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Identification of Bacteriocin Genes in Enterococci Isolated from Game Animals and Saltwater Fish

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ABSTRACT

Bacteriocins produced by enterococci, referred to as enterocins, possess great interest for their potential use as biopreservatives in food and feed, as well as alternative antimicrobials in humans and animals. In this context, the aim of the present study was to determine the antimicrobial activity and the presence of bacteriocin structural genes in fecal enterococcal isolates from animal origins. Evaluation of the direct antimicrobial activity of 253 isolates from wild boars (*Sus scrofa, n* = 69), mullets (*Liza ramada, n* = 117), and partridges (*Perdix perdix*, *n* = 67) against eight indicator bacterial strains (including *Listeria monocytogenes, Pediococcus pentosaceus*, and *Enterococcus* spp.) showed that 177 (70%) exerted antimicrobial activity against at least one indicator microorganism. From these isolates, 123 were further selected on the basis of their inhibition group, and 81 were found to be producers of bacteriocins active against *Listeria monocytogenes*. Analysis of the presence of enterocin structural genes in a subset of 36 isolates showed that 70% harbored one or more of the evaluated genes, those of enterocin P and hiracin JM79 being the most prevalent. These results show that wild animals constitute an appropriate source for the isolation of bacteriocinogenic enterococci.

The microorganisms of the genus *Enterococcus* are gram-positive, catalase-negative, and oxidase-negative cocci producing lactic acid as the major end product of glucose fermentation. Enterococci are commensals in the animal and human intestinal tract, where they are believed to play a key role in the balance of the microbiota, thereby showing great potential as probiotics *(40, 41)*. They are widespread in nature, frequently being found in wastewater, feces, and slurry, as well as in soil and on plants, and they are thus considered to be indicators for fecal contamination *(34)*. Furthermore, enterococci participate in the fermentation of foods, such as milk *(40),* vegetables *(3, 40),* and meat *(29, 40)*. In the gastrointestinal ecosystem, enterococci have to compete with other bacteria, and therefore, the production of bacteriocins may play an important role in their survival *(40, 41)*.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by gram-positive and gram-negative bacteria *(41)*. They act on sensitive bacteria by altering the permeability of the cytoplasmic membrane or by interfering with essential cell functions, such as DNA replication and translation *(2, 41)*. Although bacteriocin production has been described in different species of enterococci *(21, 30, 41, 45, 46),* enterocins are most frequently produced by *Enterococcus faecium*. According to Franz et al. *(23),* enterocins can be divided into four classes, as follows: I, lantibiotic enterocins, such as cytolysin ($cylL_{L/S}$); II, small nonlantibiotic peptides; III, cyclic enterocins, such as enterocin AS-48 (*entAS-48*); and IV, large proteins, such as enterolysin A (*entL*). Class II may be further divided into three subclasses, as follows: II.1, enterocins of the pediocin family, such as enterocin A (*entA*), enterocin P (*entP*), and hiracin JM79 (*hirJM79*); II.2, enterocins synthesized without a leader peptide, such as enterocin L50 (*entL50* [*entL50A* and *entL50B*]) and enterocin Q (*entqA*); and III.3, other linear, non–pediocin-type enterocins, such as enterocin B (*entB*). The diversity of enterocins has been attributed both to the robust nature of enterococci, which allows them to survive in a wide range of ecological niches, and to their superior genetic-exchange mechanisms *(23)*.

