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ORIGINAL ARTICLE

Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained from raw milk and cheese

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Abstract

Aims: To provide molecular and phenotypical characterization of *Enterococcus* isolates obtained from raw milk and cheese, regarding their bacteriocinogenic and virulence activity.

Methods and Results: Forty-three bacteriocinogenic enterococci isolates were identified by 16s rDNA, fingerprinted by RAPD-PCR analysis and tested by PCR for the presence of genes for lantibiotics (*lan*M, *lan*B and *lan*C) and enterocins (*ent*A, *ent*B, *ent*P, *ent*L50AB and *ent*AS48) and by phenotypical methods for bacteriocin production and inhibitory spectrum. Also, the virulence of the isolates was evaluated by PCR for genes *gel*E, *hyl*, *asa*1, *esp*, *cyl*A, *efa*A, *ace*, *van*A, *van*B, *hdc*1, *hdc*2, *tdc* and *odc* and by phenotypical tests for gelatinase, lipase, DNAse and α- and β-haemolysis. Most isolates (93·0%) harboured at least one lantibiotic or enterocin gene and were positive for several tested virulence genes, mainly *asa*1 (100%), *gel*E (93·0%) and *efa*A (83·7%). 53·5% of the isolates presented β-haemolysis.

Conclusions: *Enterococcus* spp. isolates presented an interesting potential application for food preservation because of bacteriocin production; however, virulence-related genes were identified in all RAPD profiles.

Significance and Impact of the Study: The study demonstrated the contradictory characteristics of the tested *Enterococcus* isolates: they presented a good potential for application in food biopreservation but contained several virulence factors.

Introduction

Enterococcus spp. are lactic acid bacteria (LAB) that are commensally present in the animal gastrointestinal system. They differ from other Gram-positive and catalase-negative cocci in several phenotypic traits, such as capability to survive and grow in moderately restrictive conditions: (i) between 10 and 45°C (ii) in hypersaline solutions (iii) at pH 9·6 and (iv) in 4·0% bile. In addition, they retain their viability after heated to 60°C for 30 min (Franz and Holzapfel 2004; Ogier and Serror 2008). These micro-organisms are frequently associated with many foods from animal (dairy and meat products) and vegetable origins (Franz *et al.* 2003; Giraffa 2003; Todorov and Dicks 2005; Dal Bello *et al.* 2010).

Owing to their tolerance to salts and acids, *Enterococcus* spp. are highly adapted to several food systems. They are often found in high numbers and are believed to contribute to cheese ripening and to the development of aroma, especially in cheese products made in the Mediterranean area (Giraffa 2002; Foulquié-Moreno *et al.* 2006), because of proteolysis and lipolysis and production of diacetyl (Giraffa 2003).

Some enterococci strains, especially from *Ent. faecalis*, *Ent. faecium* and *Ent. mundtii* species, are able to produce bacteriocins, active against relevant spoilage and pathogenic micro-organisms in foods, such as *Listeria monocytogenes* (Khan *et al.* 2010; Kumar and Srivastava 2010; Bayoub *et al.* 2011; Javed *et al.* 2011). Most bacteriocins produced by enterococci belong to class II (Franz

et al. 2007). Examples of well-characterized bacteriocins produced by enterococci are enterocins A, P, CRL35, 1071A and B, and L50A and B, mundticins KS, ST4V and ST15, bacteriocin 31, RC714, T8, and enterolisin A (Cintas *et al.* 1998; de Kwaadsteniet *et al.* 2005; Todorov *et al.* 2005; Franz *et al.* 2007).

Some enterococci have been investigated with regard to their potential as probiotics (Franz *et al.* 2003; Foulquié-Moreno *et al.* 2006; Todorov and Dicks 2008). However, their role as probiotics is still controversial because of their increased association with nosocomial infections and harbourage of multiple antibiotic-resistant genes, transmissible by conjugation to nonpathogenic micro-organisms (Franz *et al.* 2011; Montalban-Lopez *et al.* 2011). In addition, several putative virulence factors have been described in enterococci, such as aggregation substance protein, gelatinase, cytolysin, enterococcal surface proteins, hyaluronidase, accessory colonization factors and endocarditis antigens (Vankerckhoven *et al.* 2004; Martin-Platero *et al.* 2009).

Previous studies have shown that bacteriocinogenic LAB, including *Enterococcus* spp., are common in Brazilian dairy products (Gomes *et al.* 2008; Frazzon *et al.* 2010; Moraes *et al.* 2010; Ortolani *et al.* 2010). In this study, selected *Enterococcus* spp. isolates obtained from raw milk and cheese were better characterized for their bacteriocinogenic potential and tested for their virulence features, using genotypic and phenotypic tests for both evaluations.

