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Bacteriocinogenic Potential of Newly Isolated Strains of *Enterococcus faecium* and *Enterococcus faecalis* from Dairy Products of Pakistan

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The present study was carried out for the isolation of bacteriocin-producing enterococci from indigenous sources. Gram-positive enterococci are known for having the ability to produce enterocins with good antimicrobial potential. A total of 34 strains were isolated from processed dairy products of Pakistan and seven out of them were found to be member of genus Enterococcus on selective enumeration. Biochemical and molecular characterization revealed that four of these isolates (IJ-03, IJ-07, IJ-11, and IJ-12) were *Enterococcus faecalis* and three (IJ-06, IJ-21, and IJ-31) were *Enterococcus faecium*. Local processed cheese was the source of all enterococcal isolates, except *E. faecium* IJ-21 and IJ-31, which were isolated from indigenous yoghurt and butter samples, respectively. Bacterial isolates were sensitive to commonly used antibiotics except methicillin and kanamycin. They also lacked critical virulence determinants, mainly cytolysin (*cyl*), gelatinase (*gel*), enterococcal surface protein (*esp*), and vancomycin resistance (*vanA* and *vanB*). Polymerase chain reaction amplification identified that enterocin A and P genes were present in the genome of *E. faecium* IJ-06 and IJ-21, whereas the *E. faecium* IJ-31 genome showed only enterocin P genes. No amplification was observed for genes that corresponded with the enterocins 31, AS-48, L50A, and L50B, and *ent* 1071A and 1071B. There were no signals of amplification found for *E. faecalis* IJ-11, indicating that the antimicrobial activity was because of an enterocin different from those checked by PCR. Hence, the indigenous bacterial isolates have great potential for bacteriocin production and they had antibacterial activity against a variety of closely related species.

Keywords: Enterococci, isolation, antibiotic sensitivity, enterocin A, enterocin P

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Enterococci are lactic acid bacteria (LAB) present in a wide range of habitats including the gastrointestinal tract of animals, soils, surface waters, and plants. They are also associated with a number of fermented foods such as dry fermented sausages and traditional cheeses [5, 8, 14]. The microbial population inhabitating artisanal cheeses is very diverse, where enterococci represent an essential part, and sometimes they even dominate over lactobacilli and lactococci in ripened cheese products. The presence of enterococci was considered as an indication of fecal contamination, but now a days they are known as a normal part of food microflora [13]. Dissemination of enterococci in the environment; tolerance to heat, salt, acid, and temperature; and their biochemical properties (proteolytic and lipolytic activities, production of typical flavor components) significantly contribute to the formation of the specific organolyptic and qualitative characteristics of cheeses [3, 4, 8].

Bacteriocin-producing lactic acid bacteria (LAB), including enterococci, are also widespread in nature [12, 13], and their bacteriocins may inhibit Gram-positive food-spoilage and foodborne pathogenic bacteria, so they are an important choice as natural and nontoxic food preservatives [6]. Enterococci in food may be beneficial by producing antimicrobial agents and by improving the perceptible quality of food for consumers. On the other hand, they may also represent a concern because of nosocomial and opportunistic infections. The safety of foods containing enterococci is an issue that the food industry must carefully address [15]. A contributing factor to the pathogenesis of enterococci is their resistance to a wide variety of antibiotics, including glycopeptides such as vancomycin, in combination with the production of virulence factors like aggregation substance (*as*), gelatinase (*gel*), cytolysin (*cyl*), enterococcal surface protein (*esp*), and adhesin to collagen from *E. faecalis* (*ace*), which determine that enterococci are not considered as "generally recognized as safe" (GRAS) organisms [21].

The aims of the present study were to isolate and characterize the enterococci associated with local fermented dairy products. The investigations of critical virulence determinants, antibiotics susceptibility, and the enterocin production potential were targeted for the characterization of these enterococcal isolates for their future intended use in the food industry.

Materials and Methods

Food Products and Isolation of Enterococci

Isolation of lactic acid bacteria was done from commercially available local processed cheese, yoghurt, and butter samples from markets of Islamabad, Pakistan. Isolation was done by the serial dilution method using homogenized solutions of food products in quarter-strength Ringer's solution, followed by spreading 0.1 ml of suitable 10-fold dilutions on de Man-Rogosa-Sharpe (MRS) agar (Oxoid, Basingstoke, U.K.) and incubated at 37°C for 24 to 48 h. Predominant colony types were selected and purified by continuous streaking on the same media. Kanamycin aesculin azide medium (Oxoid, Basingstoke, U.K.) was used for selective culturing of *Enterococcus* spp. at 42°C for 18 to 24 h. Stock cultures of all the strains were kept in the appropriate medium with 15% (v/v) glycerol and stored at −20°C. *E. faecium* ATCC 27273 and *E. faecium* LMG11423^T were used as control strains.

