Identification of antimicrobial producing Enterococci isolated from Iranian raw milk cheeses using cultural methods

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***Abstract*— A collection of Enterococci spp. (about 96 isolates) were isolated from two Iranian raw milk cheeses, known as Lighvan and Koozeh cheeses and identified as *Ent. faecium, Ent. faecalis, Ent. durans, Ent. casseliflavus* and *Ent. italicus* by 16S rDNA sequencing. These 96 isolates were subjected to Agar- spot and well-diffusion assay in order to detect the bacteriocin- producing ability. According to Agar- spot method, only 48 isolates out of 96, showed bacteriocin-producing ability with clear- zone production on plates against indicator organisms. With well- diffusion Assay, these numbers decreased to 20 isolates which produced clear zone. Then, these 20 isolates (strains) were subjected to rep- PCR for typing and 15 distinct rep- PCR profiles (patterns) were identified.**

Keywords-antimicrobial compounds, Lactic flora, raw milk cheese, enterococci, bacteriocin.

I. INTRODUCTION

The enterococci genus (genera) constitutes one of the main part (genera) of Lactic acid bacteria (LAB) which distribute widely in the environment, food and different ecological niches (Kayser, 2003). Among the enterococcus species, *Ent. faecium* and *Ent. faecalis* are the two most occurring in food and related habitats. The Enterococci play an important role in the development of the sensory properties of fermented foods (Martin, M., et al., 2006). Also they can be used as starter and adjunct cultures (Giraffa, G.2003; Hugas, M. et. al., 2003).

One of the best advantages of enterococi in food is production of a diverse and heterogeneous group of ribosomally synthesized antimicrobial peptides or bacteriocins, (the enterocins) with different spectrum of antagonism activities, structure, and processing and secretion mechanisms (Cintas, L.m, et al., 2001; Franz, et al., 1999; Franz, et al., 2003).

Most enterocins belong to class II of the Klaenhammer classification (Klaenhammer, T.R., 1993), although they show a considerable diversity. Many enterococcal bacteriocins are active against the food- borne pathogen *Listeria monocytogenes*, while other enterocins like enterocin AS-48, show a much broader inhibitory spectrum. Some bacteriocins are also active against Gram- positive food-borne pathogens such as *Listeria monocytogenes*, *staphylococcus aureus*, *Bacillus subtilis* and spores of *Clostridium perfringenes* (Klaenhammer 1993; Holzapfel *et al*. 1995; Stiles 1996).

The objective of this work is to screen for enterococci isolated from two Iranian raw milk cheeses with antibacterial properties and enterocin production that may be used as bio-preservative.

Most of the enterocins classified as class II bacteriocins including enterocin A (Aymerich, et al., 1996; O'Keeffe, et al., 1999), enterocin B (Casaus, P., et al., 1997;Franz, C.M.A.P. et al., 1999),enterocin P (Cintas,L.M., et al., 1997), enterocin L50 (Cintas, L.M., et al., 1998), enterocin Q (Cintas,L.M., et al., 2000), mundticin (Bennik, M.H.J., et al., 1998) .

II. METHODS AND MATERIALS

A. *Strains, Media and culture conditions*

96 isolates of *Enterococci* spp. strains isolated during the manufacture and ripening of two Iranian traditional cheeses made from raw milk (Lighvan and Koozeh) were grouped by typing and identified with (by) ARDRA, sequencing and sequence comparison. These isolates isolated from Lighvan (52) and Koozeh (44) cheeses. 15 isolates as representative were tested for the enterocin production against indicators including gram positive and gram negative bacteria. The indicator strains included *Ent. faecalis*, *Lac. lactis* MG1363, *Staphylococcus aureus* CECT 86, *Lactobacillus sakei* CECT 906, *Listeria innocua* 4202, *Lactobacillus plantarum* 748.

All cheese isolates and indicators were recovered on BHI agar, M17 agar (for Lactococci), MRS agar (for lactobacilli), or in Trytone soy broth (TSB) (for *Listeria innocua* and *S.aureus*), from -80 °C, then the plates incubated at the corresponding optimum temperature for 24-48 h.