Materials and methods

Micro-organisms

The study was carried out with forty-three *Enterococcus* spp. isolates (named En01 to En43) selected among a LAB culture collection previously obtained from raw milk and cheese in Minas Gerais state, Brazil (Moraes *et al.* 2010; Ortolani *et al.* 2010), and capable of producing antimicrobial substances (Moraes *et al.* 2010). All isolates were submitted to identification based on 16S rDNA sequencing, according Sterr *et al.* (2009), to confirm the genus identification. Other bacteria used in this study are listed in Table 1. Enterococci strains were stored at −80°C in MRS broth (Oxoid Ltd, Basingstoke, UK) supplemented with 25% (v/v) glycerol. *Listeria* spp. and *Staphylococcus* spp. strains were stored at −80°C in tryptic soy broth supplemented with 0·6% (w/v) yeast extract (TSB-YE) (Oxoid).

Fingerprinting of *Enterococcus* spp. by RAPD-PCR

After checking the purity of isolates by streaking them on MRS agar (Oxoid) at 35°C for 24 h, isolates colonies were transferred to MRS broth and incubated at 35°C for 24 h. The obtained cultures were then diluted in MRS broth (Oxoid) until MacFarland 1 turbidity, correspondent to approximately 3×10^8 colony-forming units per millilitre (CFU ml^{-1}). The cultures were centrifuged at 14 000 ***g*** for 2 min, and DNA was extracted using ZR Fungal/Bacterial DNA kit (Zymo Research, Irvine, CA, USA). The DNA concentration in the extract was determined in a NanoDrop2000 (Thermo Scientific Inc., Waltham, MA, USA). PCR was performed using primers OPL-01 and OPL-02 (Kit L of the RAPD® 10mer kits, Operon Biotechnologies, Cologne, Germany), and amplification was performed according to Todorov and Dicks (2009). The 25 μl reaction contained 5 μl of primers, 2·5 μl of 10× *rTaq* Buffer (Takara Bio Inc., Shiga, Japan), 10 μl of 5 m l^{-1} $MgCl_2$ (Roche Group, Basel, Switzerland), 4 μl of 2·5 m l^{-1} dNTPs (Takara Bio) and 0·5 μl of r*Taq* DNA polymerase (Takara Bio). Amplification was performed using a DNA thermal cycler (GeneSystem® PCR System 7900, AB Applied Biosystems, Carlsbad, CA, USA) with the following programme: 45 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min followed by an extension of the amplified product at 72°C for 5 min. The amplified products were separated by electrophoresis on 1·4% (w/v) agarose gels in 1× TAE buffer at 100 V for 1 h. Gels were stained with TAE buffer containing 0·5 μg ml^{-1} ethidium bromide (Sigma-Aldrich Co., St Louis, MO, USA). Banding patterns were analysed using Gel Compare software (ver. 4·1; Applied Maths, Kortrijk, Belgium).

Characterization of bacteriocinogenic potential

Sensitivity to proteolytic enzymes

All *Enterococcus* spp. isolates were tested to verify the enzymatic sensitivity of their antimicrobial substance production (Lewus *et al.* 1991). Aliquots of 1 μl of each culture were spotted on the surface of plates containing MRS agar prepared with 0·5% (w/v) dextrose (modified MRS, mMRS), and the plates were incubated at 25°C for 24 h under anaerobiosis (Anaerobac, Probac do Brasil, São Paulo, SP, Ltda.). After incubation, 3-mm-diameter wells were cut adjacent to the colonies and filled with 20 μl of a solution containing α-chymotrypsin, proteinase K, trypsin TPCK, α-amylase type XII-A, papain, *Streptomyces griseus* protease, lysozyme or catalase (20 mg ml^{-1}). All enzymes were from Sigma-Aldrich. After 30 min at room temperature, the plates were overlaid with 8 ml of brain-heart infusion (BHI, Oxoid) containing 0·8% (w/v) agar and a culture of *L. monocytogenes* ATCC 7644 (10^5 CFU ml^{-1}) and incubated at 35°C for 24 h. Absence of inhibition halos around the spotted enzymatic solutions indicated the proteinaceous nature of the