Biochemical Fingerprinting and Phenotypic Characterization

Preliminary identification was done according to *Bergey's Manual of Systematic Bacteriology* [18]. The sugar fermentation profiles of these isolates were checked for glucose, lactose, mannitol, sorbitol, raffinose, arabinose, salicin, and sucrose as sole carbon sources in phenol red broth (Oxoid, U.K.) and their ability to grow in MRS broth (Oxoid, U.K.) containing 6.5% NaCl (w/v) at 10 and 45°C, pH 9.6. Hemolysis of these isolates was checked by culturing on Blood Agar Medium (Oxoid, U.K.) supplemented with 5–7% of sterile sheep blood and human blood obtained from the Quaid-i-Azam University's animal house, and University Medical Center, respectively.

Molecular Identification

Plasmid and genomic DNAs were extracted from overnight culture of MRS broth of selected enterococcal and control strains, using the Qiagen Plasmid Mini Prep. and DNeasy Blood and Tissue Kit, respectively (Qiagen Sciences, Maryland, U.S.A.). Isolated DNA was quantified by absorbance spectra on an ND-1000 spectrophotometer (NanoDrop Technologies, U.S.A.) and subsequently analyzed on 0.8% agarose (Invitrogen, U.S.A.) gel, with 1-kb DNA ladder (TarckIt Invitrogen, U.S.A.) and photo digitalized on a ChemiDoc gel documentation system (Bio-Rad, Hercules CA, U.S.A.) for qualitative purpose.

Nucleotide sequences of 16S and 23S rRNA were determined for representative enterococci strains for species identification. Primers were based on the known sequences from *E. faecium* LMG11423^T, a strain for which the complete sequences of the 16S (Accession No. AJ301830) and 23S rRNA (Accession No. AJ295305) genes are known. All primers were designed using the oligonucleotide calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). Primers were obtained from Sigma-Genosys Ltd. (Table 1). PCR amplification was done in 50-µl volumes containing 2.5 ng of template DNA, 200 mM dNTPs, 10 mM of the respective primers, 1 U *Taq* DNA polymerase, and 5 µl of standard *Taq* buffer (New England Biolabs). Amplification of 16S rRNA was carried out in 30 cycles (denaturation, 94°C for 30 sec; annealing and elongation, 72°C for 2 min; final extension, 72°C for 5 min) in an Eppendrof thermal cycler AG 22331 (Hamburg, Germany). Amplification of 23S rRNA was carried out with the same set of conditions but with 35 cycles.

The PCR products were purified using a Qiagen PCR purification kit (Qiagen Sciences, Maryland, U.S.A.) and subsequently sequenced by using the ABI PRISM BigDye Terminator cycle sequencing kit with automated capillary DNA Sequencer AB 3130XL and AB3730 DNA analyzer, using primers designed from the reported *E. faecium* LMG 11423^T and *E. faecalis* V583, whose genes sequence are available at GenBank in the National Center of Biotechnology

Table 1. Primers used for molecular identification, safety investigation, and enterocin genes amplification.

Target gene	Primer name	Primer sequence	Position	Product size
16S rRNA *Enterococcus* spp.	LMG-16S-S20 LMG-16S-AS1520	5'GACGAACGCTGGCGGCGTGCC3' 5'CGGCTACCTTGTTACGACTTCACC3'	20–41 1496–1520	1500
23S rRNA *E. faecium*	LMG-23S-S1 LMG-23S-AS2904	5'GGTTAAGT AATAAGGGCGCACGG3' 5'CCTCGATCGATTAGTATCAGTCCGCTC3'	1–24 2877–2904	2904
23S rRNA *E. faecalis*	V583-23S-S250246 V583-23S-AS253151	5'GGTTAAGTGAATAAGGGCGCACGGTGG3' 5'GTCCTCGACCGATTAGTATTGGTCCGC3'	250246–250273 253124–253151	2905
Cytolysin	*E.f-Ent.cyl*-S754 *E.f-Ent.cyl*-AS1019	5'GGGTTGGTGGCGGTATTTTTACTGGA3' 5'AATAATGCACCTACTCCTAAGCCTATGGT3'	754–780 990–1019	265
Vancomycin A	*E.f-Ent.vanA*-S227 *E.f-Ent.vanA*-AS631	5'GCATGGCAAGTCAGGTGAAGATGGAT3' 5'GGTCCACCTCGCCAACAACTAACG3'	227–253 607–631	404
Vancomycin B	*E.f-Ent.vanB*-S230 *E.f-Ent.vanB*-AS722	5'CGGAATGGGAAGCCGATAGTCTCC3' 5'TTCCCATGACCGCGCAGCCGAC3'	230–254 700–722	492
Enterocin A (*entA*)	AM746970-*EntA*-S571 AM746970-*EntA*-AS726	5'CACAACTTATCTATGGGGGTACCACTC3' 5'CCCTGGAATTGCTCCACCTAAAAAACC3'	571–598 699–726	155
Enterocin P (*entP*)	AF005726-*EntP*-S236 AF005726-*EntP*-AS344	5'GTACAAAAGTTGATGCAGCTACGCGTTC3' 5'CGATTCCTGCAATATTCTCTTTAGCTTCTC3'	236–264 314–344	108

Information (NCBI). The 16S and 23S rRNA sequences homology analyses were performed using BLASTn (NCBI, Bethesda, MD, U.S.A.) for identification purpose.

