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Identification of Anti-Microbial Producing Enterococci Isolated from Iranian Raw Milk Cheeses Using Polyphasic Approach

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Abstract

The search for new antimicrobial agents is a field of utmost importance since the prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide. This study was performed in order to isolate and identify the potential Enterococci strains exhibiting anti-microbial activity with the help of two anti-microbial detection methods namely Agar-Spot and Well-Diffusion Assay. A collection of Enterococci spp. (about 96 isolates) were isolated from two Iranian raw milk cheeses, namely Lighvan and Koozeh, and subsequently, identified as *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus durans*, *Enterococcus casseliflavus* and *Enterococcus italicus* by 16S rDNA sequencing. All of the 96 isolates were subjected to Agar-Spot and Well-Diffusion Assay in order to detect their ability to produce antimicrobial compounds. According to Agar-Spot method, 48 out of the 96 isolates produced clear zone on the plates against indicator organisms. With Well-Diffusion Assay, the positive isolates with clear zone decreased to 20 isolates. These 20 isolates (strains) were then subjected to rep-PCR for typing, and 15 distinct rep-PCR profiles (patterns) were identified, from these 15 positive strains, 11 strains were found to belong to *Enterococcus faecium*.

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1. Introduction

Enterococci belong to a genus derived from the former genus Streptococcus and considered one of the main parts (genera) of Lactic acid bacteria, which are widely distributed in the environment, food and different ecological niches [1]. Among the enterococcus species, *E. faecium* and *E. faecalis* are the two most occurring in foods and related habitats. Enterococci play an important role in the development of the sensory properties of fermented foods [2]. Also, they might be used as starter and adjunct cultures [3, 4]. Moreover, they are a part of dominant microflora in traditionally fermented cheeses made from raw milk [5]. One of the best advantages of Enterococci in food is production of a diverse and heterogeneous group of ribosomal antimicrobial peptides or bacteriocins (the enterocins) with different spectra of antagonism activities, structure, and processing and secretion mechanisms [6,7,8]. Most enterocins belong to class II of the Klaenhammer classification [9], including: enterocin A [10,11], enterocin B [7, 12], enterocin P [13], enterocin L50 [14], enterocin Q [15], and mundticin [16]. 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 *Staphylococcus aureus*, *Bacillus subtilis* and spores of *Clostridium perfringenes* [9,17,18].

The objective of this work was to screen the Enterococci isolated from two Iranian raw milk cheeses with antibacterial properties, and enterocin production as a potential natural additive to be used as bio-preservative.

2. Material and Methods

2.1 Strains, media and culture conditions

96 isolates of *Enterococci spp*. isolated during the manufacture and ripening of two Iranian traditional cheeses made from raw milk (Lighvan and Koozeh) were grouped by typing, and identified by Amplified Ribosomal DNA Restriction Analysis, sequencing and sequence comparison. 15 isolates as representatives were tested for enterocin production against the indicators including gram positive and gram negative bacteria. The indicator strains included *E. faecalis*, *L. lactis* MG1363, *S. aureus* CECT 86, *Lactobacillus sakei* CECT 906, *Listeria innocua* 4202, and *Lactobacillus plantarum* 748, Table 1 [19].

All isolates and indicator microorganisms were activated at -80°C on BHI Agar, M17 Agar (for Lactococci), MRS Agar (for Lactobacilli), or in Trytone Soy Broth (for *L. innocua* and *S. aureus*). Then, the plates were incubated at their suitable optimum temperature for 24-48h, Table 1 [19].

2.2 Identification and typing of isolates

Total genomic DNA was extracted from single, isolated colonies suspended in 50µl of molecular grade water (Sigma-Aldrich st. Louis, MO, USA), and heated at 98°C for 10 min in a thermo cycler (Bio-Rad Richmond, CA, USA). Mini-bead Beater Apparatus (Bio-Spec Products, Bartlesville, OK, USA) was used for DNA extraction of some isolates, following centrifugation for 5min at 16600 ×g.

