Production of bacteriocins by *Enterococcus* spp. isolated from traditional, Iranian, raw milk cheeses, and detection of their encoding genes

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Abstract

Strong bacteriocins, or bacteriocins with a wide range of activity against pathogens and spoilage microorganisms, are actively sought for use as natural food preservatives. This work reports the inhibitory activity of 96 enterococcal isolates from two Iranian, raw milk cheeses against five indicator organisms (including *Listeria innocua*). Forty eight isolates inhibited at least one indicator in spot agar assays. Of these, 20 isolates corresponding to 15 different strains were shown to produce bacteriocin-like substances in liquid cultures. PCR analysis revealed the genes coding for enterocins (enterococcal bacteriocins) A, B, P or X, or their combinations, in all but one of these 15 strains. In addition, the gene coding for enterocin 31 was detected in two strains. No amplification was obtained in one strain when using specific primers for all 13 bacteriocin genes sought. Three different enterocin genes were identified in most strains, and four in one strain. Although the concomitant production of bacteriocins is still to be verified, producers of multiple enterocins could be of great technological potential as protective cultures in the cheese industry.

1. Introduction

Enterococci are the dominant lactic acid bacteria (LAB) in many foods, including vegetables, meat and dairy products [*13*]. Large numbers have been repeatedly reported in curd and ripened cheeses made from raw milk (10^4-10^6 and 10^5-10^7 cfu.g^{-1} respectively) [*4, 15, 30*]. Further, enterococcal species have recently been shown to be the dominant cultivable populations of the traditional Iranian cheeses Lighvan and Koozeh [*9*].

Some authors believe enterococci responsible for producing the typical taste and flavour of certain foods [*16, 18*]. Indeed, selected strains are used as starter and ripening cultures [*3*]. A few strains even have an impressive record of safe use as probiotics, with these organisms contributing to intestinal health by improving the microbial balance of the gut [*7*]. Further, their proteolytic and lipolytic activities, their capacity to their use citrate and pyruvate as C sources, and their production of bacteriocins (known as enterocins when produced by enterococci) [*13, 16*] are all of technological interest. Although this, undesirable effects of food-borne enterococci, such as production of biogenic amines [*23*] and carry-over and spread of antibiotic resistances [*25*]. Therefore, the safety properties of all strains intended to be used in food systems should be carefully examined [*31*].

Bacteriocins are ribosomally-synthesised peptides with antimicrobial activity that are of potential use in the control of food-borne pathogens and spoilage microorganisms, and in the treatment of infections [*14*]. These inhibitory substances are produced by many bacterial groups including the enterococci [*29*]. Those produced by enterococcal strains (enterocins) include the commonly encountered enterocins A, B, P, AS-48, L50A, L50B, 1071A, 1071B and Q, as well as mundticin KS [*13, 29*]. Most enterocins are small, heat stable, non-lantibiotics with a generally strong antilisterial effect. Some enterocin-producing strains have already been successfully used in cheese trials as protective cultures against *Listeria monocytogenes* [*12, 21*]. The search continues for strains that produce broader and/or stronger inhibitory compounds, or indeed that produce multiple enterocins with synergistic activity against undesirable bacteria.

The present work reports on the screening for the production of antimicrobial compounds against five bacterial indicators by enterococcal isolates belonging to the dominant populations appearing during the manufacture and ripening of two traditional Iranian cheeses made from raw

milk. Producer strains were then screened for the presence of known bacteriocin-encoding genes via the use of the polymerase chain reaction (PCR).

2. Materials and Methods

2.1. Strains, media and culture conditions

The 96 enterococcal isolates used in this work were those previously isolated from two traditional, Iranian, raw milk cheeses: Lighvan (52) and Koozeh (44) [*10*]. These isolates have already been identified as belonging to the following species: *Enterococcus faecium* (74), *E. faecalis* (16), *E. casseliflavus* (3), *E. durans* (2), and *E. italicus* (1). *E. faecalis* JH2-2, *Lactococcus lactis* subsp. *cremoris* MG1363, *Staphylococcus aureus* CECT86, *Listeria innocua* 86/26, and *Lactobacillus plantarum* CECT748 were all used as indicators of enterocin production. *L. innocua* was used as a safer model of inhibition against the ubiquitous pathogen *L. monocytogenes*. *S. Aureus* was selected as a representative food-borne pathogen, and *Lc. lactis* and *Lb. plantarum* were chosen as indicators of technologically relevant lactic acid bacteria species.

