

Molecular investigation of enterococci isolated from different environmental sources

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The properties of 61 environmental enterococci were studied. The strains were isolated mainly from surface and waste waters and several strains from sheep manure were also included. Species identification was provided by combination of phenotypic (Micronaut System, Merlin) and molecular detection methods (ITS-PCR, ddl-PCR and the whole cell proteins electrophoresis). A good agreement between molecular methods was observed, however, several discrepancies were observed when comparing molecular and biochemical identification. Six enterococcal species were overall identified; *E. faecium* and *E. hirae* were the most abundant ones, almost 80% of isolates belonged to these two species. *E. faecalis*, *E. gallinarum* and *E. mundtii* were detected in the case of several strains and both *E. durans* and *E. casseliflavus* were present ones. The distribution of selected genes conferring virulence to enterococci (*cylA*, *gelE* and *esp*) was studied; the positive signal was obtained only in four strains of *E. faecalis*. The strains were characterized also by detection of genes coding for enterocins – small secreted peptides with antibacterial activity. The presence of five different genes (*entA*, *entB*, *entP*, *ent31*, *entL50AB*) was tested and high frequency of enterocins was observed. Thirty-five from 61 isolates contained at least one enterocin gene. Enterocins were most often detected in *E. faecium*, followed by *E. hirae*, *E. mundtii*, *E. faecalis* and *E. durans*.

Key words: enterococci, environmental isolates, molecular and biochemical identification, enterocins, virulence factors.

Introduction

The genus *Enterococcus* is a heterogeneous group

of bacteria, which includes 20 different species. The interest on enterococci is raised in the last decades mainly for two important characteristics:

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they are considered as infection agents especially in immunocompromised hosts and they are used as useful probiotics and starter cultures in various fermented food (MAKI & AGGER, 1988; PATTERSON et al., 1995; AGERHOLM-LARSEN et al., 2000; LEROY et al., 2003; MANOLOPOULOU et al. 2003).

Enterococci are causing mainly urinary tract infections and wound infections, less frequent are blood stream infections and endocarditis. The seriousness of enterococcal diseases is mainly due to their high level of intrinsic and acquired antibiotics resistance (MENDEZ-ALVAREZ et al., 2000; PORTILLO et al., 2000; SHANKAR et al., 2002; WOODFORD et al., 2002; BUJDAKOVA et al., 2003). Especially, nosocomial infections with multiresistant enterococcal strains are difficult medical problem actually solved in many hospitals worldwide. Though enterococci as opportunistic pathogens do not have strong virulence factors comparing with more virulent bacteria, several factors conferring to enhanced virulence have been identified in *E. faecalis*. The haemolytic cytotoxin (IKE et al. 1990; JETT et al., 1994) lyses a broad range of eukaryotic and prokaryotic cells and it enhances the *E. faecalis* virulence in animal models (JETT et al., 1992; CHOW et al. 1993). Aggregation substance AS is an adhesin, which mediates the formation of cell clumps that allow the highly efficient transfer of the sex pheromone plasmid on which the AS is encoded. It mediates adhesion to cultured renal tubular cells (KREFT et al., 1992) and augments the internalization of *E. faecalis* by cultured human intestinal epithelial cells (OLMSTED et al., 1994). The enterococcal surface protein (esp), a cell wall-associated peptide, was originally found in *E. faecalis* (SHANKAR et al., 1999). This protein contributes to colonization and persistence of *E. faecalis* in the urinary tract and the ability of this species to produce biofilms (SHANKAR et al., 2001; TOLEDO-ARANA et al., 2001). Recently a variant *esp* gene has been detected in glycopeptide-resistant *E. faecium* strains from hospital outbreaks (COQUE et al., 2002). The *gelE* gene encodes a Zn-metalloprotease, which could serve a wide array of functions in normal cell biology and in microbial infections (MIYOSHI & SHINODA, 2000). It has been determined that *gelE* is induced by a quorum-sensing system of *E. faecalis* encoded by the *fsr* locus (QUIN et al., 2001). Other virulence factors are represented by the *efaAfs* and *efaAfm*, cell wall adhesins expressed in serum in *E. faecalis* and *E. faecium*, respectively (SINGH et al., 1998), and by the presence of a large series of sex pheromones (cpd, cob, ccf, cad) which facilitate plasmid conjugation and are chemotactic for

human leucocytes (CLEWELL et al., 2002). Enterococcal virulence genes are often encoded on conjugative pheromone-responsive plasmids or they could be located chromosomally as a pathogenicity island of 153 Kbp inserted in chromosomal DNA which is missing in most of intestinal strains and has been detected in some infection derived strains (SHANKAR et al., 2002).