All isolates were identified by Amplified Ribosomal DNA Restriction Analysis technique, followed by sequencing of the 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 [20].

2.3 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 the method of Fleming et al. [21]. Briefly, 5µl of the overnight cultures of the strains to be tested for production of an antimicrobial compounds was spotted onto the surface of the Agar plates (BHI-Agar + 0.2% glucose, M17-Agar + 0.2% glucose) and incubated at 30°C for 24h to allow the colonies develop. The spots were then covered with 10ml of soft-Agar (0.75%) previously inoculated at 0.25% with five indicator bacteria. The plates were incubated under the required conditions for indicator bacteria, and then checked for inhibition zones. Those strains (isolates), which produced clear zone equal to 5mm or larger, were considered positive in this method (Agar-Spot). In Well-Diffusion Assay, the positive strains from previous stage were selected for evaluation of the antibacterial production. In this method, the indicator strains were first cultured overnight in the broth medium related to each indicator (MRS, M17 and BHI). Then 20ml of agar medium at 45°C was mixed with 200µl of an overnight culture of the indicator strain and poured into petri dishes. One ml of the overnight culture of each producing strain was transferred into 1.5 ml microtube and centrifuged at 13000 ×g for 2 min.

Table 1. Indicator microorganisms and their growth conditions

	Indicator microorganisms	Growth conditions
1	*Staphylococcus aureus* (ATCC 25923)	Tripticase Soy Agar (TSA)-37°C-liofilchem-Italy
2	*Listeria innocoa* (ATCC33090)	Tripticase Soy Agar (TSA)-37°C
3	*E.coli* (ATCC 25922)	Tripticase Soy Agar (TSA)-30°C
4	*Lactobacillus sakei* (ATCC15521)	DeMan Rogosa and Sharpe (MRS - Merk- Germany)-37°C
5	*Lactobacillus plantarum* (ATCC8014)	DeMan Rogosa and Sharpe (MRS)-37°C
6	*Lactococcus lactis ssp. Lactis* (ATCC 11454)	M17+1% lactose-Fluka- USA-30°C
7	*Lactococcus lactis ssp. cremoris* (ATCC 19275)	M17+1% lactose-30°C

The resulting supernatant was then adjusted to pH 6.5-7.0 with 0.1M NaOH, centrifuged at 12700 ×g for 5 min, and filtered through a 0.20µm pore membrane (Millipore, Bedford, MA, USA) for sterilization. Wells (3mm in diameter) were carved in these Agar plates and 50µl from the supernatant of each positive strain was placed into each well. The plates were incubated for 24h under appropriate conditions, and subsequently, examined for zones of inhibition [19,21].

3. Result and Discusiion

3.1 Identification and typing of Enterococci spp.

Among the 130 isolates from two Iranian traditional cheeses, namely Lighvan and Koozeh, 96 isolates were identified as Enterococci by Amplified Ribosomal DNA Restriction Analysis, 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 (*E. faecium* [38], *E. faecalis* [11], *E. durans* [1], *E. italicus* [1] and *E. casseliflavus* [1]), and Koozeh (*E. faecium* [36], *E. faecalis* [5], *E.durans* [1] and *E. casseliflavus* [2]) cheeses, respectively.