The enterococcal isolates and the indicators were recovered on either BHI agar (*E. feacalis* JH2-2 and the cheese isolates), M17 agar (*Lc. lactis*), MRS agar (*Lb. plantarum*), or Tryptone Soy agar (TSA) (*L. innocua* and *S. aureus*) from stocks held at -80°C, by incubating under aerobic conditions at the corresponding optimum temperature for 24-48 h. All media were supplied by Merck (Darmstad, Germany) except for TSA, which was made in house from its

constituent components (15% pancreatic digest of casein, 5% papain digest of soy beans, 5% NaCl, and 15% bacteriological agar; pH 7.3).

2.2. Detection of antimicrobial activity

The inhibitory activity of the isolates was evaluated successively in solid and liquid media using the agar spot test and a well diffusion assay.

2.2.1 Agar spot test

All isolates were assayed for antagonistic activity in solid media by a modification of the method described by Fleming et al. [11]. Briefly, aliquots (5 µl) from overnight cultures were spotted onto the surface of plates of modified BHI agar (BHI plus 0.2% glucose) and modified M17 (M17 without lactose, plus 0.2% glucose) and incubated at 32°C for 24 h to allow spots to develop. The spots were then covered with 10 ml of the corresponding soft agar (0.75%) for each indicator inoculated at 0.25%. Plates were incubated for 24 h under the required temperature conditions for the respective indicator, after which the plates were checked for halos of growth inhibition around the spots.

2.2.2 Well-diffusion assay

Positive strains in the agar spot test were then examined for antimicrobial activity in a well-diffusion assay. Briefly, overnight cultures of indicators were used to inoculate (at 1%) 20 ml of their corresponding agar media at 45ºC. The inoculated media were then poured into Petri dishes. After solidification, six to seven wells were made in each plate to accommodate 50 µl of neutralized (pH 6.5-7.0), filter-sterilized (through a 0.2 µm pore membrane; Millipore, Bedford, MA, USA) supernatants of the producer strains grown in BHI and M17 modified liquid media as

above. All plates were incubated under appropriate conditions and subsequently examined for zones of inhibition around the wells.

2.2.3 Confirmation of the proteinaceous nature of the antimicrobials

To judge whether the inhibitory substances were sensitive to proteolysis, a hole in the agar plates prepared as above was punched next to the wells containing the neutralized, filtered-sterilized supernatants. The hole was filled with 25 µl of a solution containing either bovine serum albumin, proteinase K, or pronase (all from Sigma-Aldrich, St. Louis, MO, USA) at a concentration each of 20 $mg.ml^{-1}$. Plates were incubated overnight at 30ºC and then examined for inhibition zones around the wells.

2.3. PCR detection of bacteriocin structural genes

Total DNA was extracted from bacteriocin-producing enterococci grown overnight in BHI broth at 32ºC for the detection of known enterocin-encoding genes. The DNA was isolated and purified using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich) following the manufacturer's recommendations, and its concentration measured at 260 nm using a spectrophotometer (Digilab, Hitachi Ltd., Tokyo, Japan). PCR amplification of the structural genes for enterocin A, enterocin B, enterocin P, enterocin L50A and L50B (amplified with the same primer pair; Table 1), bacteriocin 31, enterocin AS48, enterocin 1071A and 1071B (amplified with the same primer pair; Table 1), mundticin KS, enterocin Q, enterocin X, and pediocin PA-1 was performed using specific PCR primers as listed in Table 1. DNA from well-known enterocin-producing strains, including *E. faecium* L50 (enterocins L50A, L50B, and Q), *E. faecium* T136 (enterocins A and B), *E. faecium* AS48 (enterocin AS48), *E. faecium* P13

(enterocin 31), and *Pediococcus acidilactici* PAC1.0 (pediocin PA-1), was used to provide positive controls.

