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EVALUATION OF BACTERIOCIN ACTIVITIES AMONG ENTEROCOCCAL DAIRY ISOLATES FROM NORTH-WEST IRAN

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ABSTRACT

The objectives of the study were to isolate and characterize the bacteriocin producing enterococci in 100 enterococcal dairy isolates from north-west of Iran and to determine the prevalence of enterocin structural genes among them. The obtained isolates were assessed for antibacterial action against 6 indicator strains. The PCR method was applied to detect previously identified enterocin genes. Our results showed that 11 (11%) of enterococcal isolates were considered as potential bacteriocinogenic strains. Furthermore, genes encoding diverse bacteriocins are fairly distributed among dairy enterococci. Enterocin A, enterocin LA50A/B and cytolysin were the most abundant structural genes detected in bacteriocinogenic strains. This work is the first survey on the prevalence of bacteriocin genes among dairy enterococci in Iran.

Keywords: Prevalence, Genes, Bacteriocin, Enterococci, Dairy

INTRODUCTION

Antimicrobial peptides (AMPs) produced by a variety of microorganisms are of great importance as novel antibacterial alternatives **(Haynes et al. 1999; Overbye and Barrett 2005)** to combat antibiotic resistant bacteria **(Johnson et al. 1999; Cornelis 2008)**. A

probiotic by producing antimicrobial agents such as organic acids and bacteriocins can avoid pathogens to infect the intestinal tract. Bacteriocins have bactericidal or bacteriostatic action against bacteria closely related to producer strains **(Jack et al. 1995; Nes et al. 2007a; Shin et al. 2008)**.

The bacteriocins encompass a number of properties which make them exceptional candidates for becoming a new generation of antimicrobials, (i) their high potency, (ii) the narrow inhibition spectrum of some AMPs and their potential to develop pathogen-specific compounds (iii) yet other AMPs have broader activity spectra and can be applied in more common approaches, (iv) The high stability to many proteases, high temperature and pH variation, and (iv) the AMPs are open to bioengineering, leading to develop novel variants of the antimicrobials by peptide synthesis or DNA recombinant technology.

Overall, Bacteriocins are categorized into three main groups **(Klaenhammer 1993; Nes et al. 1996)**. Class I or lantibiotics (from lanthionine-containing bacteriocins) consist of small post translationally modified peptides (<5 kDa) and have the unusual amino acids lanthionine and methyl-lanthionine **(Guder et al. 2000)**. Class II include small (<10 kDa) heat-stable membrane active peptides which are the largest and most diverse group of bacteriocins among Gram-positive bacteria. They are typically cationic and amphiphilic and/or hydrophobic **(Nes et al. 1996)** which are further subdivided into three subclasses **(Klaenhammer 1993)**: Class IIa consists of pediocin-like peptides with a strong antibacterial activity against listeria and enterococci **(Ennahar et al. 2000)**. Class IIb are included the bacteriocins whose their optimal action is rely on the action of two different peptides **(Oppegard et al. 2007)**. Other bacteriocin subgroups are the leaderless peptide and the circular bacteriocin **(Nes et al. 2007a)**. Class III bacteriocins or bacteriolysins are big in size (>30 kDa) and heat-labile proteins **(Cotter et al. 2005; Drider et al. 2006)**.

Biosynthesis of bacteriocins generally requires four types of genes, and these are often found in the same genetic locus: (1) the structural gene(s) encoding the bacteriocin itself (2) an immunity system. The AMP producer needs to encode an immunity mechanism to keep away from being killed by its own AMP. (3) A transport system. To be active outside the cell, the AMPs should be exported across the cell envelope. 4) Genes in charge for post-translational modifications (PTMs).

The mode of action of bacteriocins differs deeply between peptides, and is often

different from classical antibiotics. In general, the bacteriocins can eradicate sensitive bacteria by two methods; in the first mechanism, they may target the cell envelope by producing pores or hindering cell wall biosynthesis. In the second method, they may act intracellularly to inhibit essential enzymatic/metabolic processes such as DNA synthesis, transcription and translation which are found only among Gram-negative bacteriocins. Conversely, both Gram-negative and Gram-positive bacteriocins are targeting the cell envelope by permeabilizing the membrane or affection the cell wall.

Enterococci from lactic acid bacteria have a significant position in human and animal health both as nosocomial pathogens and potential probiotics **(Russell and Mantovani 2002; Foulquié Moreno et al. 2006; Poeta et al. 2006)**. Their pathogenicity arises from virulence factors such as cytolysin, gelatinase, aggregation substance, extracellular surface proteins and other adhesions. Furthermore, they can harbor multiple antibiotic resistant genes and spread resistance to other bacterial strains which is another worry with regard to use of enterococci in food fermentation or as probiotics **(Franz et al. 1999; Semedo et al. 2003; Poeta et al. 2006)**. Most of the characterized bacteriocins from *Enterococcus* spp. belong to the group II bacteriocins.

Bacteriocinogenic strains from Iran, especially of *Enterococcus* species, are poorly described. In our previous work, we performed a survey on the prevalence of bacteriocins of *Enterococcus* spp. isolates originated from different clinical samples in North-Western Iran **(Hassan et al. 2012)**. In the present study we aimed to investigate the prevalence of *Enterococcus* spp. isolates from dairy products of North-Western region of Iran. The antibacterial activity of the isolates, physicochemical characteristics and presence of bacteriocin genes as well as presence of certain virulence genes and antibiotic resistance pattern of bacteriocin producers have also been studied.

