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Biochemical and Molecular Analysis of the Antilisterial Peptides Produced by *Enterococcus hirae* Strains Isolated from Raw Ewe Milk

Naheed Mojgani[1,2*], Narges Vaseji[3], Soodabeh Khalkhali[4] and Muneera Naz Baloch[5]

[1]Department of Biotechnology, Razi Vaccine and Serum Research Institute, Agriculture Research Education and Extension Organization (AREEO), Karaj, Iran.
[2]Research and Development Unit, Nature Biotech Co (Biorun), Karaj, Iran.
[3]Department of Biotechnology, Animal Science Research Institute of Iran, AREEO, Karaj, Iran.
[4]Department of Microbiology, Fars Branch, Islamic Azad University, Shiraz, Iran.
[5]Department of Microbiology, Karachi University, Karachi, Pakistan.

Authors' contributions

This work was carried out in collaboration between all authors. Author NM designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors NV and SK collected the samples and identified the isolates. Author MNB managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was conducted to identify and characterize the anti-listerial bacteriocins produced by *Enterococcus hirae* strains isolated from ewe milk.
Study Design: Bacteriocins produced by *E. hirae* strains were identified and characterized by physio-chemical methods. Bacteriocin structural genes were evaluated by molecular methods.
Place and Duration of Study: Biotechnology Department, Razi vaccine and Serum Research institute, and National institute of Animal Science, Iran, between January 2013 and March 2015.
Methodology: Two *E. hirae* were isolated from raw ewe milk samples collected from Yengi Esperan (Sfeedan) village located in East Azerbaijan Province, Iran. The isolates demonstrating

**Corresponding author: E-mail: dnmoj@yahoo.com;*

antilisterial activity were identified by 16S rRNA genes sequencing. The bacteriocinogenic potential of the isolates was evaluated using biochemical tests. The proteinaceous compounds were purified using Ammonium sulphate precipitation (40%), cation-exchange chromatography followed by SDS-PAGE analysis. Occurrence of enterocin structural genes was evaluated using a set of primers in a PCR reaction.

Results: The antilisterial compounds produced by the two *E. hirae* strains were sensitive to the proteolytic enzymes, while catalase and lipase had no effect on the activity. In contrast to the bacteriocin Eh512, enterocin Eh514 showed partial sensitivity to the enzyme lysozyme. The proteinaceous agent from the two producer isolates; Eh512 and Eh514 were single peptides of approximately 6.5 and 5.8 KDa, respectively. The enterocins in study appeared heat stable and resistant to acidic pH values. Analysis of the enterocin structural genes, showed the presence of *entA,* and *entB* genes in both the isolates whereas, *E. hirae* Eh512 additionally harbored *entP* and entQ genes. Sequence analysis of *entA* genes in both isolates indicated 95% homology with other *entA* genes in NCBI library.

Conclusion: The studied enterocins might be suitable replacement for chemical additives used in food preservations. However, further studies are required to validate these findings.

Keywords: Enterococcus hirea; anti-listerial bacteriocin; structural genes; ewe milk.

1. INTRODUCTION

Bacteriocins are one of the best studied and safest alternatives to chemically used antimicrobial agents. They are well defined as biologically active proteins displaying a bactericidal or bacteriostatic mode of action towards usually closely related species. Four classes of bacteriocins have been described based on their structural, chemical, and functional properties. These bacteriocins vary in the spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties [1,2].