Interest in bacteriocins produced by lactic acid bacteria has been stimulated by the fact that they are active against foodborne pathogens, such *Listeria monocytogenes* and *Staphylococcus aureus* and vegetative cells and spores of *Bacillus* spp. and *Clostridium botulinum (6, 41, 44)*. The use of bacteriocins as food biopreservatives would allow satisfying the consumer demand for a more ''natural'' preservation technology. Additionally, their potential application in human and animal health promotion, for example

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TABLE 1. *Indicator species and growth conditions used in this study*[a]

Indicator species	Strain	Origin	Growth conditions	
			Medium	Temp (°C)
Enterococcus faecalis	AR42	UR	BHI	37
	AR69	UR	BHI	37
Enterococcus faecium	AR1	UR	BHI	37
	AR36	UR	BHI	37
	AR58	UR	BHI	37
Enterococcus gallinarum	C86	UR	BHI	37
Listeria monocytogenes	4032	CECT	BHI	37
Pediococcus pentosaceus	C531	UR	MRS	37

[a] UR, Universidad de La Rioja (Logroño, Spain); BHI, brain heart infusion; CECT, Colección Española de Cultivos Tipo (Valencia, Spain); MRS, de Man Rogosa Sharpe.

as an alternative to antibiotics, is gaining interest *(12, 27, 33, 41).* In view of these potential biotechnological applications, the aim of this study was to determine the antimicrobial activity of and the presence of bacteriocin structural genes in enterococci isolated from fecal samples of fish, mammals, and birds in Portugal.

MATERIALS AND METHODS

Bacterial strains and growth conditions. This study included 253 enterococcal isolates obtained from fecal samples of wild boars (*Sus scrofa*, $n = 69$) *(43),* mullets (*Liza ramada*, $n = 117$), and partridges (*Perdix perdix,* $n = 67$) from Portugal. The isolates from wild boars were identified to the species level in a previous work *(43).* For identification of the isolates from mullets and partridges to the genus level, the samples were diluted and plated in Slanetz-Bartley agar (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated for 48 h at 35°C. Colonies with typical enterococcal characteristics were subjected to Gram's stain, the catalase test, and the bile-aesculin reaction (bioMérieux, La Palme, France). Species identification was carried out with PCR (see below). The indicator strains used to evaluate the antimicrobial activity of the isolates and their growth conditions are shown in Table 1. The strain *E. faecium* AR1 is vancomycin resistant and belongs to the CC17 clonal complex.

Antimicrobial activity assays. For detection of the direct antimicrobial activity of 253 isolates against eight indicator strains, the method of del Campo et al. *(14)* was used. Briefly, 50 μl of an overnight culture ($\sim 10^9$ CFU/ml) of the indicator strain was added to 5 ml of molten soft tryptic soy broth (Difco, Detroit, MI) supplemented with 0.5% yeast extract and 0.7% agar, mixed, and poured onto a yeast extract–supplemented tryptic soy agar plate (Difco). A single colony of each enterococcus to be tested for antimicrobial activity was transferred with a sterile toothpick to the agar plate seeded with the indicator species ($\sim 10^7$ CFU/ml) (Table 1). Plates were incubated at 37°C for 24 h in aerobic conditions, with the exception of *Pediococcus pentosaceus* C531, which was incubated at 30°C under a CO_2 atmosphere (5%). The antimicrobial activity was detected by the presence of growth inhibition zones of the indicator microorganism around the producer strain (only inhibition halos with diameters greater than 3 mm were considered to be positive results).

Antimicrobial activity secreted into liquid medium was assayed with the agar well-diffusion test *(7).* For this purpose, cell-free culture supernatants of the selected isolates grown in de Man Rogosa Sharpe (MRS) broth at 37°C for 16 h were obtained by centrifugation at 5,000 × *g* for 10 min at 4°C and further subjected to heat treatment at 100°C for 10 min; subsequently, 50-μl aliquots of the heat-treated, cell-free culture supernatants were placed into wells (6-mm diameter) cut in cooled soft MRS agar (0.7%, wt/vol) plates (60 ml) previously seeded (10^5 CFU ml^{-1}) with the indicator microorganism *L. monocytogenes* CECT4032. After 2 h at 4°C, the plates were incubated at 37°C for 24 h to allow the growth of the target microorganisms and then analyzed for the presence of inhibition zones around the wells. In order to determine the proteinaceous nature of the antimicrobial compounds, the supernatants showing antimicrobial activity were subjected to proteinase K treatment (10 mg/ml; Roche, Mannheim, Germany) at 37°C for 2 h. After proteinase K inactivation by heat treatment, samples were assayed for antimicrobial activity by an agar well-diffusion test as described above. The strains showing antimicrobial activity in their supernatants that was susceptible to proteinase K treatment were termed Bac^+, for bacteriocin producere, and selected for further characterization.