MATERIALS AND METHODS

Isolation and Phenotypic Characterization of the Enterococcus Genus

Enterococci were identified according to Manual of Clinical Microbiology **(Murray et al. 2003)** as follow: Milk samples collected from industrial dairy farm of West Azerbaijan provinces of Iran during 2012 to 2014 were inoculated in 20 mL of Bile Esculin Broth for 24 h at 37 ºC. Then, 20 µL of cultures which had turned black as a result of growth of the enterococcal cells, were collected and plated onto Bile-Esculin sodium azaid agar. Following the incubation at 37 ºC single black colonies were collected and kept in 30%

glycerol at -20 ºC as well as -80 ºC as stocks. The isolates were also subjected for enterococcal phenotypic characterization tests. Growth at 10 °C and 45 °C for 7 days, growth in modified Brain Heart Infusion broth (BHI, Scharlau, Spain) at apparent pH 4 and 9 containing 6.5% NaCl and Esculin hydrolysis in the presence of 40% bile salts (bile-Esculin agar, Scharlau Spain) were examined. Strains were also tested for survival at 60 °C for 30 min (Abriouel et al. 2005; Line et al. 2008).

Screening for Anti-Bacterial Activity of Enterococcal Isolates

The inhibitory activities of the isolates against a group of indicator strains (listed in **Table 1**) were evaluated by agar spot method **(Venema et al. 1995c)**. Brain Heart Infusion broth (Oxoid, Hampshire, England) supplemented with 1.5% agar (Bacto) as well as 5 mL of BHI agar 0.7% was applied as base and top agar respectively. The top agar medium was inoculated with 20 µL of an overnight culture of indicator microorganisms. The different indicator strains used for the assessment of inhibitory activity included *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Listeria monocytogenes, Enterococcus faecalis* and *E. hirea.* The strains were obtained from PTCC (Persian Type Culture Collection). A single colony of each *Enterococcus* spp. clinical isolates was spotted onto a previously seeded agar plate. Subsequently, plates were incubated at 30 °C in an upright position. Isolates that showed clear zones of growth inhibition wider than 6 mm were scored positive as potential bacteriocin producers (Bac^+) and selected for further studies. Inhibitory activity of antimicrobial substances in supernatant was measured by using a microtiter plate assay , as described previously **(Fimland et al. 2000)**. *E. hirea* were applied as indicator microorganisms during the bacteriocin activity assay.

Genotypic Differentiation of Enterococcal Isolates by PCR

Total DNA was extracted by using QiaPrep MiniPrep kit (Qiagen) according to the manufacturers' protocol. The isolated DNA was used for PCR (Polymerase Chain Reaction) template in the genotypic identification. The target strains were genotyped by 16S rDNA using *Enterococcus* specific and species-specific primers **(Ke et al. 1999; Kariyama et al. 2000)** which are listed in **Table 2**. PCR amplification for genotypic identification of the isolates performed in 25 µL of reaction mixture containing 1 µL of each primer set, 5 µL of 10 × PCR buffer (Cinnagen), 2.5 mmol of $MgCl_2$, 1 U of *Taq* DNA polymerase 5 U/mL

(Cinnagen), and 200 µmol of each deoxyribonucleoside triphosphate (Cinnagen). Deionised sterile water and DNA extracted from known *Enterococcus* species substituted as the DNA template as the negative and positive controls in each PCR reaction. PCR amplifications were performed with the following thermal cycling profile, 4 min at 95 °C and followed by 35 cycles consisting of: a denaturation step for 45 s at 95 °C, annealing for 30 s at 60 °C (for universal), 54 °C (for *Enterococcus*-specific primers), 56 °C (for species-specific primers) and extension for 1 min at 72 °C, followed by a single 5 min final extension step at 72 °C (Eppendorf AG, Hamburg, Germany). The PCR products were analyzed by 1.5% agarose gel electrophoresis (Bio-Rad Power Pac Basic Singapore) which was set at 70 V for 45 min in Tris-Acetate-EDTA (TAE) buffer. The gel staining procedure was 15 min in 0.5 µg/mL of ethidium bromide visualizated by ultraviolet light (312 nm) with (RAPD PCR).

Detection of Bacteriocin Genes by PCR

Total DNA extracted from potential Bac^+ isolates (as previously described) was applied as template for PCR to search the presence of bacteriocin genes previously found between enterococci. The specific primer sets of enterocin A (*entA*), enterocin B (*entB*), enterocin P (*entP*) (du Toit et al. 2000), bacteriocin 1071 (*bac1071*), enterocin L50A/B (*entL50A/B*), enterolysin A (*enlA*), cytolysin (*cyl*) **(Solheim et al. 2009)**, bacteriocin 31 (*bac31*), bacteriocin AS-48 (*bacAS-48*) **(De Vuyst et al. 2003, Brandao et al. 2010)** are shown in **Table 2**. PCR assay was performed using the primers **(Table 2)** and DNA templates in 25 µL reaction volume that contained 12.5 µL 2x PCR Master Mix (Cinnagen), 1 µL of each primer, 1 µL of template DNA and 10.5 µL water. Cycling parameters included 4 min initial denaturation at 94 ºC, followed by 40 cycles of 45 s at 95 ºC, 30 s at 56 ºC (for *entP*, *bacAS-48*, *bac31* and *entL50A/B*), 58 ºC in the case of (*entA*) and 60 ºC (for *entB*, *bac1071*, *cyl* and *enlA*) as annealing temperature, and 35 s at 72 ºC; this was followed by 10 min at 72 ºC. Amplified PCR fragments were resolved on 1% agarose gels. Specific enterocin sequences were verified by PCR product sequencing.