Majority of bacteria belonging to Lactic Acid bacteria (LAB) are reported as GRAS (generally regarded as safe) microorganisms and their bacteriocins are considered innocuous due to proteolytic degradation in the gastrointestinal (GI) tract. *Enterococcus* belongs to the group of LAB. They are gram positive facultative anaerobic cocci usually found in gastrointestinal tract of man and animals, in vegetables, plant material and a number of food products [3,4]. To date a number of bacteriocin (enterocin) producing enterococci has been identified and biochemically characterized. Based on the bacteriocin classification scheme, class 1 lantibiotics are rarely found in enterococci. Cytolysin, the hemolytic enterocin is the only two-peptide lantibiotic with cytolytic (hemolytic) activity and most thoroughly characterized in *Enterococcus* spp. Cytolysin is a virulence factor and consequently it is not considered useful as an antimicrobial agent [5]. Generally, most of the enterocins belongs to class II of small heat stable bacteriocins which are distinguished by their activity against *Listeria* spp [6,7]. This class of bacteriocin is further subdivided into class IIa (the pediocin-like), IIb (the two-peptide bacteriocins), the circular bacteriocins, and the leaderless enterocins. The best characterized class II enterocins include enterocins A, B, PCRL35 and bacteriocin 31 [8,9]. Enterocin A, class IIa, contains a pediocin-like structure with a YGNGVXC amino acid motif near the N-terminus of the anti-*Listeria* active peptide. Whereas, enterocin B is not pediocin-like but is similar to the class IIa bacteriocins with respect to its chemical characteristics, heat stability and anti listerial activity [10]. Two enterocins, namely Enterocin B and bacteriocin 32 are the enterocins which do not share the basic classification features of the above mentioned classes and subclasses. Enterolysin A has been characterized as a class III heat labile bacteriocin produced by *E. faecalis* [11]. *Enterococcus* species are well known for their ability to produce multiple bacteriocins and most often, enterocins A and B are found together in the same isolate. However, they may also be co-produced in conjunction with other enterocins, like enterocins P, Q, and L50 [12].

The antilisterial enterocins produced by enterococci are not only been considered for use as a potential biopreservatives in food, feed, but also as an alternative therapies to antibiotics in human and animals [13,14]. In our previous studies, we had reported a number of bacteriocin producing *Lactobacillus* species isolated from ewe milk with probiotic potential [15,16]. In this study we aimed to identify and characterize

the antilisterial bacteriocins produced by *Enterococcus* strains isolated from raw ewes' milk samples.

2. MATERIALS AND METHODS

2.1 Bacterial Isolation, Identification and Growth Conditions

Enterococcus isolates used in study were isolated from raw ewe milk samples collected from Yengi Esperan (Sfeedan) village located in East Azerbaijan Province, Iran. The ewe milk samples were cultured on Kenner Fecal Agar (KFA, Merck, USA) supplemented with 1% TTC (2,3,5- Triphenyl-tetrazolium chloride solution, Sigma, UK) and Bile Esculin Azide Agar (BEAA, Merck, USA), under aerobic and anaerobic conditions. The obtained pure colonies were tested for their Gram reactions, catalase test, growth at 10, 37 and 45ºC, and resistance in 6.5% NaCl and alkaline pH values of 9.0. The presumptive *Enteroococcus* isolates were tested for their antilisterial activity and those demonstrating significant inhibitory activity against *Listeria monocytogenes* (RTCC 1298) were identified to genus level based on their 16S rRNA genes sequence analysis [17].

The two *E. hirae* isolates identified based on their sequence similarity with other *E. hirae* strains at NCBI database, were further confirmed by specie specific primers namely mur-2 F and mur2-R [18]. The two isolates gave amplicons of 521bp and were submitted at NCBI Gene bank under accession no. KX450887 and KX450888. All cultures were stored as stock culture in Brain heart infusion (BHI, Scharlau, Spain) broth with 20% (V/V) glycerol solutions at -70ºC.

2.2 Physico-chemical Characterization of the Bacteriocins Produced

The overnight culture extract of the strains were centrifuged (15000 x g at 4ºC for 20 min) and the antagonistic activity in the collected cell free supernatant (CFS) were determined against *L.monocytogenes* RTCC 1298 by Agar well diffusion assay [7]. The indicator bacteria were used at final concentration of 10^8 CFU/mL in semisolid agar poured on solidified agar plates. The antimicrobial activity was recorded in arbitrary units per millimeter (AU/mL) by critical dilution method [19]. One AU/mL of the bacteriocin activity was defined as the reciprocal of highest dilution showing discernible activity against the indicator culture.