PCR reactions were performed in a volume of 50 µl containing 10 pmol of each primer, 25 µl of a 2x Master Mix containing DNA polymerase (Ampliqon, Skovlunde, Denmark), 100 ng of DNA from the producer strain, and molecular grade water (added up to the reaction volume) (Sigma-Aldrich). Amplifications were performed in an iCycler (Bio-Rad, Richmond, CA, USA) employing an initial denaturation cycle at 95°C for 5 min, followed by 35 cycles of denaturation (94°C for 30 s), annealing (as indicated in Table 1 for the different primer pairs) and elongation (72°C for 10 s), and a final extension step at 72°C for 7 min. Amplicons were separated by electrophoresis in 1% agarose gels, the bands stained with ethidium bromide (0.5 $\mu g.ml^{-1}$), and photographed under UV light. PCR-generated fragments were purified directly after amplification using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), or after agarose gel electrophoresis using the QIAquick Gel Extraction kit (Qiagen).

2.4. Sequencing and sequence analysis

Selected amplicons were sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were then compared to those held in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.5. Cross-protection activity test

A cross-protection activity test using all strains as both producers and indicators was performed to check the susceptibility of each strain to the inhibitory substance(s) produced by all others. This assay was performed using the agar spot test as reported above.

3. Results and Discussion

3.1. Antimicrobial activity of the cheese enterococci

The 96 isolates have already been typed by the repetitive extragenic palyndromic (rep-PCR) technique and found to consist in 57 different profiles representing distinct strains [*9*]. In spite of this, isolates were all analyzed for the production of inhibitory compounds because large phenotypic variations have been reported among genetically indistinguishable strains [*4, 27*]. Thus, in the agar spot test, 48 of the 96 isolates inhibited at least one of the indicators. *E. faecalis* OGF1 was inhibited by 38 isolates, *L. innocua* 86/26 by 32 isolates, *Lb. plantarum* CECT 748 by 27, *S. aureus* CECT86 by 20, and *Lc. lactis* subsp. *cremoris* MG1363 by 2. Differences were seen in the size of some inhibition halos, although no differences were seen in the inhibition profiles in either modified BHI or modified M17. All 48 isolates showing inhibitory activity on the agar spot test were then examined for antimicrobial production in liquid medium assay against all five indicators in a well-diffusion assay. Under these conditions, only 20 isolates showed inhibitory activities against one or more indicators. The proteinaceous nature of the antimicrobials and their susceptibility to proteases was checked by both proteinase K and pronase in a well-diffusion plate assay with bovine serum albumin as a negative control. As an example, Figure 1 shows the effect of these two proteinases on the inhibitory capacity of *E.*

faecium LR74 supernatants against *L. lactis* MG 1363. Proteolytic degradation of the inhibitory compound resulting from the presence of proteinases in the near-by wells is substantiated by a reduction of the inhibition halo (Figure 1, lanes 2 and 3), while bovine serum albumin causes no effect (Figure 1, lane 4).

On the basis of the inhibitory profiles of the isolates and their typing profiles, the producers were seen to belong to 15 different strains of three species: *E. faecium* (11 strains), *E. faecalis* (3 strains), and *E. casseliflavus* (1 strain). Table 2 summarises the inhibitory profiles of these 15 strains against all five indicators. Five to six different inhibitory profiles were observed. The most common profile (C) was characterized by the strong inhibition of *L. innocua* and the weak inhibition of *E. faecalis*. This profile was shared by seven strains belonging to both *E. faecium* (strains C20, LF44, LR75, KR30, and KR37) and *E. faecalis* (strains C35 and KR24) (Table 2). The *L. innocua* indicator was inhibited (in most cases strongly) by 13 of the 15 strains. In contrast, the *E. faecalis* strain was only weakly inhibited by all the producers except for two (*E. faecium* LR74 and *E. casseliflavus* KR47) which showed clear inhibition. The *Lb. plantarum* indicators were only inhibited by two producers, while *Lc. lactis* was inhibited by these same two plus another. Interestingly, the *S. aureus* indicator, that proved to be inhibited by approximately half of the producers on the plate assay, was inhibited by none of the strains in liquid (Table 2).