Hemolytic Activity Assay

The hemolytic activity of enterococcal isolates was assessed using blood agar supplemented with 5% (v/v) horse blood. Fresh cultures of the isolates were streaked onto the plates and incubated at 37 ºC for 48 h. the colonies with distinct lysis zones, and partial lysis (greenish zones) considered as β-hemolytic and α-hemolytic respectively, and no reaction produced γ-hemolysis.

PCR Screening of Virulence Genes and Assessment of Antibiotic Resistance

Strains with detectable antimicrobial activity were further screened by PCR for the presence of virulence genes: *ace* (collagen adhesion) **(Dupre et al. 2003)**, *agg* (aggregation substance), *esp* (enterococcal surface protein) and *gelE* (gelatinase) (Eaton and Gasson 2001). PCR primers and their amplification products are listed in **Table 2**.

Also soft agar overlay disc diffusion method was used to determine antibiotic susceptibility of Bac^+ isolates according to National Committee for Clinical Laboratory Standards (NCCLS 2003). The antibiotic-containing disk (Himedia) used was vancomycin (30 µg).

RESULTS AND DISCUSSION

Isolation and Phenotypic Identification of the Enterococcal Strains

100 dairy isolates was phenotypically classified as *Enterococcus* spp., according to the results of the identification experiments. They are shown to be Gram-positive, non-motile, catalase negative cocci. They grew in modified BHI media containing 6.5% NaCl in the temperature of 10 °C to 45 °C for up to 7 days and endured in buffered media at pH 4 and 9. All isolates were able to hydrolyze Esculin in the presence of 40% bile salts **(Murray et al. 2003)**.

Screening of the Inhibitory Activity of Bacteriocin-like Substances

Isolates of *Enterococcus* spp. were assessed for production of antimicrobial substances. Eleventh of 100 enterococcus isolates (11%) showed antimicrobial activity by producing a clear growth inhibition zone on BHI agar against at least one of the indicator strains.

All of the detected Bac^+ isolates had antibacterial activities against *E. coli, B. cereus, L. monocytogenes, E. faecalis, with no activity against E. hirea* **(Table 3)**.

All of the detectable antimicrobial activities were produced only on the solid surfaces (agar plates) and no antimicrobial activity could be found in broth medium after growth **(Table 3)**. Similar observations regarding the antimicrobial activity on solid and liquid media have also been reported in previous studies **(Qi et al. 2001; Brandao et al. 2010)**. These conflicting findings might be explained by the effect of growth conditions such as pH, medium composition and temperature on regulation of bacteriocin production **(Cintas et al. 2000; Qi et al. 2001; Rojo-Bezares et al. 2007)** as well as frequent involvement of bacteriocin-like peptides which can act as peptide pheromone in the quorum-sensing regulatory mechanism of bacteriocin gene expression **(Coburn et al. 2004; Nes et al.**

2007a; Sturme et al. 2007; Lebeer et al. 2008) might explain these conflicting results.

Genotypic identification of enterococcal isolates by PCR

Genus and species identification was accomplished by PCR using the universal bacterial 16S rDNA, *Enterococcus* genus and species specific primers. 6 out of 11 Bac^+ enterococcal isolates were identified as *E. faecalis* and the rest of isolates were *E. faecium* **(Figure 1)**. These results are in good agreement with some previous studies which show that in *Enterococcus* isolates, the bacteriocinogenic phenotype was more dominant in isolates of *E. faecalis* than *E. faecium* **(del Campo et al. 2001; De Vuyst et al. 2003)**. However, there are other research **(Solheim et al. 2009)** showing that most of bacteriocin producing enterococci among healthy babies are *E. faecium*.

Screening of Structural Bacteriocin Encoding Genes in Enterococcal Isolates

The isolates with positive antibacterial activity on agar spot test were screened by PCR for the genes encoding the known bacteriocins. The results are shown in **Table 4**. *EntA* were found as the most frequent gene encoding bacteriocins, at an incidence of 100% among bac^+ strains (Fig 2). Also *entL50A/B* (87%) and *cyl* (67%) were detected frequently. Interestingly, *entB, entP, bac31, bac1071* and *bacAS-48* were not detected in any of the Bac^+ isolates.

The PCR results **(Table 4)** suggest that all of Bac^+ isolates contained multiple structural bacteriocin genes. Two different structural genes were found in seven (7 of 11) isolates while three genes were found in five isolates. Amongst the multi-bacteriocin gene isolates *entA* was the most frequent at 100% (all of the Bac^+ isolates).

According to results of earlier studies the structural gene *entA* is widely distributed in ruminal enterococci **(Morovsky et al. 2001; Nigutova et al. 2007)**. Our results indicate that the incidence of *entA* and also *entL50A/B* are frequently found in enterococcal isolates from dairy origin and are in line with these findings. Additionally, according to our previous study, same genes of bacteriocin have been found in human blood and fecal derived enterococci; it is rational to believe that because of the extensive distribution of enterococcal contamination in nature they are likely derived from the same environment and most probably from intestine of various animals **(Hassan et al. 2012)**.

