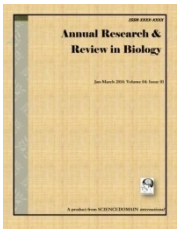

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Molecular Detection and Antimicrobial Activity of Enterolysin a Endopeptidase among *Enterococcus faecalis* Strains of Different Origins

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Authors' contributions

This work was carried out in collaboration between all authors. Author ZH designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches. Both authors managed the analyses of the study, read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The target of present study is to determine the frequency of occurrence of enterolysin A gene in *Enterococcus faecalis* strains from different sources and surveys about antibacterial effect of enl A gene positive strains.
Study Design: Enterolysin A is a class IV bacteriocin with broad spectrum of antimicrobial activity that is produced by some *E. faecalis* strains. However, research on the presence and antibacterial activity of this bacteriocin in different sources is very limited.
Place and Duration of Study: Islamic Azad University of North Tehran Branch Laboratory, between May 2011 and February 2012.
Methodology: In this study, the occurrence of enterolysin A structural gene in a target of 68 *E. faecalis* strains of three sources (animal feces, feed and surface water) was surveyed. Enterococcal strains were isolated from other feces Gram-positive and negative bacteria using Bile Aesculin Azide agar medium. After strains purification, *E.*

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faecalis bacteria were identified and the occurrence of enterolysin A gene was evaluated by using PCR method. The antimicrobial spectrum of enl A gene positive *E. faecalis* strains was assayed by deferred antagonism assay method on the some gram-positive and negative pathogen bacteria.

Results: Based on our results, 20 strains (29.4%) possessed enterolysin A structural gene. Different frequencies of the enterolysin A gene occurrence were detected in strains according to thebacterial origins; the strains from silage and lovebirds showed the highest frequency of enterolysin A gene presence. These gene-positive strains inhibited the growth of indicator bacteria such as clinical strain of *Pseudomonas aeruginosa, Listeria monocytogenes, Bacillus cereus* and *Salmonella enteric* PTCC1709.

Conclusion: Enterolysin A structural gene similar to the other enterocin genes present in enterococci of different origins. Moreover, efficiency of enterolysin A against pathogenic bacteria makes it a suitable candidate for their application in veterinary medicine, alternative antimicrobial compounds or bio-preservatives in food or feed.

Keywords: Enterococcus faecalis; PCR; Bacteriocin; Enterolysin A.

1. INTRODUCTION

Enterococci are one of the gastro-intestinal tract commensally bacteria in humans and animals. In gastrointestinal tract of animals, bacteria such as enterococci and yeast maintain a niche that produces healthy benefits to the host. Enterococci belong to the lactic acid bacteria and they are well-known to produce bacteriocins (enterocins) that are synthesized by ribosomes and released in extra cellular matrix. Several studies indicate the applicability of enterocins or enterocin-producing strains to control/reduce pathogenic bacteria in the gastrointestinal tract of animals [1,2,3].

Because of this activity against food-borne pathogens and consumer demands for more natural preservatives, enterocins have recently come into limelight for their potential as bio-preservatives in food or feed [4,5,6,7].

Enterocins produced *by Enterococcus faecium, Enterococcus faecalis* and *Enterococcus mundtii* include the well characterized enterocins A, P, CRL35, 1071A and B, mundticin, mundticin KS, bacteriocin 31, RC714, T8 and enterolysin A. Enterolysin A is a bacteriocin produced by *E. faecalis* LMG 2333 and DPC5280 [1]. It is a large (calculated molecular weight of 34,501 Da), heat labile bacteriocin and therefore meets the general characteristics of class IV bacteriocins [8]. Enterolysin A, as indicated by the similarity to moralytic enzymes, possesses cell-wall hydrolyzing activity. Although, the efficiency of enterocins against food spoilage and pathogenic bacteria (*Listeria sp., Staphylococcus sp., Bacillus sp., Clostridium sp., Escherichia coli*) in various food systems is well demonstrated, only little information is available on the role of these bacteriocins in the animal ecosystem [9].

This study provides data on the occurrence of enterolysin A structural genes and antimicrobial activity of this bacteriocin among *E. faecalis* strains isolated from different sources (animal feces, feed and surface water).

2. MATERIAL AND METHODS

2.1 Bacterial Strains and Cultures Conditions

Enterococci strains were collected from 68 samples of different sources during May 2011 until February 2012.