Enterococci frequently occur in fermented meat and dairy products (FRANZ et al. 1999; HANCOCK & GILMORE, 2000). Due to their salt and acid tolerance they grow in a variety of cheeses, especially artisanal cheeses produced in southern Europe from raw or pasteurised goat, ewe or bovine milk. In part of these cheeses enterococci play a major role in ripening and aroma development (MACEDO et al., 1995; MANNU et al., 1999; GELSOMINO et al., 2003) and some strains are successfully exploited as starter culture in different fermented food (SAMELIS et al., 1994; FRANZ et al., 1999). It was shown that different species of enterococci can produce a quite large variety of bacteriocins (enterocins), small secreted peptides which have interesting and proving ability to inhibit many kinds of food-borne pathogens (SIMONETTA et al., 1997; ENNAHAR et al., 1998; LAUKOVA & CZIKKOVA, 1999). The most studied enterocins were isolated mainly from *E. faecium* (HERRANZ et al., 1999; CINTAS et al. 2000; ENNAHAR et al., 2001), but some of them are present also in *E. faecalis* (TOMITA et al. 1996), and other are continuously identified in different enterococcal species (JENNES et al., 2000; SABIA et al., 2004).

Enterococci are widely distributed in nature. They are spread from faecal contamination and persist due to their high tolerance to the various environmental conditions. In water samples enterococci are used as indicators of faecal pollution (ŠVEC & SEDLÁČEK, 1999; TEJEDOR JUNCO et al., 2001; CUPÁKOVÁ & LUKÁŠOVÁ, 2003).

The aim of the present paper was to compare the ability of molecular and traditional methods for species identification of environmental enterococci. In addition to this, the presence of selected virulence and bacteriocin genes among enterococcal isolates was studied.

Material and methods

Bacterial strains and biochemical identification

Enterococci were isolated from different kinds of environments: Danube river (PV), Bratislava wastewater treatment plant (M), dairy and sugar factory wastewater treatment plants (respectively R and C), sheep

manure (OV). The water samples were inoculated on Slanetz-Bartley agar (SBA; Hi Media Laboratories, Bombay, India) and incubated 48 hours at 37°C. A portion 10 g (wet weight) of the sheep manure was mixed in a sterile 250 mL Erlenmeyer flask with 90 mL of physiological solution and then inoculated on SBA as for the water sample.

Strains grown on SBA were identified on genus level by PYRAtest (Pilva-Lachema, Brno, Czech Republic) for detection of pyrase, esculin hydrolysis, and glucose degradation without gas production. The isolates identified as enterococci by PYRAtest were further species-identified by commercial kit Micronaut System (Merlin, Bornheim-Hersel, Germany).

The enterococcal strains *E. casseliflavus* CCM 2478, *E. durans* CCM 5612, *E. faecalis* CCM 1875, *E. faecium* NCIMB 11181, *E. faecium* CCM 2308, *E. gallinarum* CCM 4054, *E. hirae* CCM 2423, *E. mundtii* CCM 4058 from CCM (Czech Collection of Microorganism, Brno, Czech Republic) were used as standards in ITS-PCR and whole cell proteins electrophoresis analysis. *E. faecalis* strain FI9190 kindly offered by Dr. T. J. Eaton (EATON & GASSON, 2001) was used as positive control in detection of virulence factors.

SDS-PAGE whole cell protein analysis

Cultures were grown aerobically in liquid LB medium in a rotatory shaker at 37°C to OD₄₂₀ of 0.4. The bacteria were collected by centrifugation at 4,000 rpm for 20 min. The pellet was resuspended in 200 µL

of 10 mM Tris buffer and sonificated. The supernatant after centrifugation at 3,000 rpm for 20 min was precipitated with 2 fold volume of cold acetone overnight at -20°C. The precipitated proteins were centrifuged at 10,000 rpm for 5 min, washed with 50% acetone and again centrifuged at 10,000 rpm for 5 min. The sediment was than resuspended in solubilizing buffer (LAEMMLI, 1970) and denatured by boiling for 5 min. Electrophoresis was carried out at constant current of 60 mA using Mini Protean II apparatus (Bio-Rad, Richmond, USA). For protein profile analysis 4% stacking and 12 % separating polyacrylamide gel was used. The gels were scanned and analyzed with GelCompar II software (Applied Maths, Kootrijk, Belgium).