Interestingly, the cytolysin structural gene was detected in 45.5% and 27.2% of *E. faecalis* and *E. faecium* isolates, respectively **(Table 4)**. Our results approve previously published studies that showed the presence of

the gene encoding cytolysin in *E. faecalis* as well as *E. faecium* **(De Vuyst et al. 2003; Theppangna et al. 2007; Poeta et al. 2008)**. As the cytolysin structural gene was initially found in clinical isolates of *E. faecalis* **(Booth et al. 1996)** it is most probable that this plasmid-encoded toxin may have been moved between two strains. Although the cytolysin gene was often found in our *E. faecalis* and *E. faecium* isolates, only three of eleven isolates exhibits the β-hemolytic activity. According to recent investigations, expression of cytolysin is affected by diverse factors such as expression regulation by a quorum-sensing mechanism **(Haas et al. 2002)** and/or oxygen conditions **(Day et al. 2003)** and our results can be explained based on this evidence.

ace (collagen adhesion) **(Dupre et al. 2003)**, *agg* (aggregation substance), *esp* (enterococcal surface protein) and *gelE* (gelatinase)

Virulence Factors and Antibiotic Resistance Pattern of Enterococcal Isolates

The presence of genes coding five virulence factors was evaluated by PCR for the Bac^+ strains. Interestingly, aggregation substance *(agg)* and enterococcal surface protein (*esp*) genes were detected in all of the isolates except C6 **(Figure 3)**. Only *E. faecium* M15 contains gelatinase *gel*E while (collagen adhesion) *ace*, aggregation substance (*agg)* and Cell wall adhesion genes were not detected in any of the screened isolates. *E. faecium* C6 was the only isolate not to contain the tested virulence genes.

The antibiotic resistance phenotype of all Bac^+ strains with antibacterial activity was tested. As expected all of the candidate strains were resistant to vancomycin.

The presence of multiple virulence factors in enterococcal isolates accompanied by the antibiotic resistance is a great worry about the human health **(Semedo et al. 2003; Kayaoglu and Orstavik 2004; Sood et al. 2008)**.

Table 1: Indicator bacteria used agar spot test

Staphylococcus aureus **PTCC[§] 1112**
Listeria monocytogenes **PTCC 1249**
Bacillus cereus**PTCC 1015**
Escherichia coli**PTCC 1533**
Enterococcus faecalis **PTCC 1237**
Enterococcus hirea **PTCC 1239**
[§]PTCC= Persian Type Culture Collection

Table 2: PCR primers for detection of bacteriocin genes used in this study

Primer	Sequence (5´-3´)	Fragment size(bp)	References
Universal	f: AAYATGATIACIGGIGCIGCICARATGGA	602	Ke et al (1999)

amplification	**r: AYRTTITCICCIGGCATIACCAT**		
***Enterococcus* specific**	**f: TACTGACAAACCATTCATGATG r: AACTTCGTCACCAACGCGAAC**	**112**	**Ke et al (1999)**
E.faecalis	**f: ATCAAGTACAGTTAGTCTTTATTAGr:ACGATTCAAA GCTAACTGAATCAGT**	**941**	**Kariyama et al(2000)**
Enterocin A	**f: AAATATTATGGAAATGGAGTGTAT r: GCACTTCCCTGGAATTGCTC**	**126**	**Du Toit et al (2000)**
Enterocin B	**f: GAAAATGATCACAGAATGCCTA r: GTTGCATTTAGAGTATACATTTG**	**159**	**Du Toit et al (2000)**
Enterocin P	**f: TATGGTAATGGTGTTTATTGTAA r: ATGTCCCATACCTGCCAAAC**	**121**	**Du Toit et al (2000)**
Bacteriocin 31	**f: CCTACGTATTACGGAAATGGT r: GCCATGTTGTACCCAACCATT**	**248**	**De Vuyst et al (2003)**
Enterocin L50A/B	**f: TTGGGTGGCCTATTGTTAAA r: TCTATTGTCCATCCTTGTCCA**	**224**	**Cintas et al (1998)**
Bacteriocin 1071	**f: ATGCTGTAGGTCCAGCTGC r: TTTCCAGGTCCTCCACCAGT**	**210**	**Ben Omar et al (1996)**
Bacteriocin AS-48	**f: GAGGAGTATCATGGTTAAAGA r: ATATTGTTAAATTACCAA**	**339**	**De Vuyst et al (2003)**
Enterolysin A	**f: CGCAGCTTCTAATGAGTGGT r: CATACACACTGCCATTTCCA**	**161**	**Nilsen et al (2003)**
Cytolysin	**f: TGGCGGTATTTTTACTGGAG r: TGAATCGCTTCCATTTCTTC**	**186**	**Gilmore et al (1994)**

Table 3: The inhibitory spectra of Bac^{+} strains against selected indicator bacteria

Isolate No	**Inhibition zone diameter (mm) with indicator strains**					
	***E. feacalis* PTCC 1237**	***E. hirea* PTCC 1239**	***Li. mono.* PTCC 1249**	***S. aureus* PTCC 1112**	***E. coli* PTCC *1533***	***B. cereus* PTCC *1015***
C5	**-**	**-**	**6**	**-**	**-**	**-**
C6	**-**	**-**	**7**	**-**	**-**	**-**
C7	**-**	**-**	**-**	**-**	**6**	**6**
C8	**-**	**-**	**-**	**-**	**-**	**8**
C9	**-**	**-**	**7**	**-**	**-**	**-**
M10	**7**	**-**	**-**	**6**	**-**	**-**
M11	**-**	**-**	**-**	**-**	**6**	**-**
M12	**-**	**-**	**7**	**-**	**-**	**6**
M13	**-**	**-**	**-**	**-**	**7**	**-**
M14	**9**	**-**	**8**	**-**	**-**	**-**
M15	**7**	**-**	**6**	**-**	**9**	**-**