To exclude the effect of organic acids and H_2O_2; the antilisterial activity in the supernatant fluid was determined after adjusting the pH of CFS to 6.5 using 2M NaOH and subjecting to the enzyme catalase (1 mg/L), respectively. The remaining activity was determined by Agar well diffusion assay.

The chemical nature of the antagonistic agents were determined by treating the CFS from the producer strains with enzymes like Lipase, Lysozyme, Pepsin, Pronase E, and Proteinase K (Flukaa, England) at final concentration of 1 mg/L in phosphate buffer as described earlier [7,16].

The stability of the bacteriocins at variable pH (2-11) and temperature (60, 80,100, and120℃) were determined as described by exposing the supernatant fluids to the above mentioned physical parameters and determining the residual activity [19].

Stability of the said bacteriocins in the presence of 1% V/V non-ionic (triton X100, tween 20, tween 80) and anionic (sodium dodecyl sulphate, deoxycholic acid) surfactants (Sigma, USA) was determined at different time intervals and residual activity recorded.

2.3 Growth Kinetics and Bacteriocin Biosynthesis

The growth and bacteriocin production in the selected producer strains were monitored by growing the isolates in 50 mL MRS broth at 37ºC for 24 h. The growth was monitored at hourly intervals by measuring their pH and optical density (600 nm). The viable count (CFU/mL) and antibacterial activity (AU/mL) against *L. monocytogenes* by agar well diffusion assay was also determined.

2.4 Mode of Action

Two different methods were adopted to determine the bactericidal or bacteriostatic nature of the bacteriocins in study [20,21]. In the first method, twenty mL of the neutralized CFS from the producer strains were added to 100 mL early exponential phase culture of *L. monocytogenes* and incubated at 37ºC. The optical density (OD_{600}) and viable count was determined at hourly intervals for 9 h. While in the other method, two fold dilutions of the supernatant fluids were used in critical assay using *L. monocytogenes* as indicator cells. After

overnight incubation, 5 µL of 10 mg/mL of proteinase K solution (Sigma, USA) were spotted next to the inhibition zones, and the plates further incubated at 37℃. The plates were observed for the presence or absence of growth of the indicator culture after treatment with protease. The absence of listerial growth after the destruction of the inhibitor by protease indicated that bacterial cells had been killed by the peptide, whereas absence of inhibition zones after enzymatic treatment indicated a bacteriostatic mode of action.

2.5 Adsorption Assay

The binding/adsorption of the bacteriocins produced by the two *Enterococcus* strains onto susceptible cells was evaluated by mixing equal volumes of the CSF from the producer isolates and the sensitive indicator cells (*L. monocytogenes*), and incubating at 37ºC. At every 5 min intervals, one tube was selected, chilled at 4ºC. The supernatant fluids were collected by centrifugation (20000 x g, 10 min at 4℃), neutralized and the remaining activity determined by agar well diffusion assay.

2.6 Bacteriocin Purification and Molecular Weight Determination

The CFS from *E. hirae* Eh512 and Eh514 were concentrated to one tenth of its original volume and purified by subjecting it to ammonium sulphate precipitations (20%) until saturation was achieved. The precipitates were recovered by centrifugation (15000 x g, 15 min, 4℃), dissolved in potassium phosphate buffer (pH 7.4) and dialyzed (1000 Da cut-off, Sigma) against phosphate buffer (0.1M, pH 7.0), with three buffer changes over 24 h at 4ºC. Dialyzed samples were filter sterilized (DFSB), aliquoted and stored at -70ºC.