Investigation of Critical Virulence Determinants and Enterocin Genes

Production of gelatinase, DNAse, and investigation of hemolysis were done as described previously [15]. Antimicrobial susceptibility patterns were determined by the Kirby–Bauer disc diffusion method as per National Committee for Clinical Laboratory Standards (NCCLS 2002) [17]. Antibiotics-containing discs (Oxoid) used were ampicillin (25 μg), erythromycin (15 μg), vancomycin (30 μg), teicoplanin (30 μg), ciprofloxacin (5 μg), streptomycin (10 μg), methicillin (5 μg), tetracycline (30 μg), gentamycin (10 μg), and kanamycin (30 μg). Zones of inhibition were measured using freshly grown strains after overnight incubation at 37°C. Minimum inhibitory concentrations (MIC) were recorded according to the standard broth dilution technique against the glycopeptide antibiotic vancomycin. Resistant phenotypes were recorded as recommended by NCCLS [17].

Absence of respective genes for cytolysin (*cyl*), gelatinase (*gel*), enterococcal surface protein (*esp*), vancomycin resistance type A (*van-A*), and vancomycin resistance type B (*van-B*) were confirmed by PCR amplification [7]. For PCR of the bacteriocin genes *entA*, *entB*, *entP*, *ent*31, *ent*AS-48, *ent*L50A and L50B, and *ent*1071A and 1071B, the primers were based on complete sequences available in the NCBI database. The accession numbers for these are AM746970 for *entA*; AF076604 for *entB*; AF005726 for *entP*; D7825.1 for *ent*31; Y12234.1 for *ent*AS-48; AJ223633 for *ent*L50A and L50B; and AY063485 for *ent*1071A and 1071B. All primers were designed to amplify a partial segment of the bacteriocin genes.

Table 2. Biochemical fingerprinting and identification of isolated strains of enterococci.

Tests	IJ-03	IJ-06	IJ-07	IJ-11	IJ-12	IJ-21	IJ-31	*E. faecium* Control
Source	Local cheese	Local cheese	Local cheese	Local cheese	Local cheese	Yoghurt	Butter	ATCC 27273
G. staining	G+ive	G+ive	G+ive	G+ive	G+ive	G+ive	G+ive	G+ive
Catalase test	-	-	-	-	-	-	-	-
Haemolysis test	γ	γ	γ	γ	γ	γ	γ	γ
Phenotypic tests: Growth at 10°C	+	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+	+
pH 9.6	+	+	+	+	+	+	+	+
6.5% NaCl	+	+	+	+	+	+	+	+
Sugar fermentation tests:								
Lactose, mannitol, and ribose	+	+	+	+	+	+	+	+
Raffinose and sorbitol	+	-	+	+	+	-	-	-
NCBI Accession Nos.								
16S rRNA	EU547773	EU547774	EU547775	EU547776	EU547777	EU547778	EU547779	EU547780
23S rRNA	EU547781	EU547782	EU547783	EU547784	EU547785	EU547786	EU547787	EU547788
Species identification	*Enterococcus faecalis*	*Enterococcus faecium*	*Enterococcus faecalis*	*Enterococcus faecalis*	*Enterococcus faecalis*	*Enterococcus faecium*	*Enterococcus faecium*	*Enterococcus faecium*

Screening for Bacteriocin Production and Antimicrobial Activity of Isolated Strains

Screening for bacteriocin production potential from isolated strains was done by stab-overlay and agar-well diffusion assay [2]. Bacteriocin production was checked by culturing the producer strains in MRS broth for 24 h at 37°C. The cultures were centrifuged (10,000 rpm, 5 min at 4°C) with subsequent filtration to remove any residual cells (0.2 μm pore size). These cell-free supernatant (CFS) samples were used to assess antibacterial activity against different indicator strains spread on MRS agar plates [0.7% (w/v) soft agar; 100 μl of culture; OD_{600}, 0.45], as described by De Vuyst *et al.* [6]. The 50-μL samples were placed into the well (0.5 cm diameter) on the plates inoculated with the test bacteria and were incubated at 37°C for 18–24 h. The zones of inhibition around the wells were recorded. Bacteriocinogenic activity units per milliliter (AU/ml) were measured against *E. faecalis* IJ-11, *Listeria monocytogenes* ATCC 19115, and *Bacillus subtilis* ATCC 27142 as indicator strains [11].

Results and Discussion

Food Products and Isolation of Enterococci

A total of 34 bacterial strains were isolated from dairy food products on MRS media, and further screening on selective media kanamycin aesculin azide agar revealed seven strains to be enterococci. Among these isolates, *Enterococcus* spp. IJ-03, IJ-06, IJ-07, IJ-11, and IJ-12 were isolated from local processed cheese, whereas *Enterococcus* spp. IJ-21 and IJ-31 were isolated from yoghurt and butter samples, respectively. Kanaymcin asesculine azide agar had been used successfully for selective isolation and enumeration of dairy enterococci [1].