The enterocin-producing phenotype among enterococci from different sources and ecosystems is rather common [*1, 19, 26, 32, 33, 34*]. The inhibitory range in liquid medium was different to that seen on the agar plates, in agreement with reports by many authors that inhibitory activities displayed on agar are not always observed with filtered, neutralized supernatants [*17, 24, 27, 35*]. Colony-associated metabolic compounds with antimicrobial

activity, including organic acids, hydrogen peroxide, and fatty acids are thought to account for the inhibitory effects observed in solid media [*6*]. Many enterocins have been shown to be active against the widespread food-borne pathogen *L. monocytogenes* [*19, 32, 33, 34*]. Although a positive correlation between the inhibition of *L. innocua* and *L. monocytogenes* has been repeatedly reported [*32-34*], the inhibitory activity against the real pathogen should necessarily be confirmed. The inhibition of *Listeria* spp. and other pathogens such as *S. aureus* by enterocin-producing strains has promoted their use in securing the safety of food systems [*12, 21*]. Purified enterocins could also be used for the treatment of animal and human infections, as recently proposed for the bacteriocins produced by *Lc. lactis* [*22, 28*].

3.2. Detection of enterocin structural genes by PCR

The purified DNA of all 15 enterocin-producing strains was used as a template in PCR amplifications to check for the presence of structural genes encoding 12 enterocins plus pediocin PA-1 (Table 1), all known to be readily spread among enterococci [*2, 20, 26, 33*]. Table 3 shows the amplification results. Figure 2 shows the electrophoretograms for the five enterocin structural genes for which amplifications were obtained (enterocins A, B, P, 31, and X).

To show that the amplification products corresponded to the expected enterocin-encoding genes, they were sequenced and compared to those held in the GenBank database. The gene coding for enterocin A was detected in 11 strains (Figure 2; panel A), while eight strains carried genes for enterocin P and enterocin B (Figure 2; panels B and P, respectively). The gene encoding enterocin X was found in five strains (Figure 2; panel X), and that coding for enterocin 31 in two (Figure 2; panel 31). In contrast, no genes coding for enterocins L50A, L50B, AS48, 1071A, 1071B, KS and Q or pediocin PA-1 were detected in any of the present producers,

although unspecific amplicons were obtained for some strains with enterocin L50 primers (data not shown). Representative amplicons of the different enterocin-structural genes were purified and sequenced using the forward amplification primers. Sequence comparisons showed amplicons to be identical at the nucleotide level to the corresponding gene sections in databases, except for a single, conservative nucleotide change in the gene of enterocin P from strain *E. faecium* C20, which did not alter the deduced amino acid sequence. It is worth noting that, in *E. faecium* LF44, none of the analysed genes was recorded, suggesting the presence of a gene for a new enterocin. PCR detection of more than one bacteriocin-encoding gene in the same cell is not unusual [*1, 5, 20, 26*]. In this work, up to four different genes were detected in one strain (*E. faecium* C20; Table 3). Indeed, enterococcal strains of human and animal origin carrying genes for multiple enterocins have recently been reported [*2*]. These strains might enjoy a broader range of inhibition and/or stronger inhibition against pathogens. Enterocins can act through distinct cellular targets or present cooperative antimicrobial activities. In either case, multienterocin producers may contribute to enhance the safety of fermented foods.