Table 1 Bacterial strains used in the study

Groups or genera	Description*	Observations	References
Lactic acid bacteria	43 *Enterococcus* spp. isolates (named En01 to En43)	Obtained from raw milk and soft cheese	Ortolani *et al.* 2010; Moraes *et al.* 2010.
	Ent. faecalis FAIR-E179, *Ent. faecalis* FAIR-E77, *Ent. faecium* FAIR-E178, *Ent. faecium* BFE1072	Bacteriocinogenic strains used as positive controls for identification and enterocin PCRs	Provided by Prof. Charles Franz
	Lactococcus lactis subsp. *lactis* DPC3147, *Lact. lactis* subsp. *lactis* DY13	Bacteriocinogenic strains used as positive controls for lantibiotic biosynthesis PCRs	Provided by Dr. Philip Wescombe
	Lactobacillus sakei (ATCC 15521), *Lact. lactis* subsp. *lactis* (ATCC 7962, ATCC 11007), *Lact. plantarum* (ATCC 8014), *Enterococcus faecalis* (ATCC 19433), *Lact. delbrueckii* subsp. *bulgaricus* (ATCC 11842)	Reference strains used as target in antagonism tests	–
	Enterococcus spp. (4 isolates), *Lact. plantarum* (2 isolates)	Wild isolates obtained from raw milk and cheese and used as target in antagonism tests	Ortolani *et al.* 2010;
	Lact. sakei subsp. *sakei* 2a	Isolated from pork sausage, utilized as positive control in antagonism tests	de Martinis and Franco 1998;
Listeria spp.	*Listeria inoccua* (ATCC 33090), *L. ivanovii* subsp. *ivanovii* (ATCC 19119), *L. monocytogenes* (ATCC 15313, ATCC 19112, ATCC 19117, ATCC 7644)	Reference strains used as target in antagonism tests	–
	L. monocytogenes (3 isolates), *L. seeligeri* (1 isolate), *L. inoccua* (1 isolate), *L. welshimeri* (1 isolate)	Wild isolates obtained from beef and used as target in antagonism tests	Barros *et al.* 2007;
Staphylococcus spp.	*Staphylococcus aureus* (ATCC 14458, ATCC 12598, ATCC 8095, ATCC 29213, ATTC 12600, ATCC 23235)	Reference strains used as target in antagonism tests	–
	Staph. aureus (6 isolates)	Wild isolates obtained from raw milk and cheese and used as target in antagonism tests	Viçosa *et al.* 2010

*ATCC: American Type Culture Collection, Manassas, VA, USA.

antimicrobial substance produced by the tested isolate. A culture of *Lactobacillus sakei* 2a (de Martinis and Franco 1998) and sterile Milli-Q water were used as positive and negative controls, respectively.

Spectrum of activity

All isolates were tested for their inhibitory activity against 36 target pathogenic micro-organisms and LAB listed in Table 1, according to Lewus *et al.* (1991). Aliquots of 1 μl of the cultures of each isolate in MRS were spotted on the surface of mMRS agar plates and incubated at 25°C for 24 h, under anaerobiosis (Anaerobac, Probac do Brasil Ltda.). The plates were overlaid with 8 ml of TSB-YE or mMRS containing 0·8% (w/v) agar and a culture of the target bacteria (10^5 CFU ml^{-1}) and incubated at 35°C for 24 h. The presence of an inhibition halo of at least 5 mm diameter around the spotted *Enterococcus* cultures indicated inhibitory activity against the target strain.

Tests for the presence of genes for known bacteriocins producted by enterococci

Aliquots of 1 ml of the *Enterococcus* spp. cultures were centrifuged at 14 000 ***g*** for 2 min, and the cell pellets were submitted to DNA extraction using DNA Purification Kit Wizard Genomic (Promega Corp., Madison, WI, USA). The obtained total DNA was then mixed with 20× GelRed stain (Biotium Inc., Hayward, CA, USA) at a 5 : 1 proportion and submitted to electrophoresis in 1% agarose gel in 0·5× TBE, to check the integrity of the material for molecular analysis. The extracted DNA was submitted to PCR for amplification of genes *lan*B, *lan*C and *lan*M, responsible for lantibiotics synthesis, according to Wirawan *et al.* (2006) and Hyink *et al.* (2005). For each

primer pair, the reaction was composed by 12·5 μl of PCR kit GoTaq Green Master Mix 2× (Promega), 1·0 μl of each primer (100 pMol μl^{-1}), 0·3 μl of extracted DNA and ultra-pure PCR water for a final volume of 25 μl. The PCR conditions consisted of initial denaturation at 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 40°C for 30 s and 65°C for 30 s, and the final extension step at 65° C for 10 min. The amplification products were then mixed with 20× GelRed stain (Biotium) at a 5 : 1 proportion and submitted to electrophoresis in 1% agarose gel in 0·5× TBE. The same DNAs were submitted to PCR for the detection of genes responsible for the synthesis of enterocins A, P, B, AS-48 and L50AB (Du Toit *et al.* 2000). PCR was performed using primers at 10 pMol μl^{-1} and initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, specific annealing temperature for 1 min and 72°C for 1 min, and the final extension step at 72°C for 10 min. The PCR products were analysed as described for lantibiotic biosynthesis genes. The PCR conditions are summarized in Table 2.