Table 4: Prevalence of bacteriocin structural genes and virulence factors genes in enterococcal isolates

Isolate no	**Source**	**Enterococcus spp***	**Hemolysis**	**Enterococcus Genes****	**Virulence factor Genes*****
C5	**Cheese**	**E. *fs***	**γ**	**A, LA**	**as**
C6	**Cheese**	**E. *fm***	**α**	**A,CY**	**-**
C7	**Cheese**	**E. *fs***	**α**	**A, LA, LY**	**as, sp**
C8	**Cheese**	**E. *fm***	**β**	**A,CY**	**as**
C9	**Cheese**	**E. *fm***	**α**	**A, LA**	**as, sp**
M10	**Milk**	**E. *fs***	**γ**	**A, LA**	**as, sp**
M11	**Milk**	**E. *fs***	**γ**	**A, LA**	**as, sp**
M12	**Milk**	**E. *fs***	**α**	**A, LA, LY**	**as, sp**
M13	**Milk**	**E. *fs***	**α**	**A, LA, LY**	**as, sp**
M14	**Milk**	**E. *fm***	**β**	**A, LA, LY**	**as, sp**
M15	**Milk**	**E. *fm***	**β**	**A, LA, LY**	**as, sp**

NOTE: * **E. *fm*= E. *faecium*, E. *fs*= E. *Faecalis*; ** A= *entA*, CY= cytolysin, LA= *entL50A/B*, LY= *enlA***
*** **as=aggregation substance, sp= enterococcal surface protein**

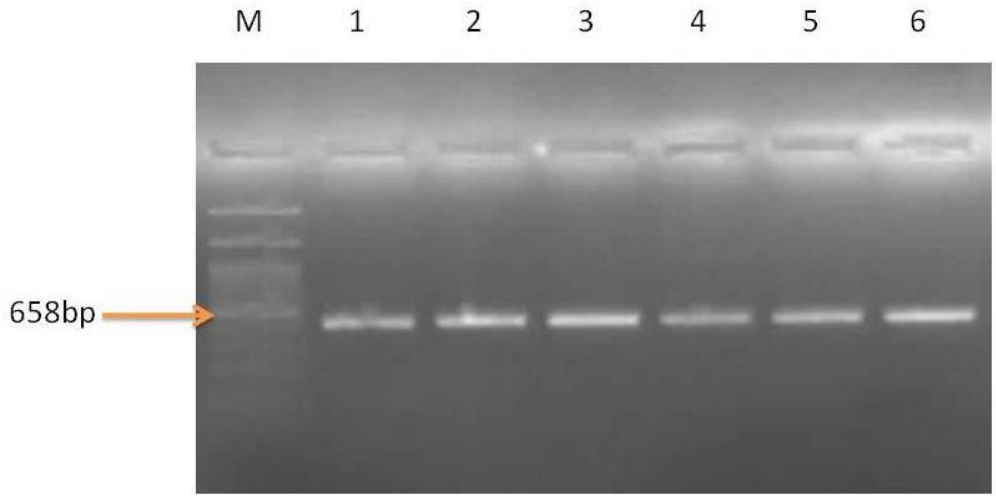

Figure 1: Agarose gel electrophoresis of PCR amplification with primers specific of specific for the *Enterococcus faecium*for 6 selected dairy isolates. (Lanes 1-6) C6, C8, C9, M14 and M15; Lane M 100-bp DNA ladder (Cinnagen).

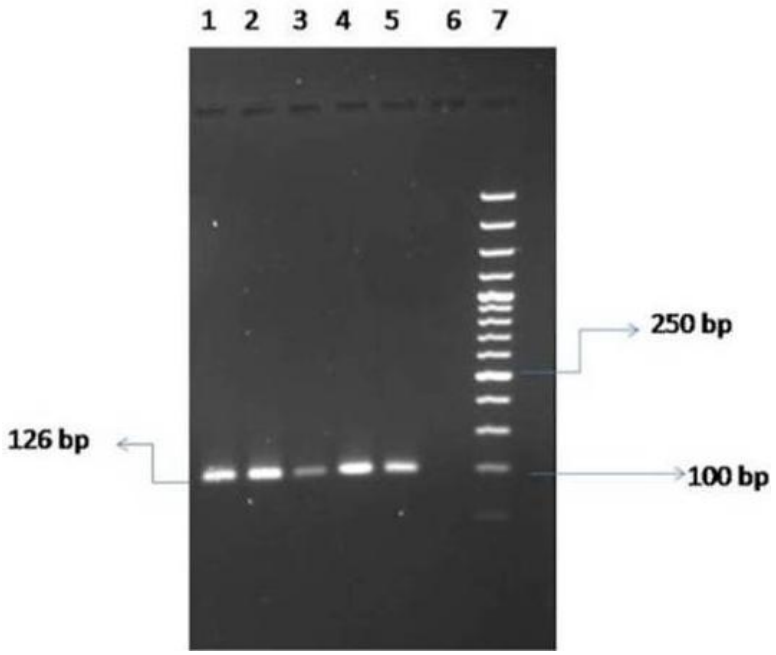

Figure 2 Agarose gel electrophoresis of PCR amplification with primers specific for enterocin Afor 5 selected dairy isolates. (Lanes 1-5) C6, C8, M12, M14 and M15; Lane M 100-bp DNA ladder (Cinnagen).