3.2 Antimicrobial activity of Enterococci spp.

The production of enterocin (bacteriocin) by the examined isolates of different strains against *L. innocua*, *S. aureus*, *E. faecalis*, *L. plantarum*, and *L. sakei* as indicators was first analyzed by an Agar-Spot test. Among the 96 isolates, 48 isolates showed inhibitory effect (clear zone) against the different indicator organisms (data not shown). *L. plantarum* CECT 748 was inhibited by 27 strains. In contrast, *S. aureus* CECT 86 was inhibited only by 20 strains. *E. faecalis*, *L. lactis* ssp. *cremoris* MG 1363, and *L. innocua* were inhibited by 38, 3 and 32 strains, respectively. Ghrairi *et al*. [22] isolated one *Enterococcus faecium* MMT21 from Tunisian rigouta cheese and evaluated its antibacterial effects against several food spoilage bacteria and food-borne pathogens. Its inhibitory activity was detected on *L. lactis*, *E. faecium*, *E. faecalis*, *L. monocytogenes and S. aureus*. Well-Diffusion Assay was then applied for all of the strains, which showed antibacterial activity against any of the above mentioned indicators. Under the conditions of Well-Diffusion Assay, the number of strains, which produced clear zone, was decreased as only 20 strains exhibited clear inhibitory effects (Table 1). These results are in agreement with the findings of some other researchers. In another study, it was found that four strains of Enterococci isolated from Argentina regional cheeses produced bacteriocin, which was active against some lactic acid bacteria. Among them, enterocin CRL35 produced by *E. faecium* CRL35 was also inhibitory to the food-borne pathogens like *L. monocytogenes* and *S. aureus* [23].

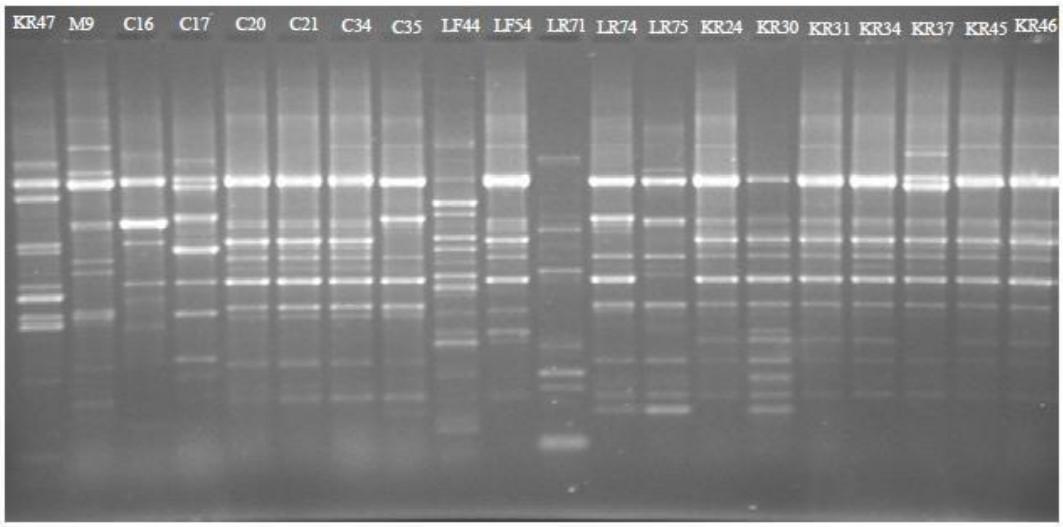

Figure 1. Rep-PCR typing of 20 Enterococci spp. isolates from two Iranian traditional cheeses (Lighvan and Koozeh). Codes: KR (Koozeh ripened), M (Milk from Lighvan), C (Curd from Lighvan), LF (Lighvan fresh cheese), and LR (Lighvan ripened cheese).

Table 2. Antibacterial producing strains of Enterococci isolated from cheese using Well-Diffusion Assay

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

Diameter of each well: 3mm, -: No clear zone was detected, +: 5mm<clear zone<7mm, ++: 7mm<clear zone<10mm, +++:10mm<clear zone<12mm, ++++: 12mm<clear zone.

Many reports suggest that conformation in liquid media of the inhibition detected by the Agar-Spot test is not always obtained [24, 25, 26, 27]. Several colony-associated antimicrobial compounds like fatty acids and H_2O_2 have been considered to be responsible for the inhibitory effects observed in solid media [28].

The numbers of strains inhibiting the indicators used in this study were as follows: *E. faecalis* (18/48 strains), *L. lactis* (3/48 strains), *S. aureus* (0 strain), *L. innocua* (25 strains) (most of them showed strong inhibition), and *L. plantarum* (2 strains) (Table 1).