The strains were collected from three sources, including: a) animal feces involving: chicken (n = 11), dog (n = 8), sheep (n = 8), horse (n = 7), goose (n = 6), lovebird (n = 5), duck (n = 4), rodent (n = 3); b) feed; grass silage, (n = 8) and c) surface water; pond water (n = 8).

Each fecal sample (1 g) was diluted 1:10 in phosphate-buffered saline (pH 7.4) and vortexed. One hundred micro liters of the diluted fecal samples, 100 micro liters of pond water specimens and 1 gram of each feed samples were enriched in Brain Heart Infusion broth tubes and incubated for 24 h at 37°C [10]. A loopful of each tube was sub cultured onto Bile Aesculin Azide agar medium (Merck, Darmstadt, Germany). After 24 hours at 37°C, the formation of dark brown to black colonies indicating aesculin hydrolysis was observed. A single colony of each positive culture was frozen in glycerol medium at -70°C.

2.2 Identification Using Standard Biochemical Tests

These black colonies were selected for Gram-staining, growth at 6.5% NaCl, Pyrrolidonyl amino peptidase (PYR) and catalase tests. Isolates exhibiting *Enterococcus* characteristics were identified to the species level using the following biochemical tests: acid production of L-arabinose, lactose, D sorbitol, D-mannitol, L-sorbose, glucose, methyl-α-D-glucopyranoside, arginine dihydrolase, motility, hippurate hydrolysis, haemolysis, pigmentation, tetrazolium 0.01% and tellurite 0.04% reduction [11,12].

2.3 Molecular Detection of Genus and Species of Isolated Bacteria

DNA extractions of Enterococci isolates were performed by mi-Bacterial Genomic DNA Kit (Metabion, Planegg/Martinsried, Germany). A single colony of each isolate was inoculated into 10 mL of Luria–Bertani broth (pH 7.5). After 18 h, two mL of each sample were used to DNA extraction with a commercial kit.

The *Enterococcus* genus primers, E_1 and E_2 (Metabion, Planegg/Martinsried, Germany), are from Deasy et al. [13] and *E. faecalis* species primers (FL_1 and FL_2) are from Jackson et al. [10]; (Table 1).

All the PCR reactions were carried out in a 25- μL volume that consisted of 18.5 μL of water, 2 μL of a 10_ reaction buffer, 0.3μL of $MgCl_2$ (50 mM), 1 μL of dNTPs (10 mM), 1 L of each primer (50 mM), 0.2μL of Taq DNA polymerase (mi-E8001S, Metabion, Planegg/Martinsried, Germany), and 1 μL of template DNA. The reaction conditions were as follows: an initial denaturation at 95°C for 4 min, products were amplified by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. Amplification was followed by a final extension at 72°C for 7 min [10]. Four micro liters of PCR products were electrophoresed on a 1% agarose gel containing 2 g of ethidium bromide/ml and visualized and photographed using a Gel Doc imaging system. DNA molecular weight marker (100 bp – 1500bp) was used as the standard (mi-E8200 Metabion, Planegg/Martinsried, Germany).

Table 1. Primers list

Primer	Sequence (5' 3') →	Taxon	Gene	Target	Product size (bp)
E_1	TCAACCGGGGAGGGT	*Enterococcus*	16S	Genus	733
E_2	ATTACTAGCGATTCCGG		rRNA		
FL_1	ACTTATGTGACTAACTTAACC	*E. faecalis*	sodA	Species	360
FL_2	TAATGGTGAATCTTGGTTTGG				
PCELF	CGATTTCTGTTGTAGGAACC	Enterolysin A	enlA	Bacteriocin	1770
PCELR	GTACATCTCCATATACTTTTCC				

E1 and E2 Primer sequences taken from Deasy et al. [13].
FL1 and FL2 Primer sequences taken from Jackson et al. [10].
PCELF and PCELR Primer sequences (forward and reverse, respectively) taken from Hickey et al. [1].

2.4 Molecular Diagnosis of Enterolysin A (Enl A) Structural Gene in Isolated *E. Faecalis* Strains

The enterolysin A structural gene primers PCELF andPCELR (Metabion, Planegg/Martinsried, Germany), are from Hickey et al. [1]. PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 5 min, products were amplified by 35 cycles of denaturation at 95°C for 1 min, annealing at 56.5°C for 1 min, and elongation at 72°C for 1 min. Amplification was followed by a final extension at 72°C for 10 min. DNA molecular weight marker (300 bp – 10000bp) was used as the standard (mi-M8201 Metabion, Planegg/Martinsried, Germany).