Biochemical Fingerprinting and Phenotypic Characterization

All the seven isolated *Enterococcus* spp., IJ-03, IJ-06, IJ-07, IJ-11, IJ-12, IJ-21, and IJ-31, showed positive growth at 10 and 45°C in 6.5% NaCl, pH 9.6. All of the isolates were able to ferment ribose, mannitol, and lactose, but only *Enterococcus* spp. IJ-03, IJ-07, IJ-11, and IJ-12 fermented raffinose and sorbitol. Further confirmation of these strains by standard morphologic, phenotypic, and biochemical tests revealed that four isolates (IJ-03, IJ-07, IJ-11, and IJ-12) were *Enterococcus faecalis* and three (IJ-06, IJ-21, and IJ-31) were *Enterococcus faecium.* Preliminary identification showed that isolated *Enterococcus* spp. were found to be a significant proportion (n=9, 21%) of the normal microbial population in indigenous dairy products of Islamabad. They mainly belonged to two genomic subgroups, *Enterococcus faecalis* (n=4, 12%) and *Enterococcus faecium* (n=3, 9%). All of the indigenous isolates of enterococci were catalase-negative and non-hemolytic on blood agar media (Oxoid, U.K.) supplemented with 5–7% of sterile sheep and human blood (Table 2). Our results are in accordance with the previous findings of isolation and identification of 64 strains of *Enterococcus* spp. from different origins, by using traditional phenotypic, biochemical, and cultural tests together with molecular characterization, where the tested strains were Gram-positive, catalase-negative cocci; 63 strains grew on kanamycin aesculin azide agar. All the strains were able to grow in an alkaline environment (pH 9.6) at 10 and 45°C [10].

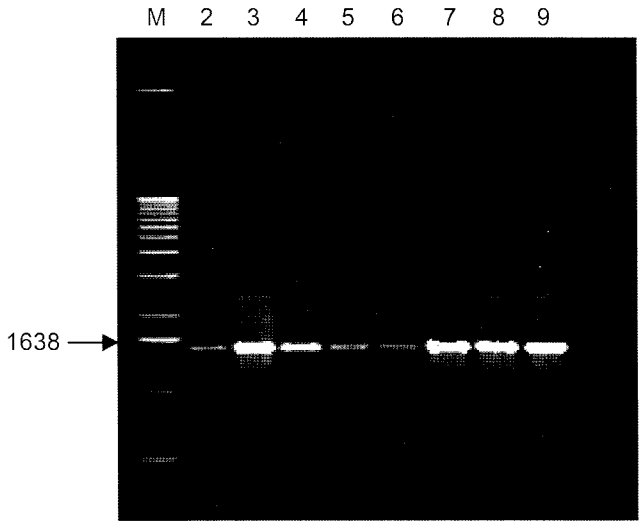

Fig. 1. PCR amplification of 16S rRNA from isolated *Enterococcus* spp. on 0.9% agarose gel.
M: 1-kb TrackIt DNA marker (Invitrogen); L2: *E. faecalis* IJ-03; L3: *E. faecium* IJ-06; L4: *E. faecalis* IJ-07; L5: *E. faecalis* IJ-11; L6: *E. faecium* IJ-12; L7: *E. faecium* IJ-21; L8: *E. faecium* IJ-31; L9: *E. faecium* ATCC 27273.

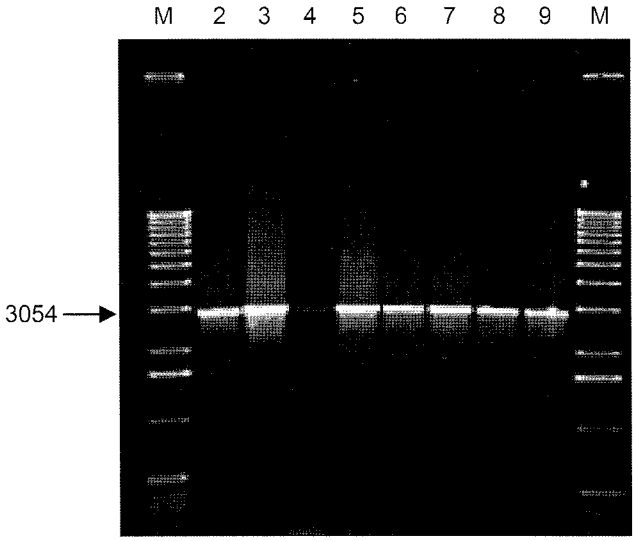

Fig. 2. PCR amplification of 23S rRNA from isolated *Enterococcus* spp. on 0.9% agarose gel M: 1kb TrackIt DNA marker (Invitrogen), L2: *E. faecalis* IJ-03, L3: *E. faecium* IJ-06, L4: *E. faecalis* IJ-07, L5: *E. faecalis* IJ-11, L6: *E. faecium* IJ-12, L7: *E. faecium* IJ-21, L8: *E. faecium* IJ-31, L9: *E. faecium* ATCC 27273.

Isolated *E. faecium* IJ-06, IJ-21, and IJ-31 were arabinose-positive and sorbitol-negative (Table 2); these enterococci were previously supposed to be indicative of a human origin, especially where enterococcal species other than *E. faecium* and *E. faecalis* are infrequent [21]. That indicates *E. faecium* strains could possibly stem from human sources and is an indicator of unsanitary conditions during handling and preparation of these fermented foods, but a more detailed study is needed to have a better idea of the phylogenetic standing of these isolated enterococci.