3.3. Cross protection activity test

Visual inspection of the inhibitory profiles of the present strains (Table 2) showed them not to correspond to those suggested by gene amplification (Table 3), a result that prompted a cross protection activity test with the present enterocin-producing strains as both producers and indicators (Table 4). The profiles obtained through the different assays were all arranged in Table 5, thus allowing easier comparison between phenotypic and genetic data. Although some coincidences were seen in the inhibition and cross protection profiles of strains carrying the same enterocin genes, large differences were also noted. In fact, the number of profiles encountered

increases from five in the inhibition range up to 12 in the cross protection activity test. It should be stressed that the presence of a structural gene does not necessarily imply its expression. Additionally, strains may also produce enterocins different to those searched for in this work by PCR. Moreover, bacteriocin resistance may be unlinked with bacteriocin production, which further complicates the phenotypic analyses. Nevertheless, the cross protection assay may be of use in checking the compatibility of strains if the design of multistrain cultures is intended.

Conclusions

In this work, 15 enterocin-producing strains belonging to *E. faecium* (11), *E. faecalis* (3), and *E. casseliflavus* (1) were identified among a set of enterococci 96 isolates recovered from two traditional, raw milk, Iranian cheeses (Lighvan and Koozeh). The genes responsible for indicator organism inhibitory activity were amplified by PCR in most strains. Four enterocin-encoding genes were readily identified in a single strain. The multiple enterocin producers detected are likely more efficient in preventing the growth of undesirable bacteria than are single bacteriocin producers. These strains are currently being examined for use as protective cultures in experimental cheese trials. The fact that a majority of the strains inhibited *L. innocua* while only a few showed an effect on lactococci (inhibited by three strains) and lactobacilli (inhibited by two strains) argue in favour of inhibiting pathogen microorganisms without disturbing species of lactic acid bacteria of technological relevance.

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Table 1.- Primers used throughout this study and their amplification details.

Name	Sequence (5'→ 3')	Target gene	Annealing temperature	Size of the amplicon	Source/reference
entAF	AAATATTATGGAAATGGAGTGTAT	Enterocin A	50	475	Du Toit et al. [8]
entAR	GCACTTCCCTGGAATTGCTC				
entBF	GAAAATGATCACAGAATGCCTA	Enterocin B	50	159	Du Toit et al. [8]
entBR	GTTGCATTTAGAGTATACATTTG				
entPF	GGTAATGGTGTTTATTGTAAT	Enterocin P	48	117	Du Toit et al. [8]
entPR	ATGTCCCATACCTGCCAAAC				
entL50F	GGAGCAATCGCAAAATTAG	Enterocins L50A, B	55	150	Du Toit et al. [8]
entL50R	ATTGCCCATCCTTCTCCAAT				
Ent31F	TATTACGGAAATGGTTTATATTG	Enterocin 31	50	122	Du Toit et al. [8]
Ent31R	TCTAGGAGCCCAAGGGCC				
EntAS48F	GAGGAGTTTCATGATTTAAAG	Enterocin AS48	50	185	Du Toit et al. [8]
EntAS48R	CATATTGTTAAATTACCAAGC				
Ent1071F	GGGGAGAGTCGGTTTTTAG	Enterocins 1071A, B	50	273	Martin et al. [26]
Ent1071R	ATCATATGCGGGTTGTAGCC				
EntKSF	CTACGGTAATGGAGTCTCATG	Mundticin KS	50	275	This work
EntKSR	CATCTGCATACAGGCTATACC				
EntQF	CAAGAAATTTTTTCCCATGGC	Enterocin Q	55	95	This work
EntQR	CTTCTTAAAAATGGTATCGCA				
EntXF	GTTTCTGTAAAAGAGATGAAAC	Enterocin X	50	500	This work
EntXF	CCTCTTAATCATTAACCATAC				
PedPAF	ACTGCGTTGATAGCGAGGTT	Pediocin PA1	50	360	Martin et al. [26]
PedPAR	TGATGCCAGCTCAGCATAAT				

Table 2.- Inhibition range of the antimicrobial(s) produced in liquid cultures by enterococci strains from Lighvan and Koozeh cheeses against food borne pathogens and indicator bacteria, as determined by a well diffusion assay.