2.5 Bacteriocin Activity Assays

Bacteriocin activity was assayed using a deferred antagonism assay, in which a colony of the enl A gene positive strains was spotted onto the MRS agar plates (pH; 6.5) included 2% glucose and incubated at 37°C overnight. These plates were then overlaid with Mueller Hinton soft agar (0.7%) seeded with 0.250 ml of indicator strains (overnight culture, OD_{600} = 1) and incubated at 37°C for 10 h. Bacteriocin sensitivity was observed as clear zones of growth inhibition surrounding the producer strains[14].

The principal indicator strains included: *Salmonella enterica*PTCC1709*, Enterococcus faecalis* PTCC1206*, Staphylococcus aureus*ATCC6538*, Escherichia coli* ATCC8739and laboratory isolates*: Bacillus cereus, Bacillus subtilis, Listeria Monocytogenesis* and *Pseudomonas aeruginosa.*

3. RESULTS AND DISCUSSION

The results of Polymerase Chain Reaction have been shown that *E. faecalis* species is present in all samples (Fig. 1).

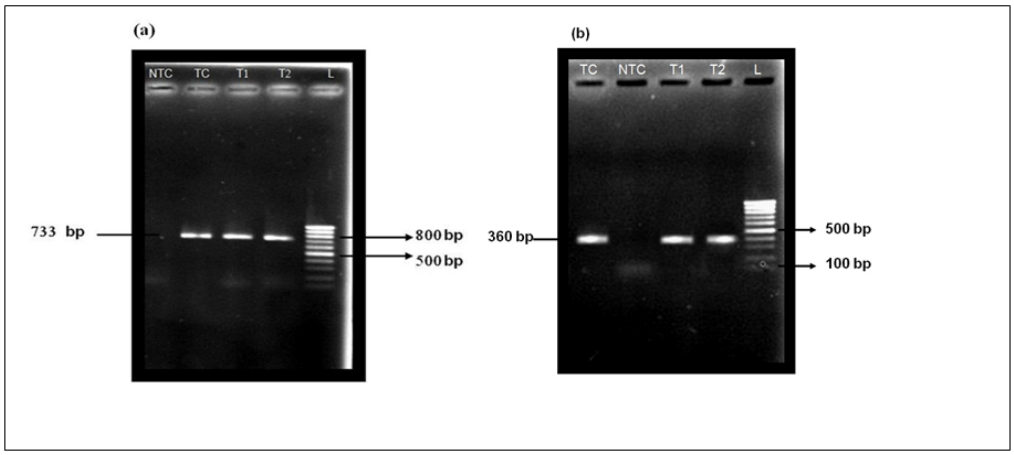

Fig. 1. Electrophoresis gel of *E. faecalis* genus and species PCR products. (a) Electrophoresis gel of Enterococcus genus PCR products. The band at 733 bp is the product of the Enterococcus genus primers. NTC, no template control; TC, template control (*E. faecalis* PTCC1237); T1, template 1; T2, template 2; L, 1000 bp ladder. (b) Electrophoresis gel of E.faecalis species PCR products. The band at 360 bp is the product of the *E. faecalis* species primers. TC, template control (*E. faecalis* PTCC1237); NTC no template control; T1, template 1; T2, template 2; L, 1000 bp ladder.

3.1 Molecular Identification of Enterolysin A Producing Isolates

According to the results of the Polymerase Chain Reaction, enterolysin A structural gene was detected in 20 strains (29.4%) from a target of 68 *E. faecalis* strains (Fig. 2). In addition, the presence of enl A structural gene was detected in all three tested sources.

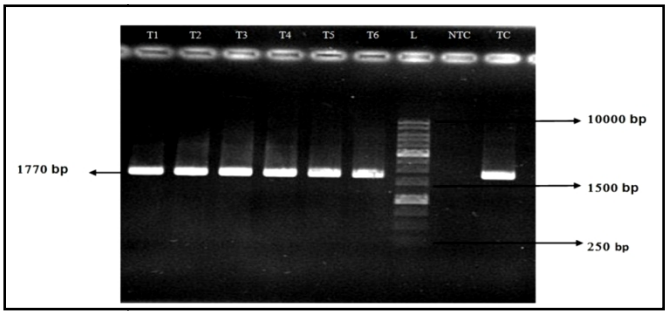

Fig. 2. Electrophoresis gel of enterolysin A structural gene PCR products. The bands at 1770 bp are the product of the enl A structural gene primers. T1- T6, templates 1-6; L, 10000 bp ladder; NTC no template control; TC, template control (*Enterococcus faecalis* LMG 2333).