DNA isolation and PCR. DNA was isolated by the alkaline lysis method as described by Baele et al. *(1).* Briefly, the isolates were grown in MRS broth at 32°C overnight and then plated on MRS agar to obtain isolated colonies. A small amount of the cells was suspended in 20 μl of lysis buffer (0.25% sodium dodecyl sulfate, 0.05 N NaOH) and heated at 95°C for 5 min. After a brief spin, 180 μl of distilled water was added to the lysed cells, which were pelleted by centrifugation at 4,000 × *g* for 5 min at 4°C. The supernatants were then transferred to clean tubes and frozen at 20°C until further use as template DNA for PCR.

PCR reactions were carried out in a Techgene DNA thermal cycler (Techne Ltd., Cambridge, UK) in 25-μl reaction mixtures containing PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 0.7 mM of each primer, 150 to 250 ng of template DNA, and 0.75 U of EcoTaq DNA polymerase (Ecogen BRL, Barcelona, Spain). All primers used for PCR were obtained from Sigma-Genosys Ltd. (London, UK). In order to evaluate the presence and integrity of DNA, a control PCR was carried out with primers plb16S and mlb16S *(36),* which amplify a region of approximately 500 bp of the 16S rRNA gene of lactic acid bacteria, according to the cycling conditions described by Jiménez et al. *(31).* PCR products were resolved and analyzed by electrophoresis on 2% (wt/vol) agarose (Pronadisa, Madrid, Spain) gels at 90 V for 1 h by using Hyperladder II (Bioline, London, UK) for DNA sizing and the Gel Doc 1000 documentation system (Bio-Rad Laboratories S.A., Madrid, Spain).

TABLE 2. *Primers and PCR conditions for detection of enterocin structural genes*

Bacteriocin (reference[s])	Target gene and primer (5′–3′)	PCR conditions			Producer strain used as PCR-positive control (reference)	PCR product length (bp)	Primer reference
		Temp (°C)	Duration	No. of cycles			
Cytolysin *(26)*	*cylL*$_{L/S}$	97	2 min	1	*E. faecalis* F2 *(18)*	324	*11*
	CylLLS-R1:	94	45 s				
	GTG TTGAGGAAATGGAAGCG	60	30 s	35			
	CylLLS-R2:	72	25 s				
	TCTCAGCCTGAACATCTCCAC	72	7 min	1			
Enterocin A *(5)*	*entA*	97	2 min	1	*E. faecium* T136 *(5)*	197	*4*
	EnterA-F:	94	45 s				
	ATGAAACATTTAAAAATTTTGTCTATTAAAG	59	30 s	35			
	EnterA-R:	72	40 s				
	TTAGCACTTCCCTGGAATTGCTCC	72	7 min	1			
Enterocin SE-K4 *(19)*	*entSE-K4*	97	2 min	1	*E. faecalis* K-4 *(19)*	146	*4*
	SEK4-FW:	94	45 s				
	GCCACGTATTACGGAAATGGTGTC	53	30 s	35			
	SEK4-RV:	72	30 s				
	TTATCTTCCACCTATACCACCTAACAC	72	7 min	1			
Enterocin 1071 *(22)*	*ent1071* (*ent1071A* and *ent1071B*)	97	2 min	1	*E. faecalis* FAIR-E309 *(22)*	403	*22*
	CFr-1:	94	45 s				
	ATAATTAGGGGGAACGATAA	51	30 s	35			
	CFr-2:	72	20 s				
	ATACATTCTTCCACTTATTTTT	72	7 min	1			
Enterocin P *(8)*	*entP*	97	2 min	1	*E. faecium* P13 *(8)*	216	*8*
	EntP1:	94	45 s				
	ATG AGAAAAAAATTATTTAGTTTAGCTCTTATTGG	64	30 s	35			
	EntP2:	72	30 s				
	TTAATGTCCCATACCTGCCAAACCAG	72	7 min	1			
Enterocin Q *(9)*	*entqA*	97	2 min	1	*E. faecium* L50 *(9)*	105	*11*
	EntQ-R1:	94	45 s				
	ATGAATTTTCTTAAAAATGGTATCGCAAAATG	57	30 s	35			
	EntQ-R2:	72	20 s				
	TTAACAAGAAATTTTTTCCCATGGCAAG	72	7 min	1			
Enterocin AS-48 *(25)*	*entAS-48*	97	2 min	1	*E. faecalis* INIA-4 *(32)*	125	*11*
	AS48-R1:	94	45 s				
	TCGGTATACCAGCAGCAGTT	59	30 s	35			
	AS48-R2:	72	15 s				
	TGCTGCAGCGAGTAAAGAAA	72	7 min	1			
Enterocin L50 *(10)*	*entL50* (*entL50A* and *entL50B*)	97	2 min	1	*E. faecium* L50 *(10)*	286	*11*
	EntL50-R1:	94	45 s				
	ATGGGAGCAATCGCAAAATTAGTAGC	65	30 s	35			
	EntL50-R2:	72	20 s				
	TTAATGTCTTTTTAGCCATTTTTCAAT	72	7 min	1			