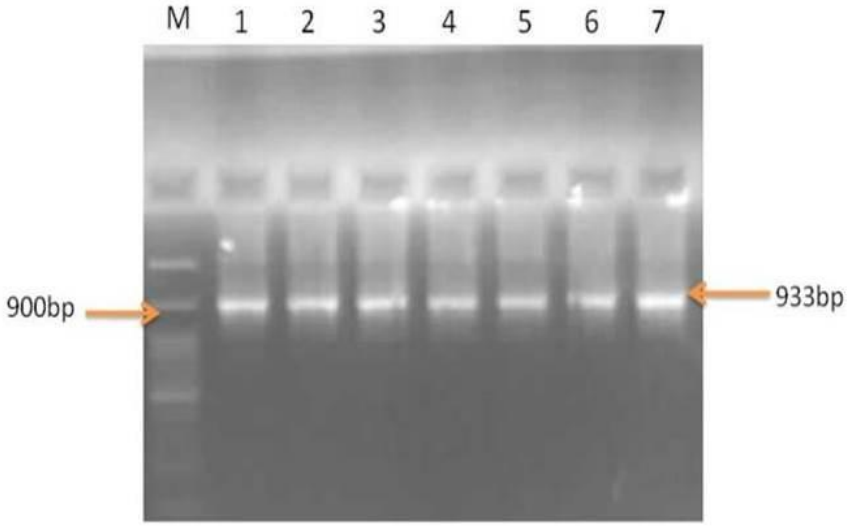

Figure 3: Agarose gel electrophoresis of PCR amplification with primers specific for enterococcal surface proteinfor 6 selected dairy isolates. (Lanes 1-6) C7, C9, M12, M13, M14 and M15; Lane M 100-bp DNA ladder (Cinnagen)

CONCLUSION

In conclusion, this work has shown that 11% of tested diary isolates of enterococci displayed antimicrobial activity against closely related species and to be considered a potential probiotic strains. *EntA*, *entL50A/B* and *cyl* were detected frequently among the strains.

Further study on their safety to human showed the presence of multiple potential virulence factors and resistance to vancomycin which could make the strains pathogenic. But, C6 isolate with antibacterial activity against indicator strain) was the only isolate with absence of the virulence genes. However, the resistance of all the tested strain to vancomycin poces alarming threat which encourage us to search for another bac^{+} strains in our future study

REFERENCES

[1] Abriouel, H., Lucas, R., Omar, N.B., Valdivia, E., Maqueda, M., Martínez-Cañamero, M., and Gálvez, A. 2005. Enterocin AS-48RJ: A variant of enterocin AS-48 chromosomally encoded by *Enterococcus faecium* RJ16 isolated from food. Syst. Appl. Microbiol. **28**(5): 383-397.

[2] Booth, M.C., Bogie, C.P., Sahl, H.G., Siezen, R.J., Hatter, K.L., and

Gilmore, M.S. 1996. Structural analysis and proteolytic activation of Enterococcus faecalis cytolysin, a novel lantibiotic. Mol. Microbiol. **21**(6): 1175-1184.

[3] Brandao, A., Almeida, T., Munoz-Atienza, E., Torres, C., Igrejas, G., Hernandez, P.E., Cintas, L.M., Poeta, P., and Herranz, C. 2010. Antimicrobial activity and occurrence of bacteriocin structural genes in *Enterococcus* spp. of human and animal origin isolated in Portugal. Arch. Microbiol. **192**(11): 927-936. doi:10.1007/s00203-010-0619-z.

[4] Cintas, L.M., Casaus, P., Herranz, C., Havarstein, L.S., Holo, H., Hernandez, P.E., and Nes, I.F. 2000. Biochemical and genetic evidence that Enterococcus faecium L50 produces enterocins L50A and L50B, the sec-dependent enterocin P, and a novel bacteriocin secreted without an N-terminal extension termed enterocin Q. J. Bacteriol. **182**(23): 6806-6814.

[5] Coburn, P.S., Pillar, C.M., Jett, B.D., Haas, W., and Gilmore, M.S. 2004. Enterococcus faecalis senses target cells and in response expresses cytolysin. Science **306**(5705): 2270-2272. doi:10.1126/science.1103996.

[6] Cornelis, P. 2008. Pseudomonas: Genomics and Molecular Biology first ed. Caister Academic Press.

[7] Cotter, P.D., Hill, C., and Ross, R.P. 2005. Bacteriocins: Developing innate immunity for food. Nat. Rev. Microbiol. **3**(10): 777-788. doi:10.1038/nrmicro1273.

[8] Day, A.M., Cove, J.H., and Phillips-Jones, M.K. 2003. Cytolysin gene expression in Enterococcus faecalis is regulated in response to aerobiosis conditions. Mol. Genet. Genomics. **269**(1): 31-39. doi:10.1007/s00438-003-0819-1.

[9] De Vuyst, L., Foulquié Moreno, M.R., and Revets, H. 2003. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. Int. J. Food. Microbiol. **84**(3): 299-318.

[10] del Campo, R., Tenorio, C., Jimenez-Diaz, R., Rubio, C., Gomez-Lus, R., Baquero, F., and Torres, C. 2001. Bacteriocin production in vancomycin-resistant and vancomycin-susceptible *Enterococcus* isolates of different

origins. Antimicrob. Agents. Chemother. **45**(3): 905-912. doi:10.1128/AAC.45.3.905-912.2001.