B. *Identification and typing of isolates*

Total genomic DNA was extracted from single, isolated colonies which were suspended in 50µl of molecular grade water (sigma- Aldrich st. Louis, MO, USA), heated at 98°C for 10 min in a thermo cycler (Bio-Rad Richmond, CA, USA), and some of the isolates, cell extracts were obtained with glass beads in a Mini bead Beater apparatus (Bio spec Products, Bartlesville, OK, USA), and centrifuged for 5 min at 16000 rpm. Isolates were identified by ARDRA, followed by sequencing of representative amplicons and comparison of the sequences obtained against those in databases. Enterococci spp. was grouped by repetitive extragenic palindromic (REP) fingerprinting employing the polymerase chain reaction (PCR) and the primer BOXA2R, as reported by koeuth et al. (1995).

C. *Detection of Antimicrobial activity*

For detection of antagonistic activity, an Agar spot test and a well-diffusion assay were applied, successively. The agar spot test was a modification of Fleming et al., method (Fleming et al., 1985). Overnight cultures of the strains to be tested for production of an antimicrobial compound were spotted 5µl onto the surface of agar plates(BHI- agar +0.2%Glucose, M17- agar +0.2%Glucose) and incubated at 30°C for 24 h to allow colonies to develop. Spots were covered with 10 ml of soft-agar (0.75%) inoculated at 0.25% with indicator bacteria. The plates were incubated under the required conditions for indicator bacteria. After incubation for 24 h at 30°C, the plates were checked for inhibition zones. Those strains (isolates) produced clear zone equal to 0.5mm or larger, considered positive in this method (agar spot). Then, in the second method, well-diffusion assay, the positive strains from previous stage, were selected for bacteriocin production evaluation. In this method, firstly, indicator strains were cultured (grown) in broth medium corressponding to each indicator (MRS, M17 and BHI) overnight.

Then, 20 ml of agar medium at 45°C were mixed with 200µl of an overnight culture of the indicator strain and poured into Petri dishes. 1 ml of overnight culture of the producing strain transferred to 1.5 ml microtube and centrifuged. The resulting supernatants were neutralized to pH 6.5-7.0 with NaOH 0.1 M, centrifuged at 14000 rpm for 5 min, and filter-sterilized through a 0.20 µm pore membrane (Milli pore, Bedford, MA, USA). Wells of 3 mm in diameter were cut into these agar plates and 50 µl of each supernatant of the potential producer strain was placed into each well. The plates were incubated for 24 h under appropriate conditions and were subsequently examined for zones of inhibition.

III. Results and Discussion

A. *Identification and typing of enterococci* spp. *isolates*

Among 130 isolates from two Iranian traditional cheeses, Lighvan and Koozeh, 96 isolates were identified as Enterococci by ARDRA and sequencing of some representatives of 16S rDNA PCR-amplicons and comparison of the sequences. From these 96 enterococci spp. 52 and 44 isolates belonged to Lighvan (*Ent. faecium* (38), *Ent. faecalis* (11), *Ent. durans* (1), *Ent. italicus* (1) and *Ent. casseliflavus* (1)) and Koozeh cheese (*Ent.faecium* (36), *Ent. faecalis* (5), *Ent. durans* (1) and *Ent. casseliflavus* (2)), respectively.

B. *Antimicrobial activity of enterococci* spp.

Firstly, the production of enterocin (bacteriocin) by representative isolates of the different strains against some indicators including *Listeria. Innocua*, *Staph. aureus*, *Ent. faecalis*, *Lb. plantarum*, *Lb. sake* was analyzed by an agar spot test. Among 96 isolates, 48 isolates showed the inhibitory effect (clear zone) against the different indicator organisms (data not shown). *Lb. plantarum* CECT 748 was inhibited by 27 strains. In contrast, S. aureus CECT 86, was inhibited only by 20 strains. *Ent. faecalis*, *Lac. lactis* ssp. *cremoris* MG 1363, *Listeria. innocua* were inhibited by 38, 3 and 32 strains, respectively. In next stage, the second method, well-diffusion assay, was applied for all strains with antibacterial activity against any of the indicators. Under the conditions of well- diffusion assay, the number of strains which produced clear zone, was decreased as only 20 strains showed clear inhibitory effects (Table 1). These results were in agreement with others' results; many authors have reported that conformation in liquid media of the inhibition detected by the agar-spot test is not always obtained (Schillinger and Lucke 1989; Larsen et al., 1993; Martinez et al., 1995; Hernandez et al., 2005). Several colony-associated antimicrobial compounds , like fatty acids and H_2o_2, have been considered to be responsible for the inhibitory for the inhibitory effects observed is solid media (de Vuyst and Leroy, 2007).