Characterization of virulence potential

Genotypic tests

The *Enterococcus* spp. isolates were tested for virulence genes *gel*E (gelatinase), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *esp* (enterococcal surface protein), *cyl*A (cytolisin), *efa*A (endocarditis antigen), *ace* (adhesion of collagen), *van*A and *van*B (both related to vancomycin resistance) and genes for amino acid decarboxylases *hdc*1 and *hdc*2 (both related to histidine decarboxylase), *tdc* (tyrosine decarboxylase) and *odc* (ornithine decarboxylase), using PCR protocols of Martin-Platero *et al.* (2009), Rivas *et al.* (2005) and Vankerckhoven *et al.* (2004). The amplified products were separated by electrophoresis on 0·8–2·0% (w/v) agarose gels in 1× TAE buffer. Gels were stained in TAE buffer containing 0·5 μg ml^{-1} ethidium bromide (Sigma-Aldrich). Primers, annealing temperatures and fragment sizes are detailed in Table 2.

Phenotypic tests

Enterococcus spp. isolates were tested for haemolytic activity and production of gelatinase, lipase and DNAse according to Barbosa *et al.* (2010). For haemolytic activity, 1 μl aliquots of the cultures were spotted onto plates containing TSA (Oxoid) added to 5% (v/v) defibrinated horse blood and incubated at 37°C for 48 h. Clear halos around the colonies indicated total or α-haemolysis, and green halos around the colonies indicated partial or β-haemolysis. Absence of halos around the colonies was interpreted as no haemolytic activity (γ-haemolysis). For gelatinase production, 1 μl aliquots of isolates cultures were spotted on plates containing Luria–Bertani agar (LB, Becton, Dickinson & Co.; Franklin Lakes, NJ, USA) supplemented with 3% (w/v) gelatine and incubated at 37°C for 48 h, followed by incubation at 4°C for 4 h. Opaque halos around the colonies were recorded as positive results. For lipase production, 1 μl aliquots of isolates cultures were spotted onto plates containing LB agar (BD) supplemented with 0·2% (w/v) $CaCl_2$ and 0·1% (w/v) Tween 80 (Sigma-Aldrich) and incubated at 37°C for 48 h. Opaque halos around the colonies were recorded as positive results. For DNAse production, 1 μl aliquots of the isolate cultures were spotted onto plates containing DNAse methyl green agar plates (BD) and incubated at 37°C for 48 h. Clear halos around the colonies were recorded as positive results. All tests were conducted in triplicate.

Results

On the basis of 16s rDNA sequencing, all isolates were confirmed as *Enterococcus*. The RAPD profiles of the isolates are presented in Table 3. The 43 isolates belonged to 20 distinct RAPD profiles, and III and IV grouped the largest number of isolates: 9 and 6, respectively.

The antimicrobial substances produced by most isolates were sensitive to α-chymotrypsin, proteinase K and trypsin, indicating their proteinaceous nature. The inhibitory spectrum of activity of the *Enterococcus* isolates is shown in Table 4. Lantibiotic and enterocin genes were present in the majority of *Enterococcus* groups (Table 5). Lantibiotic biosynthesis genes were present in distinct associations, and enterocin P gene was the most frequent (isolates of 11 profiles). Enterocin L50AB gene was not detected in any of the isolates. Several profiles presented isolates with more than one enterocin gene, and the most frequent association was for enterocins A and P genes.

With regard to evaluation of the virulence potential of the *Enterococcus* isolates, results varied in an RAPD profile–dependent format (Table 6). All profiles harboured isolates that contained the gene for aggregation substance production (*asa*1), and only isolates from profile VIII were negative for gelatinase gene (*gel*E). For amino acid decarboxylase, the gene for tyrosine descarboxylase (*tdc*) was more common, detected in the majority of profiles. Concerning vancomycin resistance genes, two profiles (V and XVI) presented *van*A, and one (XVII) presented *van*B. The isolates presented also variable results in the phenotypical testing for virulence (Table 7), and most of the profiles presented β-haemolysis.