[11] Drider, D., Fimland, G., Héchard , Y., McMullen, L.M., and Prévost, H. 2006. The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev. **70**(2): 564-582. doi:10.1128/MMBR.00016-05.

[12] du Toit, M., Franz, C.M., Dicks, L.M., and Holzapfel, W.H. 2000. Preliminary characterization of bacteriocins produced by Enterococcus faecium and Enterococcus faecalis isolated from pig faeces. J. Appl. Microbiol. **88**(3): 482-494.

[13] Dupre, I., Zanetti, S., Schito, A.M., Fadda, G., and Sechi, L.A. 2003. Incidence of virulence determinants in clinical Enterococcus faecium and Enterococcus faecalis isolates collected in Sardinia (Italy). J Med Microbiol **52**(Pt 6): 491-498.

[14] Eaton, T.J., and Gasson, M.J. 2001. Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microbiol **67**(4): 1628-1635. doi:10.1128/AEM.67.4.1628-1635.2001 [doi].

[15] Ennahar, S., Sashihara, T., Sonomoto, K., and Ishizaki, A. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24**(1): 85-106.

[16] Fimland, G., Johnsen, L., Axelsson, L., Brurberg, M.B., Nes, I.F., Eijsink, V.G., and Nissen-Meyer, J. 2000. A C-terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. J. Bacteriol. **182**(9): 2643-2648.

[17] Foulquié Moreno, M.R., Callewaert, R., Devreese, B., Van Beeumen, J., and De Vuyst, L. 2003. Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. J. Appl. Microbiol. **94**(2): 214-229.

[18] Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., and De Vuyst, L. 2006. The role and application of enterococci in food and health. Int. J. Food. Microbiol. **106**(1): 1-24. doi:10.1016/j.ijfoodmicro.2005.06.026.

[19] Franz, C.M., Holzapfel, W.H., and Stiles, M.E. 1999. Enterococci at the crossroads of food safety? Int J Food Microbiol **47**(1-2): 1-24. doi:S0168-1605(99)00007-0 [pii].

[20] Guder, A., Wiedemann, I., and Sahl, H.G. 2000. Posttranslationally modified bacteriocins -the lantibiotics. Biopolymers **55**(1): 62-73. doi:10.1002/1097-0282(2000)55:1.

[21] Haas, W., Shepard, B.D., and Gilmore, M.S. 2002. Two-component regulator of Enterococcus faecalis cytolysin responds to quorum-sensing autoinduction. Nature **3**(415): 84-87. doi:10.1038/415084a.

[22] Hassan M, Diep DB, Javadzadeh Y, Dastmalchi S, Nes IF, Sharifi Y, Yari S, Farajnia S, Lotfipour F. Prevalence of bacteriocin activities and bacteriocin-encoding genes in enterococcal clinical isolates in Iran. Can J Microbiol. 2012 Apr;58(4):359-68.

[23] Haynes, R.J., Tighe, P.J., and Dua, H.S. 1999. Antimicrobial defensin peptides of the human ocular surface. The British journal of ophthalmology **38**(6): 737-741.

[24] Jack, R.W., Tagg, J.R., and Ray, B. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59**(2): 171-200.

[25] Johnson, S., Samore, M.H., Farrow, K.A., Killgore, G.E., Tenover, F.C., Lyras, D., Rood, J.I., DeGirolami, P., Baltch, A.L., Rafferty, M.E., Pear, S.M., and Gerding, D.N. 1999. Epidemics of diarrhea caused by a clindamycin-resistant strain of Clostridium difficile in four hospitals. N. Engl. J. Med. **341** (22): 1645-1651. doi:10.1056/NEJM199911253412203.

[26] Kariyama, R., Mitsuhata, R., Chow, J.W., Clewell, D.B., and Kumon, H. 2000. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J. Clin. Microbiol. **38**(8): 3092-3095.

[27] Kayaoglu, G., and Orstavik, D. 2004. Virulence factors of Enterococcus faecalis: relationship to endodontic disease. Crit Rev Oral Biol Med **15**(5): 308-320. doi:15/5/308 [pii].

[28] Ke, D., Picard, F.J., Martineau, F., Menard, C., Roy, P.H., Ouellette, M., and Bergeron, M.G. 1999. Development of a PCR assay for

rapid detection of enterococci. J. Clin. Microbiol. **37**(11): 3497-3503.

[29] Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**: 39-85.

[30] Lebeer, S., Vanderleyden, J., and De Keersmaecker, S.C.J. 2008. Genes and molecules of lactobacilli supporting probiotic action. Microbiol. Mol. Biol. Rev. **72**(4): 728-764. doi:10.1128/MMBR.00017-08.

[31] Line, J.E., Svetoch, E.A., Eruslanov, B.V., Perelygin, V.V., Mitsevich, E.V., Mitsevich, I.P., Levchuk, V.P., Svetoch, O.E., Seal, B.S., Siragusa, G.R., and Stern, N.J. 2008. Isolation and purification of enterocin E-760 with broad antimicrobial activity against Gram-positive and Gram-negative bacteria. Antimicrob. Agents. Chemother. **52**(3): 1094-1100. doi:10.1128/AAC.01569-06.

[32] Morovsky, M., Pristas, P., and Javorsky, P. 2001. Bacteriocins of ruminal bacteria. Folia Microbiol. (Praha). **46**(1): 61-62.