The DFSB samples were further purified by cation exchange column (DEAE cellulose column) and elution was performed by using a linear gradient from citrate phosphate buffer ranging from pH 2.6 to 7.0 [22]. Protein content in the purified DFSB (P-DFSB) fraction was determined by Lowry method and the bacteriocin activity determined in AU/mL. The approximate molecular mass of the P-DFSB fractions were determined by ultrafiltration, Tricine-sodium dodecyl sulphate- polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) and Non-denaturing Gel electrophoresis. A 2 mL sample of partially purified enterocin Eh512 and Eh514 was ultrafiltered through cellulose membranes with 5, 10, 20 and 30 KDa exclusion units (Centricon, Micro concentrations, USA) and the remaining activity in filtrate and retentate determined as described earlier. The purified fractions were analyzed on SDS-PAGE as described by Tulini et al. [21]. After electrophoresis the gel was divided into two parts, one part was stained for determination of protein bands and assessment of molecular size while the other half was used for biorevelation. Initially, the unstained gel was fixed with ethanol/acetic acid/H_2O solution for 30 min, washed thrice with distilled water and placed carefully on BHI agar plates. The plates were overlaid with semisolid agar embedded with indicator strain and incubated at 4ºC for 4 h and later at 37ºC for 24 h. The antibacterial protein was located by appearance of inhibition zone around the respective band.

2.7 Determination of Bacteriocin Structural Genes

The presence of bacteriocin structural genes in the producer isolates was studied with PCR assay using a set of primers listed in Table 1. DNA template was prepared by suspending a loop full of bacterial colony in 10 µL of lysates buffer (0.25% SDS / 0.05% NaOH), heated at 95℃ for 5 min and centrifuged at 15000 x g for 5 min. The samples were diluted in 90 µL of sterile distilled water, centrifuged as above and the supernatant used as template DNA. Cycling parameters included 2 min initial denaturation at 94ºC, followed by 40 cycles of 45 s at 95ºC, 30 s at 56ºC for *entP, bac31* and *entL50A/B*, 58ºC in the case of *entA* and 60ºC for *entB, entQ,* and *cyl* as annealing temperature, and 60 s at 72ºC.

Amplified PCR fragments were resolved on 1% agarose gels, using a 100 bp ladder for size verification. Amplicons (*entA* genes) obtained from positive PCR reactions in both the isolates were further purified by use of QIA quick PCR Purification Kit (Qiagen) and then sequenced (Robin Teb, Malaysia). Analysis of the gene sequences was conducted by using Bactibase database (http://bactibase.pfba-lab-tun.org).

Table 1. Primers and their sequences used in this study

Primer	Target gene	Primer sequences (5-3)	Expected size bp	Reference
Ent A	Enterocin A	F: AAATATTATGGAAATGGAGTGTAT R: GCACTTCCCTGGAATTGCTC	126	[14]
Ent B	Enterocin B	F: GAAAATGATCACAGAATGCCTA R: GTTGCATTTAGAGTATACATTTG	159	[14]
Ent P	Enterocin P	F: TATGGTAATGGTGTTTATTGTAA R: ATGTCCCATACCTGCCAAAC	121	[14]
Cyl	Cytolysin	F: GGCGGTATTTTTACTGGAGT R: CCTACTCCTAAGCCTATGGTA	250	[23]
Ent Q	Enterocin Q	F: ATC ACA AAG TGA GCC CCT GT R: TGG TAT CGC AAA ATG GAT GA	231	[24]
Bact 31	Bacteriocin 31	F: CCTACGTATTACGGAAATGGT R: GCCATGTTGTACCCAACCATT	248	[24]
EntL 50A/B	Enterocin L50A/B	F: TGGGAGCAATCGCAAAATTAG R: ATTGCCCATCCTTCTCCAAT	130	[14]

3. RESULTS AND DISCUSSION

The importance of ewe milk and its fermentative products are quite well recognized by locals living in the rural areas in East Azerbaijan of Iran. Ewe milk made dairy products including cheese, yoghurt, and sour butter milk are highly consumed among the Iranians mainly residing in small towns and villages. These dairy products have gained high popularity in many parts of the country owing to their texture, flavor and health benefits. Owing to the mentioned facts, in the last couple of years we have performed a number of studies on the ewe milk made dairy products from these regions in order to determine their health benefits including probiotic potential and antibacterial activity against important pathogens [15,16,25,26]. In this study we explored the bacteriocin producing potential of *Enterococcus spp* isolated from the raw ewe milk collected from the East Azerbaijan Iran.

