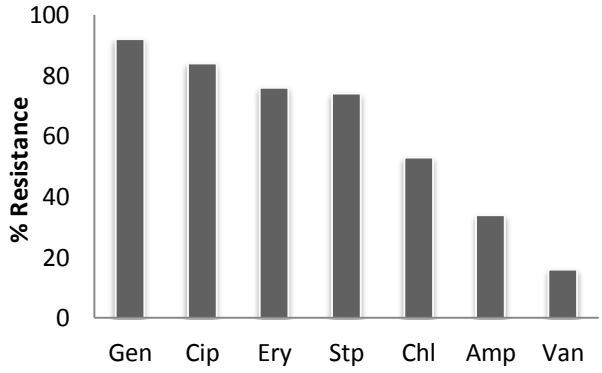


**Figure 3.** Individual antimicrobial resistance prevalence of $Bac^+$ *Enterococcus* spp. isolated from clinical samples, (Amp - Ampicillin, Chl - Chloramphenicol, Cip - Ciprofloxacin, Ery – Erythromycin, Gen – Gentamicin, Stp - Streptomycin, Van- Vancomycin)

As shown in Table 4, there were seven different resistance patterns for seven antibiotics in this study. Multi-antibiotic resistance (resistance to more than one antibiotic) was observed in 36 (94.7%) of 38 $Bac^+$ enterococcal strains. The most prevalent multi-antibiotic resistance profiles were detected for five antimicrobial agents (26.3%), followed by 21.1% to four as well as six and 13.2% to two. The lowest prevalence of multi-antibiotic resistance was 5.2% and 7.9% encountered to the combination of three and seven antimicrobial agents (Figure 4).

Emergence of enterococci which are resistant to a wide range of commonly used antibiotics as a cause of nosocomial infection is a concerning phenomenon that has developed in recent years.[39] Indeed, our study revealed that there is a high-level enterococcal resistance to aminoglycosides, erythromycin and chloramphenicol among clinical isolates of enterococci. Unfortunately, resistance to ampicillin as well as vancomycin was also noticeable.

Another important aspect was very high proportion of incidence of multi-antibiotic resistance 94.7% (36 of 38) and surprisingly, as shown in Table 4, 7.9% (3 of 38) of strains were resistant to all seven antibiotics used in the study.

**Table 4.** Most frequent resistance patterns of Enterococcus spp. isolates (Amp - Ampicillin, Chl - Chloramphenicol, Cip - Ciprofloxacin, Ery – Erythromycin, Gen – Gentamicin, Stp - Streptomycin, Van- Vancomycin)

| Resistance against antimicrobials | No. of resistant *Enterococcus* spp. isolates | Most frequent resistance patterns (isolates) |
|---|---|---|
| 1 | 1 | Gen (1) |
| 2 | 5 | Cip+ Chl (2) |
| | | Cip+Ery (1) |
| | | Gen+Stp (1) |
| | | Gen+Amp (1) |
| 3 | 2 | Cip+Ery+Chl (1) |
| | | Cip+Ery+Gen (1) |
| 4 | 8 | Cip+Ery+Gen+Stp (4) |
| | | Cip+Ery+Gen+Chl (1) |
| | | Cip+Gen+Stp+Chl (1) |
| | | Ery+Gen+Stp+Amp (2) |
| 5 | 10 | Amp+Cip+ Ery+ Gen+Stp (7) |
| | | Amp+Cip+ Ery+ Gen+Van (1) |
| | | Amp+Gen+Van+Stp+Chl (1) |
| | | Cip+ Ery+ Gen+Stp+Chl (1) |
| 6 | 8 | Amp+Chl+Cip+ Ery+Gen+Stp (4) |
| | | Amp+Chl+Ery+Gen+Stp+Van (1) |
| | | Chl+Cip+ Ery+ Gen+Stp+Van (3) |
| 7 | 3 | Amp+Chl+Cip+Ery+Gen+Stp+Van(3) |

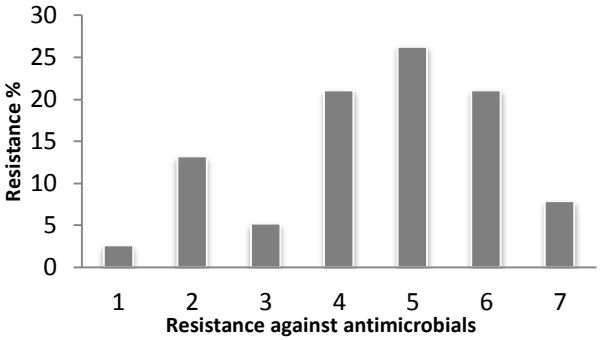


**Figure 4.** Antimicrobial resistance types of *Enterococcus* spp. isolated from clinical samples

### *In vitro evaluation of Hiracin efficacy towards multi-antibiotic resistant enterococci*

Emergence of enterococci which are resistant to a wide range of commonly used antibiotics as a cause of nosocomial infection is a concerning phenomenon that has developed in recent years.[39] Hence, there is a need for alternatives to control the dissemination of large numbers of antibiotic resistant bacteria into the environment. Interestingly, bacteriocins are one of the best candidates which tons of papers stating the potential of them to inhibit the growth of objective bacteria but few have actually evaluated them towards multi resistant nosocomial bacteria.

In this study, MIC of both partially purified and purified Hiracin were evaluated against *L. innocua* as an indicator strain and 11 antibiotic resistant enterococci by microtiter plate assay. As it is obvious from Table 5 of 11 strains, 6 isolates (resistant from one to 4 antibiotics) demonstrated acceptable sensitivity to Hiracin. Based on the obtained data, Hiracin can be an effective bacteriocin against multi-antibiotic resistant enterococcal isolates.

**Table 5.** The MIC of the partially purified and purified Hiracin against multi-antibiotic resistant enterococci

| | | Sample number[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *L. innocua* | 2 | 11 | 17 | 18 | 37 | 38 | 39 | 49 | 83 | 90 | 103 |
| Activity (BU/ml) | Partially purified | 25600 | 400 | 400 | - | 1600 | - | 6400 | 3200 | - | - | - | 800 |
| | purified | 51200 | 800 | 3200 | - | 6400 | - | 25600 | 12800 | - | - | - | 3200 |

[a]2: Cip+ Chl, 11: Gen+Stp, 13: Chl+Cip+ Ery+ Gen+Stp+Van, 17: Amp+Gen+Van+Stp+Chl, 18: Cip+Ery+Chl, 37: Amp+Cip+Ery+Gen+Van, 38: Cip+Ery+Gen, 39: Cip+Gen+Stp+Chl, 49: Amp+Chl+Cip+Ery+Gen+Stp+Van, 83: Ery+Gen+Stp+Amp, 90: Amp+Chl+Ery+Gen+Stp+Van, 103: Gen

## Conclusion

In conclusion, as the results of the presented experiment indicate an easy applicable procedure to isolate and purified an unknown proteinaceous substance with antimicrobial effect from the culture media context was described. In the next step, the antimicrobial activity of purified bacteriocin was successfully evaluated against multi-antibiotic resistant enterococci isolated from clinical samples. It would be advisable to include antibiotic resistant Listria into research. In addition, it would be interesting to check the influence of mixture of other bacteriocin plus Hiracin on pathogenic microbes. Interestingly, the obtained results could be useful in future approaches to control and treatment inflammatory conditions.

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## Ethical Issues

Not applicable.

## Conflict of Interest

The authors report no conflicts of interest in this work.

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