Molecular Identification of Isolated Enterococci

The genus-specific primer designed for the amplifications of 16S and 23S rRNAs were able to amplify these regions (~1.5 kb for 16S, ~2.8 kb for 23S rRNA) in all of the isolated strains of *E. faecium* and *E. faecalis* (Fig. 1 and 2). Amplicons were column purified and subsequently sequenced using a set of primers to identify these strains on the molecular level. Genotypic analysis confirmed four of these isolated strains (IJ-03, IJ-07, IJ-11, and IJ-12) as *Enterococcus faecalis*, whereas IJ-06, IJ-21, and IJ-31 were found to be *Enterococcus faecium*. Preliminary identification was confirmed and validated by molecular identification. These sequences were deposited in NCBI GenBank with Accession Nos. EU547773 to EU547780 (16S rRNA) and EU547781 to EU547788 (23S rRNA) for *E. faecalis* IJ-03, *E. faecium* IJ-06, *E. faecalis* IJ-07, *E. faecalis* IJ-11, *E. faecalis* IJ-12, *E. faecium* IJ-21, *E. faecium* IJ-31, and *E. faecium* ATCC 27273, respectively (Table 2). The phylogenetic standing of isolated strains of *Enterococcus faecium* IJ-06, IJ-21, IJ-31, and *Enterococcus faecalis* IJ-03, IJ-07, IJ-11, and IJ-12 on the basis of 16S rRNA is represented in the clades (Fig. 3A and 3B). Phylogenetic alnaysis, carried out by using MEGA-4 software [20], revealed that isolated *E. faecium* had close homology with a number of swine-associated enterococci isolated from Hinan Province of China and fermented dairy isolates from Tibet [http://www.ncbi. nlm.nih.gov].

Investigation of Critical Virulence Determinants

The safety investigation of the bacterial isolates revealed that all the strains were sensitive to most of the commonly used antibiotics (viz., vancomycin, teicoplanin, ampicillin, erythromycin, ciprofloxacin, tetracycline, and gentamicin), but resistant to methicillin and kanamycin, expressing a

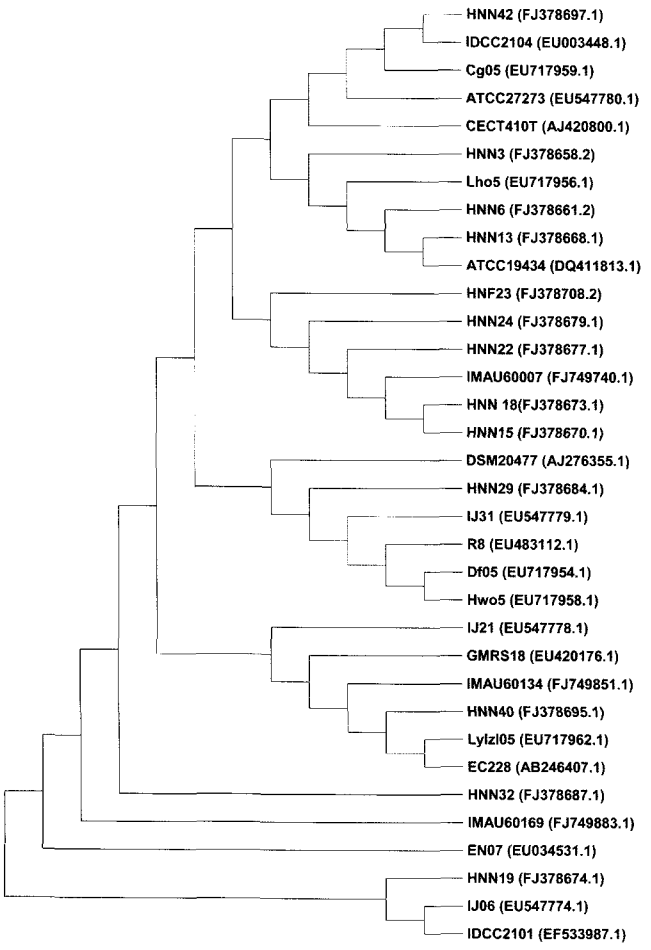

Fig. 3. Phylogenetic relationship of *Enterococcus faecium* and *Enterococcus faecalis* based on the sequences of its 16S rRNA genes.