Although, *Enterococcus* species are considered opportunistic pathogens but they have also been used as human or animal probiotics. Their role in food industry and as starter culture is also well recognized, and is considered important for aroma and flavor development and ripening of cheeses [3,6,13]. The dominant *Enterococcus* spp in the mammalian milk flora are *E. faecalis* and *E. facieum* [27-30]. In the present study, a *total of 18 Enterococcus* presumptive positive colonies appeared on supplemented KF and BEA agar. These isolates were Gram positive cocci in pairs and chains, catalase negative, grew well at 10, 37 and 45℃, and resisted 6.5% NaCl and alkaline pH values (9.0). Among these isolates only two showed anti-listerial activity and were identified to genus and species level by subjecting their genomic DNA to the mentioned universal and specie specific primers. Based on the obtained results, the two isolates were identified as *E. hirae* Eh512 and Eh514, respectively.

L. monocytogenes, the bacteria responsible for causing listeriosis is ubiquitous in the environment and is resistant to low temperatures, low pH and high salt concentrations [31,32]. Anti-listerial bacteriocin producing trait of *Enterococcus* spp is considered their useful biotechnological trait. Siragusa [32], was the first to report production of antilisterial bacteriocin (hiraecin S) by *E. hirae* isolated from bovine intestine. Since then, a number of bacteriocin producing *E. hirae* strains isolated from different sources have been reported [33-37]. However, production of metabolic end products by LAB, like acids and hydrogen peroxide might be erroneously attributed to the production of bacteriocins or bacteriocin like compounds [6,14]. Thus in order to identify bacteriocin related antibacterial activity it is essential to physico-chemically characterize the produced antibacterial agents. In our studies, the two *Enterococcus* isolate Eh512 and Eh514 demonstrated significant antlisteria activity (Fig. 1) which was recorded as 6400 and 12800 AU/ml, respectively.

During physico-chemical characterization of the anti-listerial agents produced by the two isolates, pH neutralizations and enzyme catalase appeared to have negligible effects on the antagonistic activity in the cell free supernatant fluids of the isolates (Table 2). These results

ruled out the possible effect of organic acids and hydrogen peroxide for the antimicrobial activity.

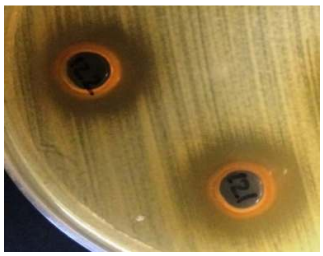

Fig. 1. Antilisterial activity of *E. hirae* Eh512 and Eh514. The clear zones around the wells indicate inhibition of the growth of *L. monocytogenes*

Table 2. Physico-chemical characterization of the bacteriocins

Treatments	Eh512	Eh514
	RA%	
Control (CFS)	**100**	**100**
NaCl		
10%	100	100
20%	100	100
30%	80	100
40%	65	70
Anions		
SDS	140	100
Deoxycholic acid	80	90
Non-anions		
Triton X100	140	150
Tween20	60	100
Tween80	150	120
Enzymes		
Catalase	100	100
Lysozyme	100	65
Lipase	100	100
Pepsin	0	0
Pronase E	0	0
Proteinase K	0	0

RA: Residual activity; CFS: Cell free supernatant fluid; N-CFS: Neutralized cell free supernatant fluid