TABLE 2. *Continued*

Bacteriocin (reference[s])	Target gene and primer (5′–3′)	PCR conditions			Producer strain used as PCR-positive control (reference)	PCR product length (bp)	Primer reference
		Temp (°C)	Duration	No. of cycles			
Enterocin B *(5)*	*entB*	97	2 min	1	*E. faecium* T136 *(5)*	126	*5*
	EntB3:	94	45 s				
	AGACCTAACAACTTATCTAAAG	50	30 s	35			
	EntB5:	72	20 s				
	GTTGCATTTAGAGTATACATTTGC	72	7 min	1			
Enterolysin A *(28, 42)*	*entL*	97	2 min	1	*E. faecalis* DBH9 *(47)*	1,770	*28*
	PCEL-F:	94	45 s				
	CGATTTCTGTTGTAGGAACC	51	30 s	35			
	PCEL-R:	72	2 s				
	GTACATCTCCATATACTTTTCC	72	7 min	1			
Hiracin JM79 *(46)*	*hirJM79*	97	2 min	1	*E. hirae* DCH5 *(46)*	250	*46*
	HNZSC-FW:	94	45 s				
	ATGAAAAAGAAAGTATTAAAACATTGTGTTATTCTAGG	61	30 s	35			
	HPJE-RV:	72	30 s				
	ATAAGTTAAGCTTGTACTACCTTCTAGGTGCCCATGGACC	72	7 min	1			
Enterococcin V583 *(50)*	*ef1097*	97	2 min	1	*E. faecalis* DBC5 *(47)*	408	*4*
	EF1097-F3:	94	45 s				
	GGCGATGGCATTACTAATGACATTAGG	65	30 s	35			
	EF1097-R3:	72	45 s				
	CTTAGCCCACATTGAACTGCCCATAAAGC	72	7 min	1			
Columbicin A *(39)*	*Cola*	97	2 min	1	*E. columbae* PLCH2 *(39)*	101	*4*
	ColA-FW:	94	45 s				
	GCAGRTCGTGGATGGATTAAG	58	30 s	35			
	ColA-RV:	72	30 s				
	TTAAGCACAATTTTTACAAGCTG	72	7 min	1			

Genotypic strain identification. In a first approach, the primers designed by Dutka-Malen et al. *(16),* targeting a region of the gene encoding D-alanine:D-alanine ligases (*ddl*) of *Enterococcus faecium* and *Enterococcus faecalis,* were used for PCR species identification. In the case of the isolates not yielding a positive result with these primer sets, a second PCR was performed with the plb16S and mlb16S primers as described above. The resulting PCR products were purified using a NucleoSpin extract II kit and then sequenced in the Genomics Unit of the Parque Científico, Universidad Complutense de Madrid (Madrid, Spain). Species identification was performed by comparing the sequences obtained with those deposited at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) by using the BLAST program.