Producing strain	Indicator strain			
	Enterococcus faecalis OGF1	*Lactococcus lactis* MG 1363	*Listeria innocua* 86/26	*Lactobacillus plantarum* CECT 748^T
E. faecium M9	-	++	-	-
E. faecium C16	-	-	++	-
E. faecium C17	-	-	++	-
E. faecium C20	(+)	-	++++	-
E. faecalis C35	(+)	-	++++	-
E. faecium LF44	+	-	+++	-
E. faecium LF54	-	-	+	-
E. faecalis LR71	(+)	(+)	-	++
E. faecium LR74	++	++	++	+++
E. faecium LR75	(+)	-	+++	-
E. faecalis KR24	(+)	-	++	-
E. faecium KR30	(+)	-	+++	-
E. faecium KR34	-	-	++	-
E. faecium KR37	(+)	-	++	-
E. casseliflavus KR47	++	-	+++	-

The number of crosses referrers to the diameter of the inhibition halo around the wells.
None of the strains inhibited *Staphylococcus aureus* CECT 86^T.

Table 3.- Presence of enterocin genes in the *Enterococcus* spp. strains analyzed in this work.

Producing strains	Amplification of the following structural bacteriocin genes				
	A	B	P	31	X
E. faecium M9	+	-	+	-	-
E. faecium C16	+	+	+	-	-
E. faecium C17	-	+	+	-	-
E. faecium C20	+	+	+	-	+
E. faecalis C35	+	-	+	-	-
E. faecium LF44	-	-	-	-	-
E. faecium LF54	+	-	+	-	-
E. faecalis LR71	-	-	-	-	-
E. faecium LR74	+	-	-	+	-
E. faecium LR75	-	-	+	+	-
E. faecium KR30	+	+	-	-	+
E. faecalis KR24	+	+	-	-	+
E. faecium KR34	+	+	-	-	-
E. faecium KR37	+	+	-	-	+
E. casseliflavus KR47	-	+	+	-	+

PA-1, pediocin PA-1.
Positive PCR amplification with specific primers for structural genes encoding bacteriocins L50A, L50B, AS48, 1071A, 1071B, KS, Q, and pediocin PA-1 was never obtained.

Table 4.- Cross protection activity using an agar-spot test and using all strains as producers and indicators.

Producing strain	Indicator strain										
	M9	C16	C17	C20	C35	LF54	LR71	KR24	KR30	KR34	KR37
E. faecium M9	-	(+)	-	(+)	-	-	-	-	+++	+++	-
E. faecium C16	-	-	(+)	(+)	-	-	-	-	+	-	-
E. faecium C17	-	(+)	-	(+)	-	-	-	-	(+)	+	-
E. faecium C20	(+)	-	-	-	-	-	-	+	+	+	-
E. faecalis C35	-	-	-	-	-	-	-	+	+	+	-
E. faecium LF44	+	-	+	-	+	+	-	-	+	+	+
E. faecium LF54	-	-	-	+	+	-	+	+	+	+	-
E. faecalis LR71	-	-	-	+	+	-	-	-	-	-	-
E. faecium LR74	-	-	-	-	-	-	-	-	+	+	-
E. faecium LR75	-	-	-	-	-	-	-	-	-	-	-
E. faecalis KR24	-	-	-	-	-	-	-	-	(+)	-	-
E. faecium KR30	-	(+)	-	(+)	(+)	-	-	-	-	-	-
E. faecium KR34	-	(+)	-	(+)	(+)	-	-	-	-	-	-
E. faecium KR37	-	-	-	-	-	-	+	-	+	+	-
E. casseliflavus KR47	-	-	-	-	-	-	-	-	+	+	-

The number of crosses relates to the inhibitory effect; (+), weak inhibition.
Under the conditions of this assay, none of the strains inhibited LF44, LR74, LR75, and KR47 when they were used as indicators.

Table 5.- Phenotypic and genetic profiles of enterocin-producing *Enterococcus* spp. strains from Iranian traditional Lighvan and Koozeh cheeses.