PCR identification of strains

Enterococci isolates were identified on species level by ITS-PCR, which amplifies the internal transcribed region of rRNA operon (DRAHOVSKA et al., 2002). The species *E. faecalis* and *E. faecium* were specific identified by the amplification of *ddl* genes (DUTKA-MALEN et al., 1995) in a multiplex PCR. The chromosomal DNA was isolated by DNeasy purification kit (Qiagen, Hilden, Germany) following the instruction of the manufacturer. The PCR experiments were performed in 25 µL of mix-reaction containing 25 pmol of each primer (Table 1), 200 µM of each dNTP (Invitrogen, Gaithersburg, Maryland, USA), 1.5 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 2.5 µL of 10x PCR

Table 1. PCR primers used in this study.

Target	Sequence	Product bp	Source
Enterocin A	entA-F – ttagtgaggagcaattccagg entA-R – ccagcagttcttccaatttca	294	This work
Enterocin B	entB-F – cgaagagatgaacaaattatcg entB-R – catgctagtggctcttttggg	151	This work
Enterocin P	entP-F – gatgcagctacgcttcata entP-R – ggggtggctaagtctgtttca	206	This work
Enterocin 31	ent31-F – tgtggcattattgggattgg ent31-R – ttttggccctcttttagtcaca	448	This work
Enterocin L50AB	ent50AB-F – ggagcaatcgcaaaattagtag ent50AB-R – tgtccaataaattgcatgatttg	237	This work
Cytolysin	cylA-F – atggatgggacagatggaaa cylA-R – agctgcgcttacttctggag	519	This work
Enterococcal surface protein	esp-F – ttgctaagtctagtcacgacc esp-R – gcgtcaacacttgcatgcccga	954	SHANKAR et al., 2001
Gelatinase	gel-F – gatgcaatcggtgaagaagg gel-R – gtatccgcaccaatttctgg	750	This work
<i>ddl E. faecium</i>	ddlEfm-F – tagagacattgaaatgccc ddlEfm-R – ctaacatcgtgtaagct	550	DUTKA-MALEN et al., 1995
<i>ddl E. faecalis</i>	ddlEfs-F – atcaagtacagtagtct ddlEfs-R – acgattcaagctaactg	941	DUTKA-MALEN et al. 1995
ITS-PCR	L1 – caagcatccaccgt G17 – gtgaagtcgtaacaagg		JENSEN et al., 1993

buffer, 2.5 mM of MgCl₂, and 1.5 μL of DNA. Reactions were performed in a T1 thermal cycler (Biometra, Goettingen, Germany) using a programme consisting of the initial denaturation at 94°C for 3 min, 30 cycles with a denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and polymerization at 72°C for 1 min, and a final polymerization at 72°C for 8 min. The amplified products were detected by electrophoresis in a 1.5 % agarose gel, stained with ethidium bromide and visualized by transillumination by UV-light. The PCR products of the ITS-PCR were analyzed by 4% native polyacrylamide gel electrophoresis using Mini Protean II apparatus (Biorad) and visualized by ethidium bromide staining.

Detection of virulence and enterocin genes

The PCR primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the sequences from public databases (National Center for Biotechnology Information, Bethesda, Maryland, USA), *esp* primers were taken from previous publication (Table 1).

Three PCRs were used for enterocin detection, the first for *entA* and *entB*, the second for *entP* and *ent31*, and the last one for *entL50AB* alone. The specificity of detection was confirmed by sequencing of selected PCR products. These products were used as positive controls in subsequent reactions. The virulence factors were tested by a multiplex PCR, which included three pairs of primer designed for the *esp*, *gel*, *cylA* genes. The positive control was the *E. faecalis* strain F19190.