PCR detection of bacteriocin structural genes. The presence in the selected Bac^+ strains of structural genes encoding previously described bacteriocins was evaluated by PCR. The specific oligonucleotide primers, cycling conditions, and positive control strains used in the PCR reactions are shown in Table 2 *(4).* PCR products were resolved and analyzed by gel electrophoresis as described above.

RESULTS

Antimicrobial activity assays. Analysis of the direct antimicrobial activity of the 253 enterococcal isolates against eight indicator strains showed that 177 strains clearly inhibited at least one indicator microorganism (Fig. 1). Of these strains, 38 were obtained from partridges, 99 from mullets, and 40 from wild boars. The most sensitive indicator was *E. faecium* AR58, followed by *E. faecium* AR1 and *L. monocytogenes* CECT4032. Conversely, the most resistant indicator was *Enterococcus gallinarum* C86.

Analysis of the extracellular antimicrobial activity of a subset of 123 enterococcal isolates displaying direct antimicrobial activity against *L. monocytogenes* CECT4032, comprising 33, 85, and 5 isolates from partridges, mullets, and wild boars, respectively, showed that 83 strains secreted active antimicrobial compounds. Interestingly, 81 of these strains were classified as Bac^+, since their extracellular antimicrobial activity was abolished after proteinase K treatment (Fig. 2). These 81 isolates were classified into 5 different groups on the basis of their inhibitory spectrum, which ranged from inhibition of one to eight of the tested indicator strains (Table 3).

Genotypic identification and detection of enterocin structural genes. In order to investigate the presence of known enterocin genes, a further selection of 36 of the 81 isolates displaying bacteriocin activity was performed. The selection was done taking into consideration the following: (i) all animal species of origin were represented; (ii) all the inhibition groups were also represented; and (iii) for the selection in each inhibition group, both the most active isolates and those showing heterogeneity in their antimicrobial potency were selected. Genotypic identification of 30 of these isolates was achieved by using primers based on *E. faecium* and *E. faecalis ddl*, resulting in 29 *E. faecium* isolates (6 from partridges, 20 from mullets, and 3 from wild boars) and 1 *E. faecalis* isolate (from a partridge). The remaining six isolates were identified by BLAST analysis of the nucleotide sequences of the PCR products obtained with primers mlb16S and plb16S as *Enterococcus hirae* (three isolates: T56, T59, and T62), *Enterococcus durans* (one isolate: T29), *E. faecium* (one isolate: T71 VRE B), and *Enterococcus sanguinicola* (one isolate: T41).

The results of the analysis of the presence of known enterocin structural genes in the selected isolates are shown in Table 4. The presence of one or more enterocin genes was detected in 25 strains (69.4%). The most frequently occurring structural gene was that of enterocin P (*entP*), followed by that of hiracin JM79 (*hirJM79*). Conversely, the less abundant genes were those of enterocin Q (*entqA*), enterolysin A (*entL*), and enterococcin V583 (*ef1097*). The cytolysin A and columbicin A genes ($cylL_{L/S}$ and *colA*, respectively) were not detected in any of the enterococci analyzed in this study. Concerning the presence of several enterocin structural genes in a single isolate, 10 isolates (40.0%) contained two enterocin genes, the combinations *entP* plus *hirJM79* and *entL50* plus *entP* being the most frequently found. None of the isolates contained three enterocin genes, while 2 isolates (8.0%) contained the combination *entL50* (*entL50A* and *entL50B*) plus *entP, entA,* and *entB*.