A. 16S rRNA gene clade of isolated *E. faecium* strains. **B.** 16S rRNA gene clade of isolated *E. faecalis* strains.

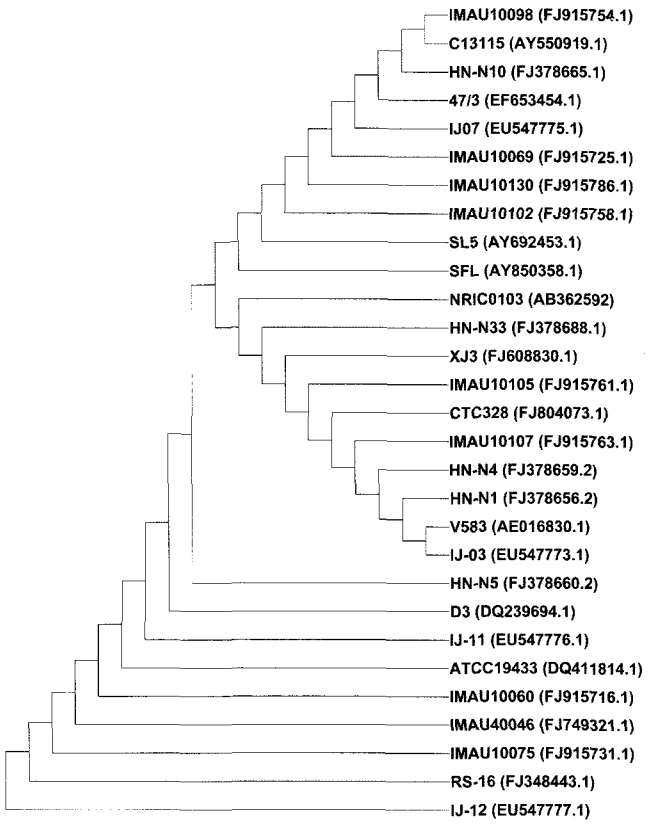

Fig. 3. Continued.

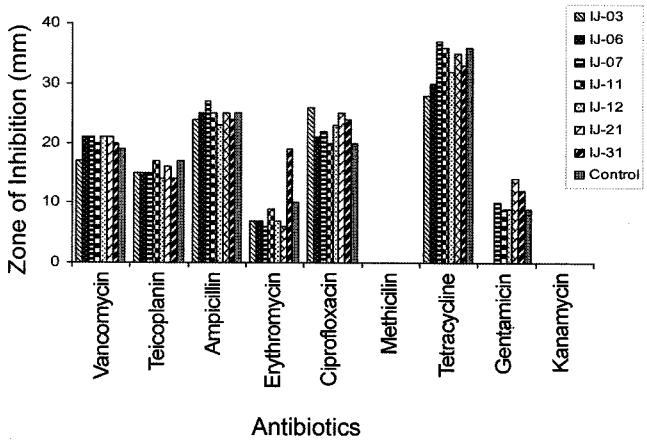

Fig. 4. Antibiotics susceptibility of isolated strains against commonly used antibiotics.

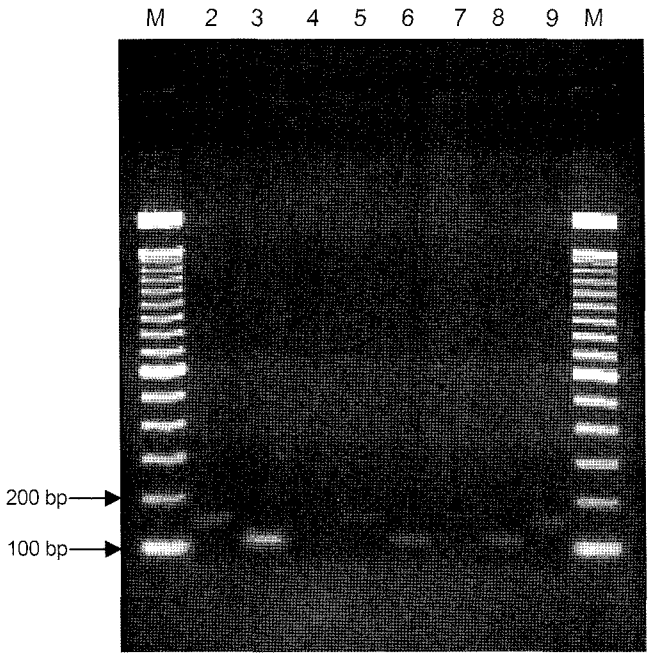

Fig. 5. Amplifications of bacteriocin genes from isolates on 2.0% agarose gel.
M: 100 bp DNA marker (Invitrogen, U.S.A.); L2: *E. faecium* IJ-06 *entA*; L3: *E. faecium* IJ-06 *entP*; L4: *E. faecalis* IJ-12 *entA*; L5: *E. faecium* IJ-21 *entA*; L6: *E. faecium* IJ-21 *entP*; L7: *E. faecium* IJ-31 *entA*; L8: *E. faecium* IJ-31 *entP*; L9: *E. faecium* ATCC 27273 *entA*.

natural resistance phenotype (Fig. 4). The minimum inhibitory concentration (MIC) of vancomycin for the isolates was in the range of 1–4 μg/ml. Vancomycin sensitivity was also confirmed by PCR amplifications of known glycopeptides resistance genes, *vanA* and *vanB*, using different sets of primers for the bacterial isolates. The plasmid extraction confirmed the absence of extrachromosomal DNA in the isolated strains. *Enterococcus faecalis, E. faecium*, and *E. durans* of dairy food origin did not possess vancomycin resistance and genes for other common virulence factors, which are prerequisites if the strain is to be used in food [15, 16, 21], whereas vancomycin-resistant enterococci (VRE) were reported from some foods of non-dairy origin [18, 19].

The absence of known virulence determinants was confirmed by amplification of critical virulence genes, mainly cytolysin (*cyl*), hemolysin, enterococcal surface protein (*esp*), and gelatinase (*gel*), by PCR. The absence of the virulence determinants among *E. faecium* strains was in agreement with earlier studies, in that the incidence of virulence factors among *E. faecalis* strains from foods was by far greater than for *E. faecium* strains from non-food sources [7, 9]. The absence of *esp* in the enterococcal strains was not

Table 3. PCR amplification of the bacteriocin gene and virulence determinants of isolated enterococcal strains.