Producing strain	Phenotypic or genetic profile		
	Inhibition range	Amplification of enterocin-encoding genes	Cross protection activity
E. faecium M9	IR-1	GC-1	CP-1
E. faecium C16	IR-2	GC-2	CP-2
E. faecium C17	IR-2	GC-3	CP-1
E. faecium C20	IR-3	GC-4	CP-3
E. faecalis C35	IR-3	GC-1	CP-4
E. faecium LF44	IR-3	GC-5	CP-5
E. faecium LF54	IR-2	GC-1	CP-6
E. faecalis LR71	IR-A	GC-5	CP-7
E. faecium LR74	IR-5	GC-6	CP-8
E. faecium LR75	IR-3	GC-7	CP-9
E. faecalis KR24	IR-3	GC-8	CP-10
E. faecium KR30	IR-3	GC-9	CP-11
E. faecium KR34	IR-2	GC-10	CP-11
E. faecium KR37	IR-3	GC-9	CP-12
E. casseliflavus KR47	IR-3	GC-10	CP-8

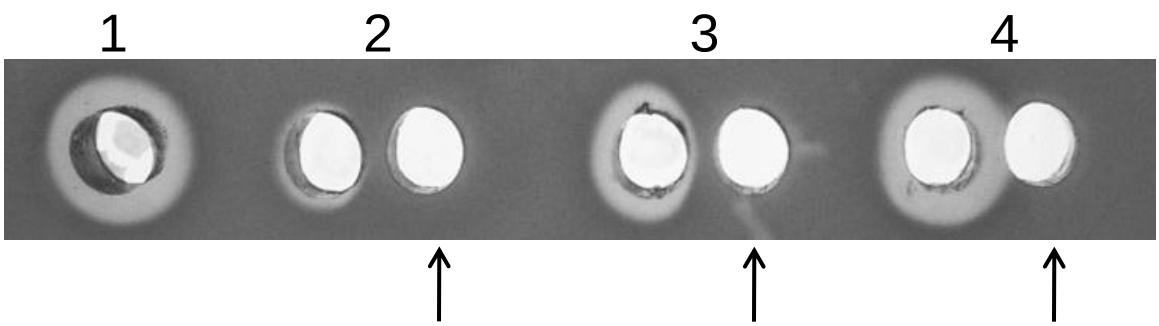

Figure 1

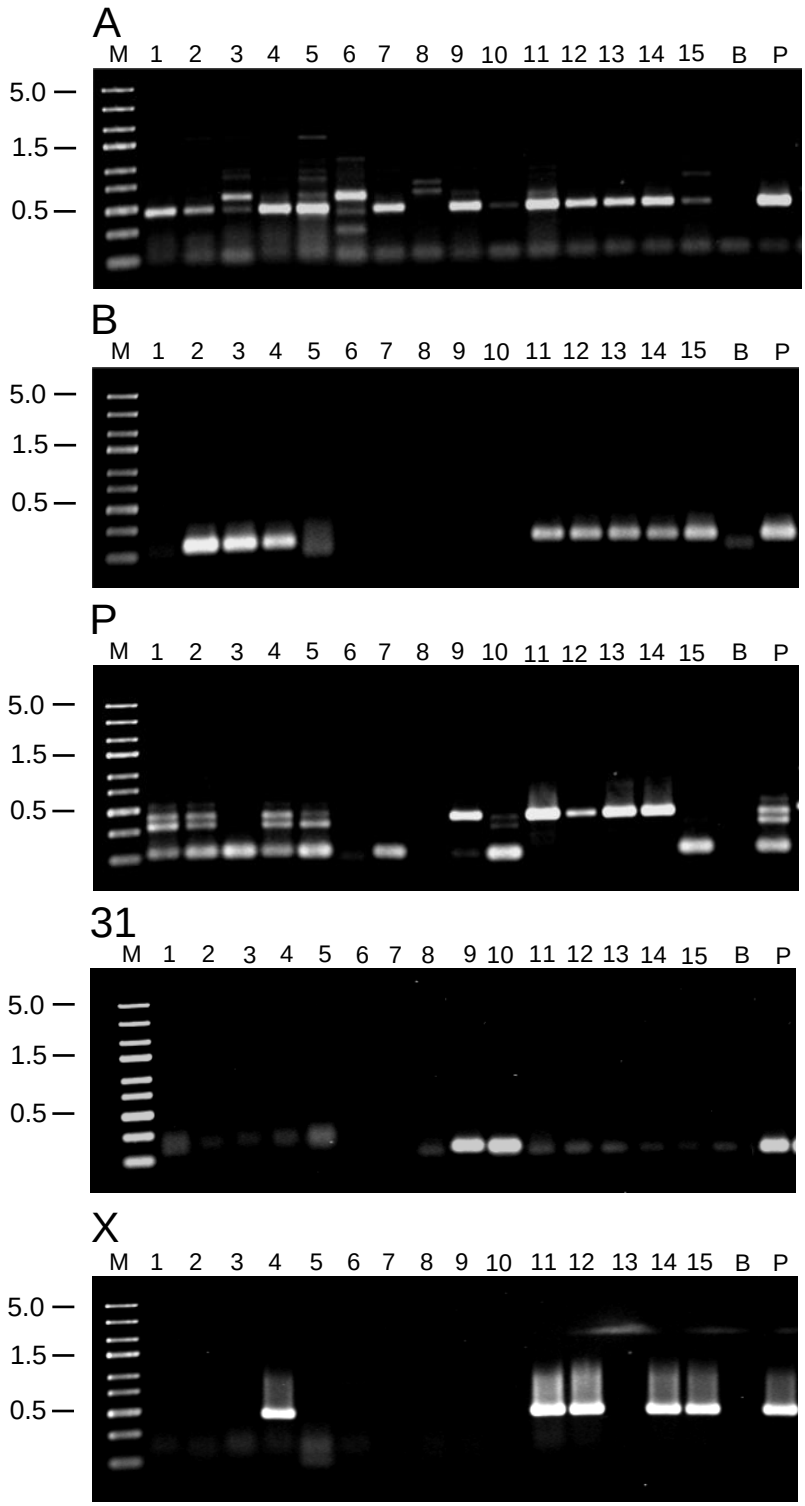
A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 B P
5.0
1.5
0.5
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 B P
5.0
1.5
0.5
P
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 B P
5.0
1.5
0.5
31
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 B P
5.0
1.5
0.5
X
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 B P
5.0
1.5
0.5

Figure 2

Figure captions