Results and discussion

The bacteria belonging to the genus *Enterococcus* are normal inhabitants of the gastrointestinal tract of many animals and men. They have controversial properties, as opportunistic pathogenic enterococci are able to cause human infections (HUNT, 1998) but on the other hand they are useful for the ripening of various raw milk cheeses and some enterococcal strains are able to inhibit pathogen growth in food (LAUKOVA & CZIKKOVA, 1999). Enterococci are widely spread in nature; they are used as indicators of faecal contamination in environmental waters.

Properties of 61 environmental enterococci have been analyzed in our work. Strains were predominantly isolated from waste water (35 isolates) and river water (7 isolates); sample of strains was supplemented with 19 isolates from sheep manure.

Strains were species identified by combination of phenotypic and molecular detection methods. Bacterial colonies grown on selective Slanetz-Bartley medium with pyrrolidylarylamidase activity were preliminary considered to be enterococci. The biochemical identification was carried out by a

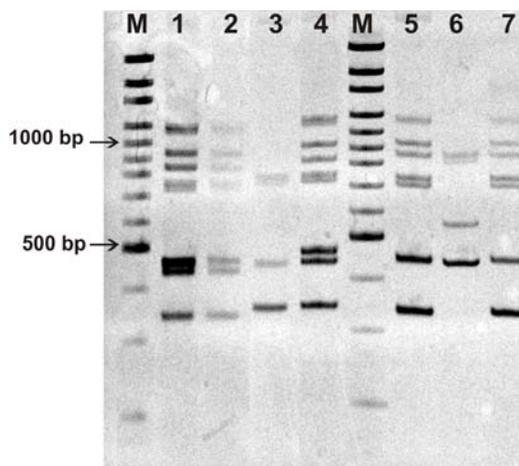


Fig. 1. ITS profiles of different enterococci strains. 1, 2, 3, *E. hirae* profile P7B; 4, *E. faecalis* profile P1; 5, 7 *E. faecium* profile P3; 6, *E. faecium* profile P2; M, 100 bp DNA ladder.

commercial kit (Merlin, Micronaut system), which includes a panel of 40 biochemical substrates. Three independent molecular identification methods were also used: (i) ITS-PCR based on the amplification of internal spacer region of ribosomal operon (DRAHOVSKA et al., 2002); (ii) ddl-PCR which specifically detects strains belonging to *E. faecalis* and *E. faecium* (DUTKA-MALLEN et al., 1995); and (iii) electrophoretic analysis of whole cell proteins (ANDRIGHETTO et al., 2001). The results of species identification are summarized in Table 2. A good agreement between molecular methods was observed, however, several discrepancies were noted when comparing molecular and biochemical identification. By ITS-PCR 54 strains showed identical electrophoretic profiles with some reference collection strain from the database profiles of the previous work (Fig. 1; DRAHOVSKA et al., 2002). Seven strains had unique ITS-PCR profile and these strains could not be species identified by this method. Twenty-four and 7 strains were detected as *E. faecium* and *E. faecalis*, respectively, by species specific ddl primers. Hundred % of concordance was observed in identification of *E. faecium* and *E. faecalis* between ITS-PCR and ddl-PCR. However, a relevant discrepancy was noted between the biochemical and the molecular identification methods mainly for the recognition among the species *E. hirae*, *E. mundtii*, *E. durans* and *E. faecium*, while many isolates identified as *E. hirae*, *E. durans* and *E. mundtii* by biochemical kit were clearly identified as *E. faecium* by the ddl-

Table 2. Concordance of the four identification methods used in our study: Micronaut system (M), ITS-PCR, ddl PCR and electrophoresis of whole cell proteins (SDS-PAGE).

Species identified as	M	Identification method		SDS-PAGE	No of strains
		ITS ^a	ddl ^b		
<i>E. hirae</i>	<i>E. hirae</i>	<i>E. hirae</i>	–	<i>E. hirae</i>	19
	<i>E. hirae</i>	U	–	<i>E. hirae</i>	1
	<i>E. hirae</i>	U	–	<i>E. gallinarum</i>	1
<i>E. faecium</i>	<i>E. hirae</i>	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>	15
	<i>E. mundtii</i>	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>	2
	<i>E. durans</i>	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>	7
<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	6
	<i>E. faecalis / mundtii</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	1
<i>E. gallinarum</i>	<i>E. gallinarum</i>	U	–	<i>E. gallinarum</i>	4
<i>E. mundtii</i>	<i>E. mundtii</i>	<i>E. mundtii</i>	–	<i>E. mundtii</i>	3
<i>E. casseliflavus</i>	<i>E. casseliflavus</i>	U	–	<i>E. hirae</i>	1
<i>E. durans</i>	<i>E. durans</i>	<i>E. durans</i>	–	<i>E. durans</i>	1

^a U: ITS-PCR profile did not match with any collection strain.