Discussion

Enterococci are relevant as starter cultures in several artisanal foods, being responsible for the production of dis-

Table 2 Primers sequences utilized in the investigation of positive results for genes for lantibiotics, enterocins, virulence factors, vancomycin resistance and biogenic amine production

Target	Genes*	Primers	Annealing temperature	Fragment size (bp)	References
Lantibiotics biosynthesis	*lan*M	ATGCWAGWYWTGCWCATGG CCTAATGAACCRTRRYAYCA	40°C	200–300	Hyink *et al.* 2005
	*lan*B	TATGATCGAGAARYAKAWAGATATGG TTATTAIRCAIATGIAYDAWACT	40°C	400–500	Wirawan *et al.* 2006
	*lan*C	TAATTTAGGATWISYIMAYGG ACCWGKIIIICCRTRRCACCA	40°C	200–300	Wirawan *et al.* 2006
Enterocins	A	CATCATCCATAACTATATTTG AAATATTATGGAAATGGAGTGTAT	56°C	126	Du Toit *et al.* 2000
	B	GAAAATGATCACAGAATGCCTA GTTGCATTTAGAGTATACATTTG	58°C	162	Du Toit *et al.* 2000
	P	TATGGTAATGGTGTTTATTGTAAT ATGTCCCATACCTGCCAAAC	58°C	120	Du Toit *et al.* 2000
	L50AB	STGGGAGCAATCGCAAAATTAG ATTGCCCATCCTTCTCCAAT	56°C	98	Du Toit *et al.* 2000
	AS48	GAGGAGTITCATGATTTAAAGA CATATTGTTAAATTACCAAGCAA	56°C	340	Du Toit *et al.* 2000
Virulence	*gel*E	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	47°C	213	Vankerckhoven *et al.* 2004
	hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	53°C	276	Vankerckhoven *et al.* 2004
	*asa*1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	50°C	375	Vankerckhoven *et al.* 2004
	esp	AGATTTCATCTTTGATTCTTG AATTGATTCTTTAGCATCTGG	47°C	510	Vankerckhoven *et al.* 2004
	*cyl*A	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	52°C	688	Vankerckhoven *et al.* 2004
	*efa*A	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	57°C	688	Martin-Platero *et al.* 2009
	ace	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	48°C	1008	Martin-Platero *et al.* 2009
Antibiotic resistance	*van*A	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT	52°C	377	Martin-Platero *et al.* 2009
	*van*B	GCTCCGCAGCCTGCATGGACA ACGATGCCGCCATCCTCCTGC	60°C	529	Martin-Platero *et al.* 2009
Biogenic amines	*hdc*1	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	46°C	367	Rivas *et al.* 2005
	*hdc*2	AAYTCNTTYGAYTTYGARAARGARG ATNGGNGANCCDATCATYTTRTGNCC	50°C	534	Rivas *et al.* 2005
	tdc	GAYATNATNGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNGTRTG	55°C	924	Rivas *et al.* 2005
	odc	GTNTTYAAYGCNGAYAARCANTAYTTYGT ATNGARTTNAGTTCRCAYTTYTCNGG	54°C	1446	Rivas *et al.* 2005

**lan*M, *lan*B, *lan*C (lantibiotics biosynthesis), *gel*E (gelatinase), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *esp* (enterococcal surface protein), *cyl*A (cytolisin), *efa*A (endocarditis antigen), *ace* (adhesion of collagen), *van*A and *van*B (vancomycin resistance), *hdc*1 and *hdc*2 (histidine decarboxylase), *tdc* (tyrosine decarboxylase) and *odc* (ornithine decarboxylase).

tinct typical characteristics (Giraffa 2002, 2003; Martin-Platero *et al.* 2009). They are also present as autochthonous microbiota from distinct foods and are capable of producing bacteriocins (Dal Bello *et al.* 2010; Khan *et al.* 2010; Javed *et al.* 2011). However, the virulence potential of enterococci determines a proper characterization of wild strains, to verify their adequacy to be used as bio-preservatives (Foulquié-Moreno *et al.* 2006; Franz *et al.* 2011).

The bacteriocinogenic activity of the isolates was confirmed by the enzymatic sensitivity of their produced antimicrobial substances. Testing the sensitivity of bacteriocins to digestive enzymes does not indicate the type of bacteriocin produced by an isolate, but evaluates its

Table 3 Distribution of the *Enterococcus* spp isolates obtained from raw milk and cheese grouped according to the RAPD profile

RAPD profile	*n*	Isolates
I	5	En01, En02, En04, En14, En15
II	2	En02, En27
III	9	En05, En09, En11, En12, En32, En36, En37, En39, En42
IV	6	En06, En08, En22, En28, En35, En38
V	1	En07
VI	1	En10
VII	2	En13, En17
VIII	3	En16, En18, En19
IX	1	En20
X	1	En21
XI	1	En23
XII	1	En24
XIII	1	En25
XIV	1	En28
XV	1	En29
XVI	1	En30
XVII	1	En31
XVIII	2	En33, En34
XIX	2	En40, En43
XX	1	En41

n, number of isolates.

potential applicability for food biopreservation, as food preservatives should be destroyed during the passage through the gastrointestinal system (Sharma *et al.* 2006; de Arauz *et al.* 2009). The isolates presented antimicrobial activity against most *Listeria* spp., different species of LAB and *Staphylococcus* spp. (Table 4); similar results were reported in other Brazilian studies (Moreno *et al.* 2000; de Martinis *et al.* 2001; Bromberg *et al.* 2005; Gomes *et al.* 2008).