DISCUSSION

In recent years, there has been a great interest in investigating the ability of enterococci to produce bacteriocins that inhibit the growth of pathogenic and/or food spoilage bacteria, including members of the genus *Listeria,* and the inhibitory spectrum, physicochemical properties, and possible food applications of enterocins have been thoroughly studied *(2, 4, 5, 8–10, 17, 23, 43, 46).*

Although previous European Food Safety Authority opinions concluded that species of the *Enterococcus* genus could not be recommended for the qualified presumption of safety list, some strains of *E. faecium* are authorized for use as feed additives *(20).* Nevertheless, the risk assessment was carried out on a case-by-case basis because it is not possible to distinguish between virulent and nonvirulent strains without resorting to the level of investigation used in a strain-level risk assessment *(37).*

The present work describes the antimicrobial activity of a collection of 253 enterococcal isolates of animal origin. Clear antimicrobial activity against at least one of the eight indicator strains tested in this work was observed in 177 strains (70%).

Analyzing each individual species, it appears that mullets have a higher percentage of strains with direct antimicrobial activity, since 99 (84.6%) of 177 strains studied showed this activity, while in wild boars and partridges, these percentages were only 57.9 and 56.7%, respectively. However, these values are considerably higher than those detected by other authors. In this respect, Ferreira et al. *(21)* analyzed the antimicrobial activity of 70 strains of *Enterococcus mundtii* and found that only 15 strains (21.4%) displayed antimicrobial activity. Moreover, du Toit et al. *(17)* conducted a study of 92 enterococci isolated from

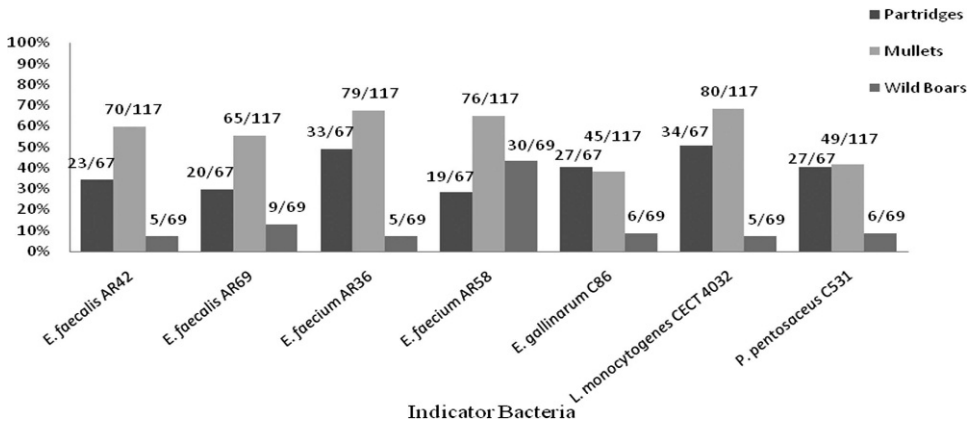

FIGURE 1. *Distribution of the strains showing direct antimicrobial activity against different indicator bacteria according to their origin and with respect to the total number of strains from each origin.*

pig feces, of which only 7 had direct antimicrobial activity (7.6%).

Direct antimicrobial activity might be due to competition for nutrients, formation of organic acids (mainly lactic acid and acetic acid) with a concomitant decrease in pH, and the production of other antimicrobial substances, such as ethanol, carbon dioxide, diacetyl, acetaldehyde, hydrogen peroxide and other metabolic derivatives of oxygen, and bacteriocins *(40)*. In this respect, the sensitivity to proteinase K of the extracellular antimicrobial activity of 81 of 123 supernatants of strains displaying direct antimicrobial activity showed that their antimicrobial activity is due to proteinaceous compounds (i.e., bacteriocins).