Different frequencies of the enterolysin A gene occurrence was detected in the strains according to their origin (Table 2). The strains from silage and lovebirds showed the highest frequencies of enterolysin A gene presence (50% and 40%, respectively). Only strains isolated from rodents did not possess the enl A structural gene. In summary, the ecosystems

with the highest frequencies of the enterolysin A gene occurrence were silages (50% of PCR+ strains) followed by lovebirds (40%) and chickens (36.4%). On the contrary, the ecosystems of rodents (0%) and pond waters (12.5%) showed the lowest frequencies of the structural enterolysin A gene occurrence. Finally, the general comparison of the strains in connection with the presence of enl A in three different ecosystems (animals feces, feed and surface water) showed that the strains from the feed origin possessed enl A structural gene with the highest frequency (50), followed by the strains from animal feces origin (28.8%) and the strains from the surface water origin (12.5%).

Table 2. The occurrence of enterolysin A structural gene among enterococci according their origin

Source/Origin	Number of tested E. faecalis strains	Number of strains possessing the enl A gene	Percentage of strains possessing the enl A gene
Animal feces			
Chicken	11	4	36.4%
Dog	8	2	25%
Duck	4	1	25%
Goose	6	2	33.3%
Horse	7	2	28.6%
Lovebird	5	2	40%
Rodent	3	0	0%
Sheep	8	2	25%
Feed			
Grass silage	8	4	50%
Surface water			
Pond water	8	1	12.5%

3.2 The Inhibitory Spectrum of Enl A Gene + *E. faecalis* Strains Supernatants According Their Origins

A number of bacteria were tested for sensitivity to inhibitory substance(s) produced by enl A gene + strains using the overlay assay. The average inhibition zone of enl A gene + *E. faecalis* cultures in each origin have been shown at Table 3.

3.3 The Distribution of Enterolysin a Structural Gene

Most bacteria in every ecosystem produce an extraordinary array of microbial defense systems that their production in difficult conditions (e.g. un-rich environment or with competitive micro-organisms) will be increased. These systems include classical antibiotics, metabolic by-products, lytic agents, numerous types of protein toxins and bacteriocins [15].

The bacteriocins are found in almost allbacterial species and more than ten kinds of bacteriocins can be produced in a bacterial species. The bacteriocins produced by Lactic Acid Bacteria have a narrow inhibitory spectrum [16] however the bacteriocins of *Enterococcus* sp. isolates exhibited a broad inhibitory spectrum including Gram negative bacteria. So much interest in enterococci has been raised in the last decades and most of in vitro studies deal with the ability of enterococci to produce bacteriocins (spectrum of inhibitory activity, physicochemical properties of bacteriocin, their application in food products, etc.) [4].

Table 3. The antimicrobial activity of enlA gene + *E. faecalis* cultures against indicator bacteria by overlay assay

Source/ origin	Indicator bacteria								
	Strain	***B. cereus***	***B. subtilis***	***E. coli* ATCC8739**	***E. faecalis PTCC1206***	***L. Monocytogenesis***	***P. aeruginosa***	***S. enterica PTCC1709***	***S. aureus ATCC6538***
Chicken	*E. faecalis* N3	++	++	NZ	++	+++	+++	++	+
	E. faecalis N4	++	+	NZ	+	+++	++	++	+
	E. faecalis N8	++	++	NZ	+	++	+++	++	NZ
	E. faecalis N11	+	++	NZ	++	++	++	++	NZ
Dog	*E. faecalis* N17	+	++	NZ	++	++	++	+	NZ
Duck	*E. faecalis* N20	++	NZ	NZ	+	+++	++	++	NZ
	E. faecalis N23	+	+	NZ	NZ	++	++	++	NZ
Goose	*E. faecalis* N25	NZ	++	NZ	++	+++	++	++	+
	E. faecalis N28	NZ	++	NZ	+	+++	++	++	NZ
Horse	*E. faecalis* N33	+	++	NZ	+	+++	++	++	NZ
	E. faecalis N35	+	+	NZ	NZ	++	++	+	NZ
Lovebird	*E. faecalis* N37	+	++	NZ	++	+++	+++	+	NZ
	E. faecalis N41	+	+	NZ	+	++	+++	+	NZ
Sheep	*E. faecalis* N48	NZ	+	NZ	++	++	++	++	+
	E. faecalis N51	+	+	NZ	+	++	++	+	NZ
Grace silage	*E. faecalis* N54	++	++	NZ	++	+++	+++	+	+
	E. faecalis N56	+	++	NZ	+	+++	++	++	NZ
	E. faecalis N58	+	++	NZ	+	++	++	+	NZ
	E. faecalis N59	++	++	NZ	NZ	+++	++	++	+
Pond water	*E. faecalis* N64	+	NZ	NZ	++	++	++	++	NZ

NZ, No zone; +, 1–5 mm; ++, 5–10 mm; +++, 10 mm and over.