It is well known that the bacteriocins produced by different bacteria might differ in their chemical nature. According to reports, bacteriocins might be either simple proteins or proteins linked to lipid or carbohydrate moieties [16,22]. The antagonistic agent produced by the two isolates showed slight differences in their physico-chemical nature. The antibacterial compounds of both the isolates was sensitive to the action of the tested proteolytic enzymes and complete loss of antibacterial activity was observed after exposure of the supernatant fluids to the mentioned enzymes. However, the antagonistic agents were resistant to the action of lipase but lysozyme resulted in partial loss of activity in Eh514 and a loss of approximately 35% was observed after treating the neutralized supernatant fluid of the mentioned isolate with the said enzyme. These results showed that while lipid moiety is not critical for the actions in EH514, but the carbohydrate component had an essential role to play in the bioactivity of the mentioned bacteriocin. In contrast, enterocin Eh512 appeared a simple protein molecule rather than conjugated protein, a character similar to Enterocin LD_3, and Enterocin F420 produced by *E. hirae* strains [33,34].

Most of the anti-listerial enterocins identified to date are thermo–stable, pediocin-like bacteriocins which have been of considerable interest as biopreservative [38,39,40]. Heat stability of bacteriocins is considered an important characteristic for their potential application as a biological food preservative [41,42]. In this study, we observed enterocin Eh512 to be more heat tolerant than the other enterocin in study. The mentioned enterocin resisted 100℃ for more than 90 min and also autoclaving temperatures. At 60 and 80ºC both the enterocins retained their activity for 160 minutes. Enterocin Eh514 resisted 100℃ for only 60 min and completely lost its antibacterial activity at 121℃ within 15 min. Similarly, the bacteriocin produced by *E. hirae,* bacteriocin ST15 produced by *Enterococcus mundtii* ST15, bacteriocin N15 produced by *Enterococcus faecium* N15 has been shown previously to be completely demolished after treatment at 121℃ for 20 min [43].

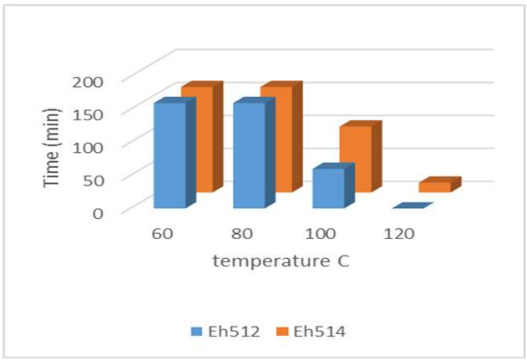

Fig. 2. Thermo-stability of the bacteriocins produced by *E. hirae* Eh512 and 514 at different time intervals

During pH stability studies, enterocin Eh512 appeared more pH stable and resisted pH values of 3 to 10 while complete loss of their activity was observed at pH 2.0 and 11.0. Enterocin Eh514 was active at a pH range and resisted pH values ranging from 5 to 10 only. However, maximum antilisterial activity by both the enterocins in study was seen at pH values of 5 to 9. Both the bacteriocins appeared more pH stable then the other studied *E. hirae* bacteriocins [44].

Both the enterocins produced by mentioned producer strains were able to tolerate variable concentrations of NaCl and only partial loss of activity was observed at 40% of the salt concentrations. Slight differences were observed in the antimicrobial properties of the two isolates after subjection to different anionic and non-anionic detergents. SDS, Triton X100 and Tween 80 resulted in enhanced activity of Eh512, while tween 20 demolished almost half of the activity in this isolate. Similar to these studies, Gupta and Taiwari [45] showed the stability of the bacteriocin produced by *E. hirae* LD_3 in the presence of different organic solvents, detergents and surfactants.

Bacteriocin production in both the producer isolates appeared growth related as increase in growth resulted in an enhanced activity (Fig. 3).

The production of enterocin Eh512 started in early log phase within 4 hrs (100 AU) indicating the peptides to be primary metabolites. The activity significantly increased during mid-logarithmic phases (6-8 hrs) and reached its peak during 10 to 14 hrs. Gradual decrease in activity started from the end of stationary phase (18-22 h). Production of Eh514 bacteriocin started a bit later in mid phase log phase (5-6 h) and similar to enterocin Eh512 continued till late stationary phase. Reciprocally, enterocin LD3 produced by *E. hirae* LD_3 isolated from dosa batter was shown to be produced during mid-log phase. Similarly, Jennes and his colleagues [46] reported synthesis of enterocin 012 initiated in the middle log phase, reaching a first maximum at the end of this phase.