[33] Murray, P.R., Baron, E.J., Jorgenson, J.J., Pfaller, M.A., and Yolken, R.H. 2003. Manual of Clinical Microbiology. 8th ed. ASM Press: Washington, DC.

[34] NCCLS. 2003. Performance standards for antimicrobial disk susceptibility tests. NCCLS document M2-A8. Approved standard, 8th ed. NCCLS, Wayne, PA: .

[35] Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink, V., and Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology **70**(2-4): 113-128.

[36] Nes, I.F., Diep, D.B., and Holo, H. 2007a. Bacteriocin diversity in Streptococcus and Enterococcus. J. Bacteriol. **189**(4): 1189-1198. doi:10.1128/JB.01254-06.

[37] Nes, I.F., Yoon, S.S., and Diep, D.B. 2007b. Ribosomally synthesiszed antimicrobial peptides (bacteriocins) in lactic acid bacteria: A review. Food Science and Biotechnology **16**(5): 675-690.

[38] Nigutova, K., Morovsky, M., Pristas, P., Teather, R.M., Holo, H., and Javorsky, P. 2007. Production of enterolysin A by rumen Enterococcus

faecalis strain and occurrence of enlA homologues among ruminal Gram-positive cocci. J. Appl. Microbiol. **102**(2): 563-569. doi:10.1111/j.1365-2672.2006.03068.x.

[39] Oppegard, C., Rogne, P., Emanuelsen, L., Kristiansen, P.E., Fimland, G., and Nissen-Meyer, J. 2007. The two-peptide class II bacteriocins: structure, production, and mode of action. J. Mol. Microbiol. Biotechnol. **13**(4): 210-219. doi:10.1159/000104750.

[40] Overbye, K.M., and Barrett, J.F. 2005. Antibiotics: where did we go wrong? Drug. Discov. Today. **10**(1): 45-52. doi:10.1016/S1359-6446(04)03285-4.

[41] Poeta, P., Costa, D., Rodrigues, J., and Torres, C. 2006. Antimicrobial resistance and the mechanisms implicated in faecal enterococci from healthy humans, poultry and pets in Portugal. Int J Antimicrob Agents **27**(2): 131-137. doi:S0924-8579(05)00302-X [pii]

[42] 10.1016/j.ijantimicag.2005.09.018 [doi].

[43] Poeta, P., Igrejas, G., Costa, D., Sargo, R., Rodrigues, J., and Torres, C. 2008. Virulence factors and bacteriocins in faecal enterococci of wild boars. J. Basic. Microbiol. **48**(5): 385-392. doi:10.1002/jobm.200700327.

[44] Qi, F., Chen, P., and Caufield, P.W. 2001. The group I strain of Streptococcus mutans, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. Appl. Environ. Microbiol. **67**(1): 15-21. doi:10.1128/AEM.67.1.15-21.2001.

[45] Rojo-Bezares, B., Saenz, Y., Navarro, L., Zarazaga, M., Ruiz-Larrea, F., and Torres, C. 2007. Coculture-inducible bacteriocin activity of Lactobacillus plantarum strain J23 isolated from grape must. Food Microbiol. **24**(5): 482-491. doi:10.1016/j.fm.2006.09.003.

[46] Russell, J.B., and Mantovani, H.C. 2002. The bacteriocins of ruminal bacteria and their potential as an alternative to antibiotics. J. Mol. Microbiol. Biotechnol. **4**(4): 347-355.

[47] Semedo, T., Santos, M.A., Lopes, M.F., Figueiredo Marques, J.J., Barreto Crespo, M.T., and Tenreiro, R. 2003. Virulence factors in food, clinical and reference Enterococci: A

common trait in the genus? Syst Appl Microbiol **26**(1): 13-22.

[48] Shin, M.S., Han, S.K., Ji, A.R., Kim, K.S., and Lee, W.K. 2008. Isolation and characterization of bacteriocin-producing bacteria from the gastrointestinal tract of broiler chickens for probiotic use. J. Appl. Microbiol. **105**(6): 2203-2212. doi:10.1111/j.1365-2672.2008.03935.x

[49] Solheim, M., Aakra, Å., Snipen, L.G., Brede, D.A., and Nes, I.F. 2009. Comparative genomics of Enterococcus faecalis from healthy Norwegian infants. BMC. Genomics **24**(10): 194. doi:10.1186/1471-2164-10-194.

[50] Sood, S., Malhotra, M., Das, B.K., and Kapril, A. 2008. Enterococcal infections & antimicrobial resistance. Indian J. Med. Res. **128**(2): 111-121.

[51] Sturme, M.H.J., Francke, C., Siezen, R.J., de Vos, W.M., and Kleerebezem, M. 2007. Making sense of quorum sensing in lactobacilli: a special focus on Lactobacillus plantarum WCFS1. Microbiology **153**(12): 3939-3947. doi:10.1099/mic.0.2007/012831-0.

[52] Theppangna, W., Murase, T., Tokumaru, N., Chikumi, H., Shimizu, E., and Otsuki, K. 2007. Screening of the enterocin genes and antimicrobial activity against pathogenic bacteria in Enterococcus strains obtained from different origins. J. vet. Med. Sci. **69**(12): 1235-1239.

[53] Venema, K., Venema, G., and Kok, J. 1995c. Lactococcal bacteriocins: mode of action and immunity. Trends Microbiol. **3**(0966-842X): 299-304.