Most of these inhibitory strains were shown to belong to *E. Faecium*; C34, C35, LR71, KR24 were also proved to be *E. faecalis* and KR 47 was the only *E. casseliflavus*. Then, all of these 20 inhibitory (positive clear zone) strains (isolates) were subjected to rep-PCR for typing with primer BOXA2R (Figure 1). According to the rep-PCR profiles, 15 out of the mentioned 20 strains showed distinct typing profiles (Figure 2).

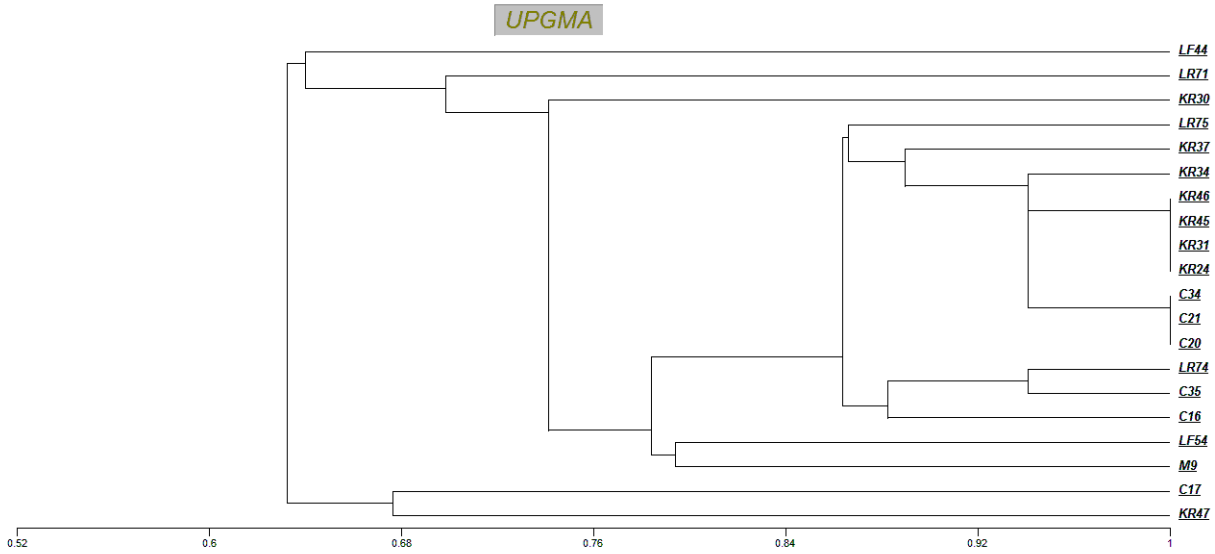

Figure 2. Homology tree dendogram of the carbohydrate fermentation profiles obtained with the API 20 STREP system. The similarity matrix of the profiles was subjected to cluster analysis by the non-weighted pair groups' average linkage analysis clustering method (using the simple matching coefficient). Vertical lines of the dendogram represent the degree of similarity shared by the groups connected by the lines.

As depicted in Table 2 (Well-Diffusion Assay), some strains could inhibit *E. faecalis* but with weak inhibition (clear zone). Only two strains showed the inhibitory effects against *L. lactis*. Most of the strains showed inhibitory influence towards *L. innocua*. Among these strains, LR74 (*E. faecium*) showed the widest spectrum of inhibitory; as it produced clear zone against *E. faecalis*, *L. lactis*, *L. innocua* and *L. plantarum*. None of the strains showed antimicrobial activity against *S. aureus.*

4. Conclusion

In conclusion, using Agar-Spot and Well-Diffusion Assays, we detected 20 isolates (producing strains), which produced clear-zone (bacteriocin-like subtances). Among these 20 isolates, 15 different distinct isolates were detected according to the rep-PCR profiles (patterns); 11 of these 15 bacteriocinogenic strains were found to belong to *E. faecium*.

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