In our study, most of the bacteriocinogenic enterococci were identified as *E. faecium,* which agrees with previous reports on bacteriocin production by fecal enterococci *(35)*. Interestingly, 11 strains (10 *E. faecium* and 1 *E. sanguinicola*—8 from mullets, including the *E. sanguinicola* isolate, 2 from partridges, and 1 from a wild boar) showing

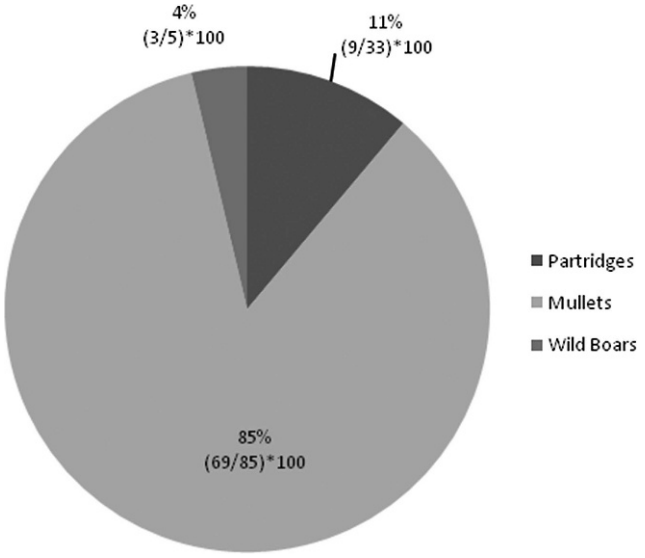

FIGURE 2. *Distribution of the strains showing extracellular antimicrobial activity against* L. monocytogenes *CECT4032 according to their origin. Figures in parentheses refer to percentages with respect to the number of strains of each origin showing direct antimicrobial activity against this indicator.*

antimicrobial activity in their supernatants against at least *L. monocytogenes* CECT4032 did not harbor any of the tested bacteriocin structural genes, suggesting that they may produce bacteriocins that have not been previously characterized. However, the occurrence of one or more known bacteriocin structural gene(s) was detected in the remaining 25 (69.4%) strains. Our screening of the presence of known enterocin genes indicated that *entP* was the most frequently detected enterocin gene (44.4%), in agreement with the results obtained by other authors *(38, 48, 49)* with enterococci from different sources (animals, food, and feed). In this study, the presence of a single enterocin gene was the most frequent trait, occurring in 52.0% of the isolates, while the presence of two or four different enterocin genes, being present in 10 and 2 isolates, respectively, was found in 48% of the isolates. Among the combinations of two enterocin genes, the combination *entP* plus *hirJM79* was the most frequently found, in contrast to the results of Strompfová et al. *(49),* who found that the most frequent combination was that of *entA* plus *entP*. Although the different enterocin genes contained in a single strain might not be expressed simultaneously, the production of multiple bacteriocins in a single strain has been demonstrated by the use of biochemical tests and specific antibodies *(9, 13)*. Even though *entA* is generally found in association with *entB* and the enterocins encoded by these genes act synergistically *(5, 15, 24), entA* was not associated with *entB* in two of the tested isolates, while in another two isolates, it was associated with *entB*, *entL50* (*entL50A* and *entL50B*), and *entP*. The structural genes of cytolysin A and columbicin A were not detected in any of the enterococci analyzed.

In conclusion, the results presented in this article show the presence of bacteriocinogenic enterococci with different inhibitory spectra in partridges, wild boars, and mullets. The PCR screening of enterocin structural genes showed that more than half of the tested isolates possess at least one enterocin gene, *entP* being the most frequently found. On the whole, wild animals constitute an appropriate source for the isolation of bacteriocinogenic enterococci producing either known or hitherto-uncharacterized bacteriocins which may be suitable for evaluation as probiotic cultures for the promotion of human and animal health.