^b -: no PCR product was created with ddl primers specific for *E. faecalis* and *E. faecium*.

PCR and the ITS-PCR. These kinds of anomalies are also reported in other works (ANGELETTI et al., 2001; HUDSON et al., 2003), which treated other kind of biochemical kits.

In order to better characterize enterococcal isolates the SDS-PAGE of whole cell proteins was performed (ANDRIGHETTO et al., 2001) and protein profiles were compared with those of reference collection strains. All *E. faecium* isolates showed a similar SDS-PAGE profile aside from the fact that two ITS-PCR profiles were observed for this species. Isolates of *E. hirae* had two types of profiles. Among the *E. faecalis* strains the protein profiles showed a high degree of similarity with exception of the strain 1/3PV. The unique *E. durans* isolate (1/4PV) had identical profile with the *E. durans* collection strain, which supported identification of this strain done by ITS-PCR. The three *E. mundtii* isolates presented protein profiles with a high degree of mutual similarity as well as similarity with the *E. mundtii* collection strain. By the comparison of the protein profiles it has been possible to identify some isolates, which were not recognized by ITS-PCR (6/1PV, 2R, 3R, 4R, 6R, 9R, and 22R). The strain 6/1PV has a protein profile similar to *E. hirae* profile, which is in contrast with identification by the Micronaut system. The strain R2 was identified by Micronaut system as *E. hirae*, but it has a protein profile similar to *E. gallinarum*. Strains 3R, 4R, 6R, 9R were identified as *E. gallinarum*, and the strain 22R as *E. hirae* by both the Micronaut system and protein profile analysis.

In our strain collection six enterococcal species were overall identified; *E. faecium* and *E. hirae*

were the most abundant ones, almost 80% of isolates belonged to these two species. *E. faecalis*, *E. gallinarum* and *E. mundtii* were detected in the case of several strains and both *E. durans* and *E. casseliflavus* were present ones. The same enterococcal species were isolated also by other authors, who monitored the enterococci presence in surface and/or waste waters (TEJEDOR JUNCO et al., 2001; ČUPÁKOVÁ & LUKÁŠOVÁ, 2003), assuming that enterococci from waste water environments are mostly from faecal pollution of human or animal origin.

After species identification strains were further characterized by a series of PCR analysis. The distribution of selected genes conferring the virulence to enterococcal strains was studied with the aim to assess the pathogenic potential of environmental strains. The presence of virulence genes *cylA*, *gelE* and *esp* coding for haemolytic cytolysin, gelatinase and enterococcal surface protein, respectively, was monitored in 61 isolates; the positive signal was obtained only in four strains of *E. faecalis* (Table 3). These results may belong to the few attempts to determine the distribution of virulence genes in environmental enterococci, and they confirm the most accepted opinion that *E. faecalis* is the species which harbours more virulence determinants than other enterococci (EATON AND GASSON, 2001; DRAHOVSKÁ et al., 2003; DUPRE et al., 2003).

The characterization of strains involved also detection of genes coding for enterocins – small secreted peptides with antibacterial activity. The presence of five different genes (*entA*, *entB*, *entP*, *ent31*, *entL50AB*) was tested and high frequency

Table 3. Presence of the virulence genes in enterococci.^a

Strain	<i>esp</i>	<i>cylA</i>	<i>gel</i>
<i>E. faecalis</i> M17	-	-	+
<i>E. faecalis</i> M20	+	-	+
<i>E. faecalis</i> C5	+	+	+
<i>E. faecalis</i> C6	+	+	+

^a All strains were tested for the presence of *esp*, *cylA*, and *gelE* genes by PCR. Only strains positive in any factor are listed in the table.