Test	*E. faecalis* IJ-03	*E. faecium* IJ-06	*E. faecalis* IJ-07	*E. faecalis* IJ-11	*E. faecalis* IJ-12	*E. faecium* IJ-21	*E. faecium* IJ-31	*E. faecium* LMG 11423^T (Control)
Bacteriocin activity AU/ml[a]	0	3200	0	1600	0	6400	6400	3200
Bacteriocin structural genes:								
ent A	–	+	–	–	–	+	–	+
ent B	–	–	–	–	–	–	–	–
ent P	–	+	–	–	–	+	+	–
ent AS48	–	–	–	–	–	–	–	–
ent 31	–	–	–	–	–	–	–	–
ent L50A & L50B	–	–	–	–	–	–	–	–
ent 1071A & 1071B	–	–	–	–	–	–	–	–
Virulence determinants Hemolysin, *cyl*, *gel*, *esp*	–	–	–	–	–	–	–	–
Vancomycin resistance *vanA* and *vanB*	–	–	–	–	–	–	–	–

[a]Activity units per ml (AU/ml) were measured by using *E. faecalis* IJ-11as the indicator strain.

Table 4. Antimicrobial activity of cell-free culture supernatant of isolated strains against different indicator strains.

Indicator species	*E. faecalis* IJ-11	*E. faecium* IJ-06	*E. faecium* IJ-21	*E. faecium* IJ-31	Control
E. faecium LMG11423^T	+	+	+	+	+
E. faecium ATCC27273	+	+	+	+	+
E. faecalis IJ-07	+	+	+	+	+
L. monocytogenes ATCC19115	-	-	+	+	+
B. cereus ATCC 10987	-	-	+	+	-
B. subtilis ATCC27142	-	+	+	+	-
S. aureus ATCC 6538	-	-	-	+	-
E. coli ATCC 10536	-	-	-	-	-

American Type Culture Collection (ATCC, Manassas, VA, U.S.A.); LMG, Gent Culture Collection (Gent, Belgium); IJ-strains, our own laboratory isolates collection.

surprising as this determinant is generally associated with pheromone responsive plasmids, which are mostly associated with *E. faecalis* strains [8], and the strains under study also showed no plasmid when plasmid isolation was done.

Amplification of Enterocin Genes

A PCR fragment of 108 bp was amplified from genomic DNA of *E. faecium* IJ-06, *E. faecium* IJ-21, and *E. faecium* IJ-31, which corresponds to the enterocin P. Although the PCR fragment for enterocin A (155 bp) was detected in the case of *E. faecium* IJ-06, *E. faecium* IJ-21, and *E. faecium* ATCC 27273 (Fig. 5), no signals were obtained that corresponded with the enterocins 31, AS-48, L50A and L50B, and *ent* 1071A and 1071 B. This revealed that *E. faecium* IJ-06 and *E. faecium* IJ-21 have the potential to produce both enterocin A and P, whereas *E. faecium* ATCC 27273 and *E. faecium* IJ-31 were able to produce enterocin A and P, respectively. No signal was obtained for DNA of *E. faecalis* IJ-11 (lane 5 in Fig. 5), indicating the antimicrobial activity was because of an enterocin different from those checked by PCR. Enterocins A, B, and P were reported from enterococcal isolates of different sources [4, 11]. However, this is the first report of bacteriocinogenic enterococci isolated from fermented dairy products of Islamabad, Pakistan.

Screening for Bacteriocin Production Potential of Isolated Strains

Stab overlay, cross streak, agar well diffusion assay results showed that *E. faecium* IJ-06, *E. faecium* IJ-21, and *E. faecium* IJ-31 along with *E. faecalis* IJ-11 had antibacterial activity against the indicator strains tested. Amplification of enterocin genes also confirmed the genes from *E. faecium* IJ-06, IJ-21, and IJ-31, but no gene was amplified from *E. faecalis* IJ-11, revealing that antibacterial activity is due the enterocin other than checked through PCR.

Assessment of the antibacterial activity of isolated enterococcal strains in cell free supernatant showed that *E. faecium* IJ-21 and *E. faecium* IJ-31 had good activity against other enterococcal isolates as well as the other food-spoilage and pathogenic microorganisms *L. monocytogenes*, *B. cereus*, and *B. subtlis*, whereas *E. faecium* IJ-06 and *E. faecalis* IJ-11 had a narrow spectrum of activity, mainly against closely related species (Table 4). *Enterococcus faecium* IJ-21 and IJ-31 showed 6,400 AU/ml bacteriocin activity, whereas *E. faecium* IJ-06 and the control displayed 3,200 AU/ml when checked against a lawn of sensitive strains (Table 3).