TABLE 3. *Grouping of the 81 Bac$^+$ isolates according to their direct antimicrobial activity spectrum against eight indicator strains*

Inhibition group	No. of strains in group	Origin of strain(s)	Antimicrobial activity against:
1	53	14 Mullets from the Mondego River 27 Mullets from the Douro River 3 Wild boars from Northern Portugal 9 Partridges from Trás-os-Montes e Alto Douro	*L. monocytogenes* CECT4032 *P. pentosaceus* C531 4–6 enterococci
2	2	1 Mullet from the Mondego River 1 Mullet from the Douro River	*L. monocytogenes* CECT4032 *P. pentosaceus* C531 2–3 enterococci
3	22	22 Mullets from the Douro River	*L. monocytogenes* CECT4032 4–6 enterococci
4	3	3 Mullets from the Douro River	*L. monocytogenes* CECT4032 *P. pentosaceus* C531
5	1	1 Mullet from the Mondego River	*L. monocytogenes* CECT4032

TABLE 4. *Occurrence of bacteriocin structural genes among 36* Enterococcus *spp. isolates*

Species	Source	Strain	Inhibition group	Occurrence of bacteriocin structural gene(s)								
				entL50A and *entL50B*	*entP*	*entqA*	*entA*	*entB*	*entL*	*entAS-48*	*Ef1097*	*hirJM79*
E. faecium	Mullet	TA9	1	−	+	−	−	−	−	−	−	−
E. durans	Mullet	T29	1	−	+	−	−	−	−	−	−	−
E. faecium	Mullet	T71 VRE B	1	−	−	−	−	−	−	+	−	−
E. faecium	Wild boar	J26A	1	−	−	−	−	−	−	−	−	−
E. faecium	Partridge	P2CS	1	+	−	−	−	−	−	−	−	−
E. faecium	Partridge	P3CS	1	−	−	+	−	−	−	−	−	+
E. faecium	Partridge	P8CC	1	−	−	−	−	−	−	−	−	−
E. faecium	Partridge	P31LS	1	−	+	−	−	−	−	−	−	−
E. faecium	Mullet	T35	1	−	−	−	−	−	−	−	−	+
E. faecium	Mullet	T8	1	−	−	−	−	−	−	−	−	+
E. faecium	Wild boar	JN4A	1	+	+	−	+	+	−	−	−	−
E. faecium	Wild boar	JN9A	1	+	+	−	+	+	−	−	−	−
E. faecium	Mullet	T20	1	−	−	−	−	−	−	−	−	−
E. faecium	Mullet	T32	1	−	−	−	−	−	−	−	−	−
E. faecium	Partridge	P8LS1	1	−	−	−	−	−	−	−	−	−
E. faecium	Mullet	T22	1	+	+	−	−	−	−	−	−	−
E. faecium	Mullet	T71VREA	1	−	−	−	+	−	−	−	−	−
E. faecalis	Partridge	P13CS2	1	−	−	−	−	−	+	−	+	−
E. faecium	Partridge	P21CS	1	−	−	−	−	−	−	−	−	−
E. faecium	Mullet	TA32	1	−	+	−	−	−	−	−	−	+
E. faecium	Mullet	T51	1	−	+	−	−	−	−	−	−	−
E. faecium	Mullet	TA34	1	+	+	−	−	−	−	−	−	−
E. hirae	Mullet	T59	1	−	+	−	−	−	−	−	−	+
E. faecium	Mullet	T39	1	−	−	−	−	−	−	−	−	−
E. faecium	Mullet	T69	1	−	−	−	−	−	−	−	−	−
E. faecium	Mullet	T2	1	−	+	−	−	−	−	−	−	−
E. faecium	Mullet	T57	1	−	−	−	−	−	−	−	−	−
E. faecium	Mullet	TA9VRE	2	−	−	−	+	−	−	−	−	−
E. hirae	Mullet	T56	2	−	+	−	−	−	−	−	−	+
E. faecium	Mullet	T30	3	+	−	−	−	−	−	+	−	−
E. faecium	Mullet	T13	3	−	+	−	−	−	−	−	−	−
E. faecium	Mullet	T53	3	+	+	−	−	−	−	−	−	−
E. faecium	Mullet	T16	3	−	−	−	−	−	−	−	−	−
E. sanguinicola	Mullet	T41	3	−	−	−	−	−	−	−	−	−
E. hirae	Mullet	T62	4	−	+	−	−	−	−	−	−	+
E. faecium	Mullet	TA36	5	−	+	−	−	−	−	−	−	−

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