The number of strains which inhibiting the indicators used in this study were as follows: *Ent. faecalis* (18/48) strains, *Lac. lactis* (3/48) strains, *Staph. aureus*- 0 strain, *Listeria innocua*- 25 strains (most of them showed strong inhibition), and *Lb. plantarum*- 2 strains (Table1).

Most of these inhibitory strains were shown to belong to *Ent. faecium* and C34, C35, LR71, KR24 proved to be *Ent. faecalis* and KR 47 was the only *Ent. casseliflavus*. Then, all these 20 inhibitory (positive clear zone) strains (isolates) were subjected to rep-PCR for typing with primer BOXA2R (Figure 1).

According to rep-PCR profiles, 15 out of 20 strains showed distinct typing profiles.(Figure 2).

As we can see in table 1 (well-diffusion assay), some strains can inhibit the *Ent. faecalis* but with weak inhibition (clear zone), only 2 strains showed the inhibitory effects against *Lac. lactis*. Most of strains showed the inhibitory influence towards *Listeria. innocua*. Among all these strains, LR74 (*Ent. faecium*) showed the widest spectrum of inhibitory since it produced clear zone against *Ent. faecalis*, *Lac. lactis*, *Listeria innocua* and *Lb. plantarum*. None of the strains showed the antimicrobial activity against *Staph. aureus.*

In conclusion, we detected 20 isolates (producing strains) which produced clear-zone (bacteriocin-like subtances) using Agar- spot and well-diffusion assay. Among these 20 isolates, we detected 15 different distinct isolates according to rep-PCR profiles (patterns). From these 15 bacteriocinogenic strains, 11 of which belonged to *Ent. faecium* .

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TABLE I. BACTERIOCIN PRODUCTION FROM ENTEROCOCCI ISOLATES FROM CHEESE SAMPLES IN WELL DIFFUSION ASSAY

	Indicators / Isolates code	*Ent.faecalis*	*Lac.lactis*	*Staphylococcus.aureus*	*Listeria.innocoa*	*Lb.plantarum*
1	M1:Ent.faecium	-	-	-	-	-
2	M9:Ent.faecium	-	++	-	-	-
3	M13:Ent.faecium	-	-	-	-	-
4	C15:Ent.faecium	-	-	-	-	-
5	C16:Ent.faecium	-	-	-	+++	-
6	C17:Ent.faecium	-	-	-	+++	-
7	C19:Ent.faecium	-	-	-	-	-
8	C20:Ent.faecium	+weak	-	-	++++	-
9	C21:Ent.faecium	+weak	-	-	++++	-
10	C32:Ent.faecium	-	-	-	-	-
11	C34:Ent.faecalis	+weak	-	-	++++	-
12	C35:Ent.faecalis	+weak	-	-	++++	-
13	LF37:Ent.faecium	-	-	-	-	-
14	LF41:Ent.faecium	-	-	-	+weak	-
15	LF43:Ent.faecium	+weak	-	-	++	-
16	LF44:Ent.faecium	+	-	-	+++	-
17	LF53:Ent.casseliflavus	-	-	-	-	-
18	LF54:Ent.faecium	-	-	-	++	-
19	LR59:Ent.faecium	-	-	-	+weak	-
20	LR66:Ent.faecium	-	-	-	-	-
21	LR67:Ent.faecium	-	-	-	-	-
22	LR71:Ent.faecalis	+weak	+weak	-	-	++
23	LR74:Ent.faecium	++	++	-	++	+++
24	LR75:Ent.faecium	+weak	-	-	+++	-
25	LR82:Ent.faecalis	-	-	-	-	-
26	LR83:Ent.faecalis	-	-	-	-	-
27	KR10:Ent.faecium	-	-	-	-	-
28	KR16:Ent.faecalis	-	-	-	-	-
29	KR21:Ent.faecium	-	-	-	-	-
30	KR22:Ent.faecalis	-	-	-	-	-
31	KR24:Ent.faecalis	+weak	-	-	++	-
32	KR26:Ent.faecium	-	-	-	-	-
33	KR29:Ent.durans	-	-	-	-	-
34	KR30:Ent.faecium	+weak	-	-	+++	-
35	KR31:Ent.faecium	+weak	-	-	++	-
36	KR32:Ent.faecium	-	-	-	+weak	-
37	KR34:Ent.faecium	-	-	-	++	-
38	KR36:Ent.faecium	-	-	-	-	-
39	KR37:Ent.faecium	+weak	-	-	++	-
40	KR38:Ent.faecium	-	-	-	+	-
41	KR40:Ent.faecium	+	-	-	+	-
42	KR41:Ent.faecium	-	-	-	-	-
43	KR42:Ent.faecium	+weak	-	-	+	-
44	KR43:Ent.faecium	-	-	-	-	-
45	KR44:Ent.faecalis	-	-	-	-	-
46	KR45:Ent.faecium	+weak	-	-	+++	-
47	KR46:Ent.faecium	+weak	-	-	+++	-
48	KR47:Ent.casseliflavus	++	-	-	+++	-