Table 4. Presence of the bacteriocin genes in enterococci. ^a

Strain	<i>ent A</i>	<i>ent B</i>	<i>ent P</i>	<i>ent 31</i>	<i>ent L50AB</i>
<i>E. hirae</i> OV5	-	+	-	-	-
<i>E. hirae</i> OV6	-	+	-	-	-
<i>E. hirae</i> OV7	-	+	-	-	-
<i>E. hirae</i> OV8	-	+	-	-	-
<i>E. faecium</i> OV9	+	+	-	-	-
<i>E. hirae</i> OV11	-	+	-	-	-
<i>E. hirae</i> OV12	+	+	-	-	-
<i>E. hirae</i> OV15	-	+	-	-	-
<i>E. mundtii</i> OV17	-	-	+	+	-
<i>E. mundtii</i> OV18	-	-	+	+	-
<i>E. mundtii</i> OV19	-	-	+	+	-
<i>E. faecium</i> R1	-	-	+	-	-
<i>E. faecium</i> M3	+	+	-	-	-
<i>E. faecium</i> M4	+	+	-	-	-
<i>E. faecium</i> M5	+	+	-	-	-
<i>E. faecium</i> M6	+	+	-	-	-
<i>E. faecium</i> M7	+	+	-	-	-
<i>E. faecium</i> M10	+	+	-	-	+
<i>E. faecium</i> M11	+	+	-	-	-
<i>E. faecium</i> M12	-	+	-	-	-
<i>E. faecium</i> M13	+	+	-	-	-
<i>E. faecium</i> M14	+	+	-	-	-
<i>E. faecium</i> M15	+	+	-	-	-
<i>E. faecium</i> M16	+	+	-	-	-
<i>E. faecium</i> M19	+	+	-	-	-
<i>E. faecalis</i> M20	-	-	+	-	+
<i>E. faecium</i> M21	-	-	+	-	+
<i>E. hirae</i> C1	-	-	+	-	-
<i>E. hirae</i> C3	-	-	+	-	-
<i>E. faecium</i> C4	-	+	+	-	+
<i>E. hirae</i> C5	-	-	+	-	-
<i>E. faecalis</i> PV1/3	-	-	+	-	-
<i>E. durans</i> PV1/4	-	+	-	-	-
<i>E. faecalis</i> PV3/2	-	+	-	-	-
<i>E. faecium</i> PV3/5	-	-	+	-	+

^a All strains were tested for the presence of enterocins *entA*, *entB*, *entP*, *ent31* and *ent50AB* by PCR. Only strains positive in any enterocin are listed in the table.

of enterocins was observed in our isolates. Thirty-five from 61 isolates contained at least one enterocin gene (Tab. 4). *EntB* gene was the most abundant one; it was present in 24 strains, the least frequent enterocin *ent31* being detected only 3 times. Enterocins were most often detected in *E. faecium*, followed by *E. hirae*, *E. mundtii*, *E. faecalis* and *E. durans*. No enterocin genes were present in *E. gallinarum* and *E. casseliflavus*. This is in accordance with the results of many other works, which found these enterocin factors mainly in *E. faecium* (HERRANZ et al., 1999; CINTAS et al. 2000; ENNAHAR et al., 2001) and proposed the *E. faecium* strains as probiotic for the inhibition of food-borne pathogens (GIRAFFA, 2003; HUGAS et al, 2003). The high frequency and variability of enterocin genes in enterococcal strains detected in our study is probably facilitated by the horizontal gene transfer among enterococcal strains and by the well known ability of enterococci to incorporate DNA material which could help them to survive in different kinds of environments. In future work it will be interesting to compare the content of enterocin genes with the inhibition properties of the strains.

In conclusion we can say that the members of the genus *Enterococcus* are difficult to identify on species level by the use of a unique identification system. The biochemical tests are useful, but only for a preliminary identification. It is necessary to couple them with the molecular methods for reliable results. The PCR based methods are the most promising and the most reliable although in some cases they are not able to recognize the isolates well. We have also investigated the distribution of virulence and enterocin genes in environmental enterococci. The larger presence of the enterocin determinants comparing with virulence factors is perhaps connected to the inhibition properties of the enterocins against the antagonist bacteria. The PCR screening again confirms the ability of enterococci to uptake various useful genes and the ambiguous nature of enterococcal strains, capable to be used as probiotic bacteria but also ready to cause human infections.

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