Figure 1.- Analysis of the proteinaceous nature of the antimicrobials produced by the enterococci strains studied in this work. In the picture, effect of proteinase K (2), pronase (3), and bovine serum albumin (4) (arrowed wells) on the inhibitory activity against *L. lactis* MG 1363 of neutralized, filter sterilized supernatants from an overnight culture of *E. faecium* LR74 (1 through 4, non-arrowed wells).

Figure 2.- Amplification results for genes encoding enterocin A (panel A), enterocin B (panel B), enterocin P (panel P), enterocin 31 (panel 31) and enterocin X (panel X). Order of strains in all panels: Line 1, *E. faecium* M9; Line 2, *E. faecium* C16; Line 3, *E. faecium* C17; Line 4, *E. faecium* C20; Line 5, *E. faecalis* C35; Line 6, *E. faecium* LF44; Line 7, *E. faecium* LF54; Line 8, *E. faecalis* LR71; Line 9, *E. faecium* LR74; Line 10, *E. faecium* LR75; Line 11, *E. faecalis* KR24; Line 12, *E. faecium* KR30; Line 13, *E. faecium* KR34; Line 14, *E. faecium* KR37, and Line 15, *E. casseliflavus* KR47. M, molecular weight marker. B, blank, reaction without template DNA. P, positive reaction using purified DNA from a producer strain, as indicated in the text.