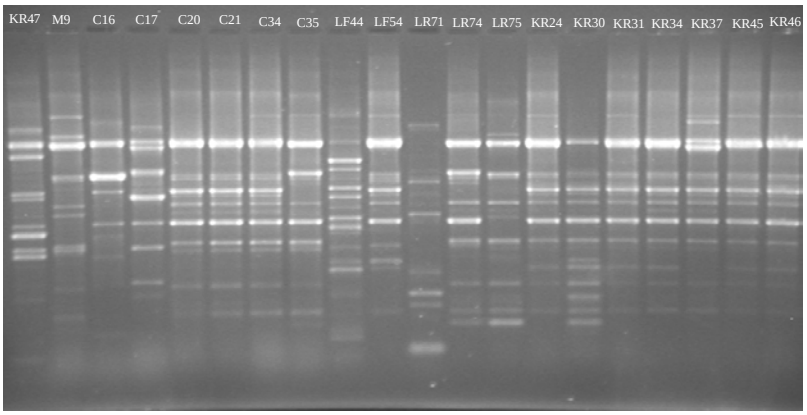

Fig1. Rep-PCR typing of 20 Enterococci spp. isolates from two Iranian traditional cheeses (Lighvan and Koozeh). Codes: KR (Koozeh ripened), M (Milk from Lighvan), C (Curd from Lighvan), LF (Lighvan fresh cheese), LR (Lighvan ripened cheese

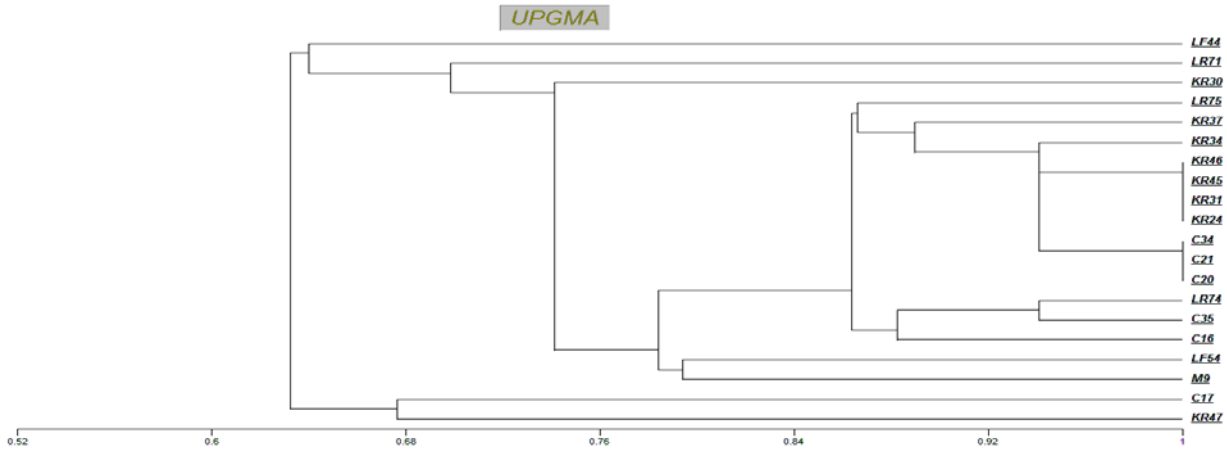

Fig 2. Dendogram of rep- PCR of 20 isolates for enterocins