Considering the results for lantibiotic biosynthesis genes (Table 5), *lan*B was the most frequent gene among the RAPD profiles, present in 12. This gene was also present is association with *lan*C (four profiles) and *lan*M (one profile). According to Hyink *et al.* (2005) and Wirawan *et al.* (2006), positive result for any of the three tested genes for lantibiotics biosynthesis is enough to indicate the capacity of the isolate to produce these bacteriocins. Despite not being the best characterized and known bacteriocins produced by *Enterococcus* spp. (de Vuyst *et al.* 2003), lantibiotics are the main class of bacteriocins produced by LAB and can be applied in several food systems to control foodborne pathogens and spoilage micro-organisms (McAuliffe *et al.* 2001; Riley and Wertz 2002).

As shown in Table 5, isolates belonging to 11 RAPD profiles contained at least one of the tested enterocin genes. It is possible to verify the distinct association of positive results for enterocin genes among the RAPD profiles and also presence of enterocins genes in four profiles that did not present any of the lantibiotic biosynthesis genes (VI, X, XVI and XVII). Several isolates, from 7 RAPD profiles, presented more than one enterocin gene, and the most frequent association was for enterocins A and P genes. Other authors have also observed that enterococci may contain multiple enterocin genes (de Vuyst *et al.* 2003; Dal Bello *et al.* 2010; Javed *et al.* 2011). Genetic transfer mechanisms, owing to the presence of conjugative transposons and plasmids, can explain the observed variability of multiple enterocin genes in isolates presenting the same RAPD profile (Franz *et al.* 2007).

The presence of more than one gene does not mean that all will be expressed simultaneously and that an isolate is capable of producing multiple bacteriocins at the same time (Cintas *et al.* 1998; Javed *et al.* 2011). Seven isolates (En13, En14, En15, En18, En20, En34 and En35) did not produce antimicrobial substances with sensitivity to at least one of the tested enzymes (data not shown), but belonged to RAPD profiles that contained isolates presenting one or more bacteriocins genes (I, IV, VII, VIII, IX and XVIII), suggesting that these genes were not expressed in those seven isolates.

The virulence potential (Table 6) and activity (Table 7) of the isolates belonging to distinct RAPD profiles were variable and present in distinct associations as well. In general, the frequency of positive results for the studied virulence factors was similar to those reported in other studies on *Enterococcus* isolated from foods (Semedo *et al.* 2003; Gomes *et al.* 2008; Barbosa *et al.* 2010), but the frequency of positive results was lower when compared to studies with clinical isolates (Eaton and Gasson 2001; Semedo *et al.* 2003; Barbosa *et al.* 2010). Despite being less relevant in food isolates, verification of virulence factors in *Enterococcus* spp. by molecular and phenotypic procedures is important because of the risk of genetic transfer, because these genes are usually located in conjugative plasmids (Eaton and Gasson 2001).

The investigation of virulence factors in *Enterococcus* with potential application in food preservation is of foremost importance as enterococci may contain several determinants of pathogenicity. Virulence factors may be either colonization factors, such as those that promote the adhesion of bacteria to the host cells, or invasion factors that promote the invasion of epithelial cells, which disorder the immune system (de Sousa 2003). Several cell wall–anchored surface proteins are implicated in enterococcal pathogenicity, including aggregation substance, enterococcal surface protein, collagen-binding components (Hendrickx *et al.* 2009). Some secreted products, such as hyaluronidase, may interact with lymphocyte receptors and induce autoimmune diseases (de Sousa

Table 4 Inhibitory activity of *Enterococcus* isolates according to the RAPD profile and the number of tested target micro-organisms