LAB Bacteriocins are known to exert bactericidal or bacteriostatic action on the sensitive cells, depending greatly on factors such as bacteriocin dose and degree of purification, physiological state of the indicator cells and experimental conditions [12]. In this study, the mode of action of the two enterocins appeared bactericidal as addition of highest concentrations of the bacteriocin lead to complete loss of growth of the sensitive cells within an hour of incubation. However, at lower concentrations (640AU/mL) a bacteriostatic effect was recorded which indicates the emergence of bacteriocin resistant cells. The number of viable counts appeared directly related to the concentrations of the bacteriocin used.

Adsorption of the bacteriocin to the target cells is considered highly significant for defining the mode of action of the concerned bacteriocin. According to reports, adsorption process intermediates the insertion of the bacteriocin in the cell membrane causing pore formation which leads to the cell death [20]. The two enterocins in study were studied for their adsorption ability to the sensitive indicator cells (*L. monocytogenes*) at different time intervals. A decrease in the activity within 30 min of the exposure of the neutralized supernatant fluids of the producer organisms to the indicator cells was recorded. Complete loss of activity of both the enterocins was recorded after 35 min of incubation. The decrease in activity might be due to increased adsorption of the sensitive cells to the respective enterocin molecules.

A number of purification strategies are employed to purify different bacteriocins [37]. Among these strategies, ammonium sulphate precipitation and ion exchange chromatography are often used [21,22]. We also used multiple purification protocols to purify the enterocins in study. Table 3 describes the recovery of bacteriocins during different purification stages. Significant increase in the activity of the enterocins was observed after 40% ammonium sulphate precipitations of the cell free fractions of the isolates. During ion exchange chromatography (DEAE cellulose), the active fraction was eluted (single 309 peak) with pH 5.0 citrate phosphate buffer, with significant increase in their percent yield. During ultra-filtration studies, the antagonistic peptides produced by the two isolates Eh512 and Eh514 were retained in 5 KDa membranes, while collected in filtrates of 10 KDa membranes. These results indicated the approximate molecular size of the peptides to be in the range of 5 to 10 KDa. On SDS-PAGE the two enterocins Eh512 and Eh514 were resolved as a single peptide band of ~ 6.5 and ~5.8 KDa, respectively.