In summary, isolated *Enterococcus* spp. from local dairy products were in a good proportion of lactic acid bacteria and mainly belonged to *E. faecalis* and *E. faecium.* All of the isolated strains were susceptible to most of the commonly used antibiotics, and these strains lack the major virulence traits. Further, the *E. faecium* strains IJ-06, IJ-21, and IJ-31 were potential producers of enterocins and had very good antimicrobial activity against their closely related strain *E. faecalis* IJ-11, including food-spoilage and pathogenic microorganisms. Isolated enterococci are promising candidates for further investigation of their bacteriocin production potential in relation to their safe use in dairy food products.

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References

1. Andrighetto, C., E. Knijff, A. Lombardi, S. Torriani, M. Vancanneyt, K. Kerster, J. Swings, and F. Dellaglio. 2001. Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. *J. Dairy Res.* **68:** 303–316.

2. Bhunia, A. K., M. C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of bacteriocin produced by *Pediococcus acidilactici*. *J. Appl. Bacteriol.* **65:** 261–268.
3. Choho, G, H. Abriouel, N. B. Omar, R. L. Lopez, E. Ortega, M. Martinez-Canamero, A. Laglaoui, S. Barrijal, and A. Galvez. 2008. Characterization of bacteriocin producing strain *Enterococcus faecalis* from cow's milk used in the production of Moroccan traditional dairy foods. *World J. Microbiol. Biotechnol.* **24:** 997–1001.
4. Cocolin, L., R. Foschino, G Comi, and M. G Fortina. 2007. Description of the bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian goat milk. *Food Microbiol.* **24:** 752–758.
5. Cogan, T. M., M. Barbosa, E. Beuvier, B. Bianchi-Salvadore, P. H. Coconcelli, P. S. Fernandez, *et al.* 1997. Characterizations of the lactic acid bacteria in artisanal dairy products. *J. Dairy Res.* **64:** 409–421.
6. Cotter, P. D., C. Hill, and R. P. Ross. 2005. Bacteriocins: Developing innate immunity for food. *Nat. Rev. Microbiol.* **3:** 777–788.
7. Eaton, T. J. and M. J. Gasson. 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl. Environ. Microbiol.* **67:** 1628–1635.
8. Franz, C. M. A. P., W. H. Holzapfel, and M. E. Stiles. 1999. Enterococci at the crossroad of food safety? *Int. J. Food Microbiol.* **47:** 1–24.
9. Franz, C. M. A. P., A. B. Muscholl-Silberhorn, N. M. K. Yousaf, M. Vancanneyt, J. Wings, and W. H. Holzapfel. 2001. Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Appl. Environ. Microbiol.* **67:** 4385–4389.
10. Franzetti, L., M. Pompei, M. Scarpellini, and A. Galli. 2004. Phenotypic and genotypic characterization of *Enterococcus* spp. of different origins. *Curr. Microbiol.* **49:** 255–260.
11. Foulquié-Moreno, M. R., R. Callewaert, B. Devreese, J. Van-Beeumen, and L. De Vuyst. 2003. Isolation and biochemical characterization of enterocins produced by enterococci from different sources. *J. Appl. Microbiol.* **94:** 214–229.
12. Foulquie-Moreno, M. R., P. Sarantinopoulos, E. Tsakalidou, and L. De Vuyst. 2005. The role and application of enterococci in food and health. *Int. J. Food Microbiol.* **106:** 1–24.
13. Klevin, G 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and gastro-intestinal tract. *Int. J. Food Microbiol.* **88:** 123–131.
14. Leroy, F., M. R. Foulguie-Moreno, and L. De Vuyst. 2003. *Enterococcus faecium* RZS C5, an interesting bacteriocin produce to be used as a co-culture in food fermentation. *Int. J. Food Microbiol.* **88:** 235–240.
15. Martin, M., J. Gutierrez, R. Criado, C. Herranz, L. M. Cintas, and P. E. Hernandez. 2006. Gene encoding bacteriocins and their expression and potential virulence factors of enterococci isolated from wood pigeons (*Columba palumbus*). *J. Food Prot.* **69:** 520–531.
16. Morandi, S., M. Brasca, C. Andrighetto, A. Lombardi, and R. Lodi. 2006. Technological and molecular characterization of enterococci isolated from north-west Italian dairy products. *Int. Dairy J.* **16:** 867–875.
17. National Committee for Clinical Laboratory Standards. 2002. *Performance Standards for Antimicrobial Susceptibility Testing.* Twelfth Informational Supplement, Vol 21.
18. de Vos, P., G Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K. H. Schleifer, and W. B. Whitman (eds.). 2009. *Bergey's Manual of Systematic Bacteriology. The Firmicutes,* 2nd Ed. Vol. 3. Springer, New York.
19. Sanchez, J., A. Basanta, B. Gomez-Sala, C. Herranz, L. M. Cintas, and P. E. Hernandez. 2007. Antimicrobial and safety aspects, and biotechnological potential of bacteriocinogenic enterococci isolated from mallard ducks (*Anas platyrhynchos*). *Int. J. Food Microbiol.* **117:** 295–305.
20. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24:** 1596–1599.
21. Yousaf, N. M. K., P. Dawyndt, H. Arioue1, A. Wijaya, U. Scillinger, M. Vancanneyt, *et al.* 2005. Molecular characterization, technological properties and safety aspects of enterococci from 'Hussuwa', an African fermented sorghum product. *J. Appl. Microbiol.* **98:** 216–228.