	Target bacteria (number of tested strains)											
RAPD profile	*Staphylococcus aureus* (12)	*Listeria monocytogenes* (7)	*L. innocua* (2)	*L. ivanovii* subsp. *ivanovii* (1)	*L. seeligeri* (1)	*L. welshimeri* (1)	*Enterococcus faecalis* (4)	*Enterococcus* spp. (1)	*Lactobacillus plantarum* (3)	*Lact. lactis lactis* (2)	*Lact. delbrueckii* subsp. bulgaricus (1)	*Lact. sakei* (1)
I	1–10*	1–6	0–2	0–1	0–1	0–1	2–3	1	0–1	0	0	0–1
II	5–7	5–6	2	0–1	1	1	3	1	0–1	0	0	1
III	1–11	3–7	1–2	0–1	0–1	1	3–4	1	0–3	0–2	0–1	1
IV	2–10	5–7	0–2	0–1	0–1	1	2–4	0–1	1–3	0–2	0–1	1
V	12	5	2	1	1	1	3	1	0	0	0	1
VI	4	7	2	1	1	1	4	1	3	2	0	1
VII	2–3	0–3	0	0	0	0	1–2	0–1	0	0–1	0–1	1
VIII	1–8	3–6	2	0	1	1	3	1	0	0	0–1	1
IX	4	7	2	1	1	1	3	1	0	0	0	0
X	2	4	1	0	1	1	3	1	0	0	0	1
XI	8	6	2	1	1	1	4	1	1	1	0	1
XII	10	7	2	2	2	1	1	0	3	1	0	1
XIII	10	6	2	2	2	0	1	1	3	1	1	2
XIV	0	3	1	1	1	0	0	0	2	0	0	0
XV	11	7	2	2	2	1	1	1	3	1	0	0
XVI	10	6	1	1	1	1	1	1	2	1	0	0
XVII	1	7	2	2	2	0	1	1	4	0	2	2
XVIII	6–12	6	2	2	2	1	1	1	4	1	2–3	2
XIX	5–7	5–7	1–2	1–2	1–2	1	1	1	2–3	1	0–2	0–2
XX	8	4	2	2	2	1	1	1	4	0	1	2

*Variability of the number of strains inhibited by *Enterococcus* spp isolates belonging to the same RAPD profile (Table 3).

Table 5 Positive results (+) for genes for lantibiotics biosynthesis and enterocins in *Enterococcus* spp isolates belonging to 20 RAPD profiles

	Lantibiotic genes			Enterocins genes				
RAPD profile	*lan*B	*lan*C	*lan*M	L50AB	AS48	P	A	B
I	+	–	–	–	–	+	+	+
II	+	–	–	–	–	+	–	–
III	+	+	–	–	+	+	+	–
IV	+	+	–	–	+	+	+	–
V	+	–	+	–	–	–	–	–
VI	–	–	–	–	+	+	–	–
VII	+	–	–	–	–	+	+	–
VIII	+	+	–	–	–	+	+	–
IX	+	–	–	–	–	–	–	–
X	–	–	–	–	–	+	–	–
XI	–	–	–	–	–	–	–	–
XII	+	–	–	–	–	–	–	–
XIII	–	–	–	–	–	–	–	–
XIV	–	+	–	–	–	–	–	–
XV	+	–	–	–	–	–	–	–
XVI	–	–	–	–	–	–	+	–
XVII	–	–	–	–	+	+	–	–
XVIII	+	+	–	–	–	–	–	–
XIX	+	–	–	–	+	–	–	–
XX	–	–	–	–	–	–	–	–

2003). Cytolysin is an exotoxin with bifunctional bacteriocin and haemolytic effects (Haas *et al.* 2002). Cytolysin causes the invading organism to evade the host immune system (Franz and Holzapfel 2004), and this toxin can lyse human, rabbit and horse erythrocytes (Chow *et al.* 1993). Enterococcal surface proteins include aggregation substance, *Enterococcus* surface protein, adhesins and other adhesive molecules, such as *Enterococcus* endocarditis antigen. Expression of the aggregation substance protein enables close contact between cells for conjugation and subsequent transfer of virulence plasmids (Hendrickx *et al.* 2009). The aggregation substance protein may have a role in translocation of enterococci into epithelial cells (Franz and Holzapfel 2004). Enterococcus surface protein is a cell wall–anchored protein characterized by its ability to form biofilms and may, therefore, be implicated in enterococcal infections that are associated with biofilm (Hendrickx *et al.* 2009). Angiotensin-converting enzyme (ACE) proteins facilitate the binding of *Enterococcus* spp. to collagen and are expressed during human infections (Franz and Holzapfel 2004). The expression of endocarditis antigens produced by *Ent. faecalis* has been shown to be essential for the growth of this species and to be bound to fibrinogen, collagen, fibronectin and laminin, damaging host cell structure (Franz and Holzapfel 2004).