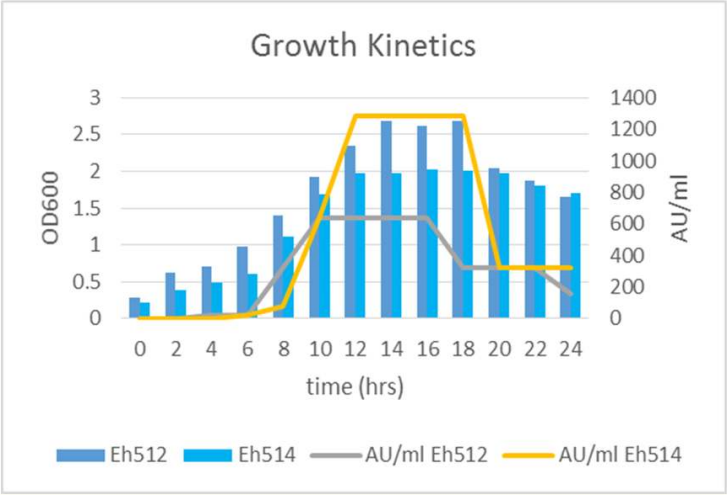

Fig. 3. Growth and bacteriocin production by *E. hirae* Eh512 and Eh514

Table 3. Bacteriocin recovery during different stages of purification

Isolates	Purification stages	Volume (ml)	Activity (AU/ml)	Total protein (mg)	Specific activity	Yield (%)	Purification (fold)
E. hirae Eh512	CFS	1000	6400	21.90	73.05	100	1.00
	C-CFS	100	1280	9.91	129.16	80.0	1.76
	40% AS-CFS	60	10240	12.21	838.65	640	11.48
	D-CFS	20	9830	4.9	2006	614	27.46
	DC 52	5	10400	0.84	12380	650	169
E. hirae Eh514	CFS	1000	12800	23.14	69.14	100	1.00
	C- CFS	100	960	12.11	79.27	60.0	1.14
	40% AS-CFS	45	7168	16.43	436.27	448	6.31
	D-CFS	12	10240	5.6	1828.57	640	26.44
	DC 52	3	14700	1.2	12250	918	177

CFS: Cell free supernatant fluid; C-CFS: Concentrated CFS; AS-CFS: Ammonium sulphate precipitated CFS; DF-CFS: dialyzed and filter sterilized fractions; DC52: DEAE cellulose 52 fractions.
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

A number of structural enterocin genes have been reported in *Enterococcus spp.* Henning and colleagues [14] reported presence of more than one bacteriocin structural genes in *Enterococcus* species. Based on their reports, *entA* gene was the dominant bacteriocin gene in *Enterococcus* isolates. Often, enterocin A gene which belongs to class IIa bacteriocins, is produced in conjunction with other bacteriocins like enterocin B. Majority of bacteriocin positive *E. facieum* isolates have been shown to possess *entA* or *entB* or both *entA* and *entB* genes [35]. Similar to these reports, we observed multiple bacteriocin genes in the studied *Enterococcus* isolates. Table 4, shows presence of multiple enterocin structural genes in the two producer isolates. The two *E. hirae* isolates in study harbored *entA* and *entB* genes as seen by amplicons of 126 and 159 bp, respectively. However, additional enterocin structural genes were seen in *E. hirae* Eh512 as it also harbored class IIa bacteriocins, *entP* (121bp) and the leaderless enterocin *entQ (231bp)* genes. Presence of *entP* genes in *E. hirae* was reported for the first time by Achmechem and his colleagues [33]. Similar to their studies, we also report the presence of *entP* genes in *E. hirae* Eh514. However, none of the isolates carried class I hemolytic cytolysin genes, class IIa bac31 and classII leaderless peptide enterocin *entL50A/B* genes.

Purification and sequencing of 126 bp *entA* genes from *E. hirae* Eh512 and Eh514 showed 95 and 98% similarity with the known enterocin A (*entA*) sequences in Bactibase databank, respectively (data not shown).

Table 4. Bacteriocin structural genes in *E. hirae* Eh512 and Eh514

Bacterial isolates	*entA (126bp)*	*entB (159bp)*	*entP (121bp)*	*entQ (231bp)*	*Bac31*	*cyl*	*Ent50A/B*
Eh512	+	+	+	+	-	-	-
Eh514	+	+	-	-	-	-	-

4. CONCLUSION

Biochemical and genetic characterization of the two bacteriocins revealed great similarity and minor differences between the two produced enterocin. Both the enterocins in study were similar with respect to their sensitivity to proteolytic enzymes, resistance to enzyme catalase and lipase, presence of structural genes *entA* and *entB.* However, slight differences existed between the two enterocins including the glyco-protein nature of the enterocin Eh514 compared to pure proteinaceous nature of enterocin Eh512, presence of additional *entP* and *entQ* genes in Eh512, and difference in the molecular weight of the peptide enterocins.

To conclude*, E. hirae* Eh512 and Eh514 produced class IIa heat stable anti-listerial enterocins active at wide range of pH values. These characteristics make them highly suitable for the control of *L. monocytogenes* mainly in dairy products. However, prior to recommending them for application as a bio-preservative in food products it is essential to evaluate their safety and stability in food matrix (*In-situ*).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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