Several studies have revealed that different strains of a species (e.g. *E. faecalis*) can produce similar types of bacteriocins. Despite the importance of bacteriocin-producing strains with vitality in the gastro-intestinal systems of humans and animals, little information has been recorded about the distribution of a class IV bacteriocin "enterolysin A" gene in a target of strains from different sources [17].

Our report on Enterolysin A production by environmental and feces *E. faecalis* strain demonstrates the diversity of production of this bacteriocin among these bacteria and for the first time among bacteria from different ecosystems as well.

According to this study, enterolysin A structural gene is prevalent in enterococcal isolates of all three tested ecosystems and also enl A gene abundance is different based on their ecosystem origin. *E. faecalis* strains of feed samples, animal feces, and surface water had thehighest frequency of enl A structural gene, respectively. Based on our results, the strains with the origin of animal feces showed 28.8% presences of enl A structural gene.

Despite the lack of information, Nigutova et al. [17] investigated the occurrence of enlA homologues among ruminal Gram-positive cocci and reported that enterolysin A structural genes were detected in approximately one/sixth (16.7%) of tested Gram-positive ruminalcocci strains.

These differences between reports are because of their use of a collection of Gram-positive ruminalcocci strains instead of our use of just *E. faecalis* strains in experiments.

The distribution of enterolysin A structurel gene is less than that of enterocin A (a small heat-stable class II bacteriocin) structural gene. The structural gene for enterocin A was detected in nearly 70% of tested strains by Morovsky et al. [18].

Less distribution of enl A gene can be due to large size and chromosomal encoding of enterolysin A structural gene in comparison to enterocin A structural gene that located in plasmid [19]. On the contrary, the wide enterocin A gene distribution may be due to remarkable ability of enterococci to disseminate and receive genetic material between strains but also between genera (e.g. between staphylococci and enterococci) [20] and use of gene transfer mechanisms include conjugative (pheromone responsive with high-frequency of transfer) and non-conjugative plasmids, as well as conjugative transposons [21].

To achieve reliable results examination of larger collections of strains from different geographic areas and comparative independent studies are needed.

3.4 Antibacterial activity of Enterolysin A

All of the strains having enterolysin A structural gene were selected for screening of bacteriocin activity. A prescreening for bacteriocin production in enterolysin A structural gene positive strains showed that 100% of the strains demonstrated inhibitory activity.

In our experiments we have observed different levels of sensitivity of tested indicator strains towards the enl A gene + *E. faecalis* strains, which could be explained by coproduction of other antimicrobial components, expression of the associated immune function by these strains, penetration rate of bacteriocin to the solid medium, growth rate of indicator bacteria and plasmid stability in each strain.

The bacteriocins produced by lactic acid bacteria have a narrow inhibitory spectrum and a few reports have shown the antimicrobial activity of bacteriocin produced by LAB against Gram-negative bacteria that probably arises from the presence of the outer membrane on the cytoplasmic membrane in Gram-negative bacteria [22]. However the bacteriocins of *Enterococcus* sp. isolates exhibited a broad inhibitory spectrum including Gram negative bacteria.

According to the results of this research, enterolysin A structural gene positive strains have growth inhibitory spectrum on Gram-positive bacteria such as *L. monocytogenesis*, *B. cereus, B. subtilis* and Gram-negative bacteria such as *Salmonella enterica*PTCC1709*, Erwinia amylovora and P. aeruginosa.*

4. CONCLUSION

The bacteriocins of *Enterococcus* sp. isolates "such as Enterolysin A"exhibited a broad inhibitory spectrum including Gram positive and Gram negative bacteria. So it seems that the presence of *Enterococcus* sp. and the other Lactic Acid Bacteria in animals have a significant role inprotecting the health system from attacking pathogens. It is appealing to the food industry from the point of view that these strains exhibit such a wide range of inhibitory effects on various food borne and clinical pathogens.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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