Table 6 Positive results (+) for genes for virulence and biogenic amines in *Enterococcus* spp isolates belonging to 20 RAPD profiles

	Virulence genes*							Antibiotic resistance genes		Biogenic amines genes*			
RAPD profile	*gel*E	*hyl*	*asa*1	*esp*	*cyl*A	*efa*A	*ace*	*van*A	*van*B	*hdc*1	*hdc*2	*tdc*	*odc*
I	+	–	+	–	–	+	–	–	–	–	–	+	–
II	+	–	+	–	–	–	–	–	–	–	–	–	–
III	+	–	+	–	–	+	+	–	–	–	–	+	+
IV	+	–	+	–	+	+	+	–	–	–	–	+	–
V	+	–	+	–	–	–	+	+	–	–	–	+	+
VI	+	+	+	–	+	+	–	–	–	+	–	–	–
VII	+	–	+	+	–	+	–	–	–	–	–	+	–
VIII	–	–	+	–	–	–	–	–	–	–	–	–	–
IX	+	–	+	–	–	–	+	–	–	–	–	–	+
X	+	+	+	+	–	+	–	–	–	–	–	+	–
XI	+	–	+	+	–	+	–	–	–	+	–	+	–
XII	+	+	+	+	–	+	–	–	–	+	–	+	–
XIII	+	–	+	–	–	+	–	–	–	–	–	–	–
XIV	+	–	+	–	–	+	–	–	–	–	–	–	–
XV	+	–	+	+	–	+	–	–	–	+	–	+	–
XVI	+	–	+	+	–	+	+	+	–	+	–	–	–
XVII	+	–	+	+	–	+	–	–	+	–	–	+	+
XVIII	+	+	+	+	–	+	–	–	–	–	–	–	–
XIX	+	–	+	+	–	+	–	–	–	–	–	+	–
XX	+	+	+	+	–	+	–	–	–	–	–	–	–

**gel*E (gelatinase), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *esp* (enterococcal surface protein), *cyl*A (cytolisin), *efa*A (endocarditis antigen), *ace* (adhesion of collagen), *van*A and *van*B (vancomycin resistance), *hdc*1 and *hdc*2 (histidine decarboxylase), *tdc* (tyrosine decarboxylase) and *odc* (ornithine decarboxylase).

Table 7 Positive results (+) for virulence factors in *Enterococcus* spp isolates belonging to 20 RAPD profiles

RAPD profile	Virulence factors				
	Gelatinase	Lipase	DNAse	β-haemolysis	α-haemolysis
I	–	–	–	+	–
II	–	–	–	+	–
III	+	–	–	+	–
IV	+	–	–	+	–
V	+	–	–	+	–
VI	+	–	–	+	–
VII	+	–	–	+	–
VIII	+	–	–	+	–
IX	–	–	–	+	–
X	–	–	–	+	–
XI	–	–	–	+	–
XII	–	–	–	+	–
XIII	+	–	–	–	–
XIV	–	–	–	+	–
XV	–	–	–	+	–
XVI	–	–	–	+	–
XVII	–	–	–	–	–
XVIII	+	–	–	+	–
XIX	+	–	–	+	–
XX	–	–	–	+	–

Gelatinase also plays an important role in pathogenicity as it is a protease involved in the hydrolysis of gelatine, casein, collagen and haemoglobin, and small bioactive proteins, such as *Ent. faecalis* sex pheromone-related peptides (Archimbaud *et al.* 2002). Gelatinase production is usually associated with enterococci from clinical samples, but it has also been detected in enterococci isolated from dairy and meat products (Silva Lopes *et al.* 2006).

The role of hyaluronidase in infections has been reviewed by Girish and Kemparaju (2007). Hyaluronidase facilitates the spread of bacteria and toxins throughout the host tissue by causing tissue damage (Kayaoglu and Orstavik 2004). Microbial hyaluronidase production is linked to enterococcal virulence primarily because the enzyme is linked to pathogenicity through enzymatic degradation of host tissue in other organisms (Franz and Holzapfel 2004).

Enterococci are often the causative agents of infections in hospitalized patients and nosocomial bloodstream infections (Vankerckhoven *et al.* 2008). In enterococci, six vancomycin resistance types have been phenotypically and genotypically identified, and two of them, VanA and VanB, may be located in transferable plasmids (Courvalin 2006).

Enterococcus spp. isolates, obtained from milk and cheeses in Minas Gerais state, Brazil, presented an interesting potential application for food preservation because of the production of protease-sensitive bacteriocins, with a good inhibitory spectrum of activity. Most isolates harboured genes responsible for synthesis of known lantibiotics (*lan*M, *lan*B and *lan*C) and enterocins (*ent*A, *ent*B, *ent*P, *ent*L50AB and *ent*AS48). However, most isolates presented genes for virulence factors, such as production of aggregation substance (*asa*1), gelatinase (*gel*E), endocarditis antigen (*efa*A) and tyrosine descarboxylase (*tdc*). Phenotypic tests indicated that a great part of isolates presented partial or β-haemolysis. The present study demonstrated the contradictory characteristics of these *Enterococcus* isolates.

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