Bacteriocin Activity of Enterococci and Presence of Genes Related to Pathogenesis

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Abstract

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In total 228 enterococci strains isolated from food were studied. *Enterococcus faecalis*, *E. faecium*, and *E. casseliflavus* were found to be the dominant strains while *E. durans* and *E. mundtii* were present in a smaller extent. Antimicrobial activity determined by double layer technique revealed that 150 (65.7%) strains showed antimicrobial activity against the individual tested pathogenic strains of *Listeria monocytogenes*, *Staphylococcus aureus*, and methicilin resistant *S. aureus* (MRSA). Cell-free neutralised supernatants (CFNS) were prepared from 150 potential bacteriocin producers. Of these 150, CFNS 107 (71.3%) strains were active in the bacteriocin production against one or more pathogenic strains tested. *S. aureus* and MRSA were found to be more sensitive to the antimicrobial substances than *L. monocytogenes*. Multiplex PCR for the detection of seven virulence genes in bacteriocin producing strains showed that 47.6% of strains were able to amplify one or more virulence genes. *E. faecalis* was the most virulent species. The presence of *tyrdc* gene was seen in all bacteriocin producing strains. None of the strains carried genes encoding the resistance to vancomycin.

Keywords: food; antimicrobial activity; virulence genes; *tyrdc* gene; *Listeria monocytogenes*; *Staphylococcus aureus*; methicilin resistant *Staphylococcus aureus*

Enterococci can be found in many foods of both animal and vegetable origins (Murray 1990; Muller *et al.* 2001; Ben Omar *et al.* 2004). They are able to survive the heat treatment and other adverse conditions during the food processing. On the other hand, they can be used to extend the shelf life and improve the hygienic safety of foodstuffs because they produce several antimicrobial substances such as lactic acid, hydrogen peroxide, and bacteriocins (Martín-Platero *et al.* 2009). Bacteriocins are ribosomally synthesised peptides or proteins produced by bacteria that exhibit antimicrobial activity against other more or less related bacteria (Klaenhammer 1993). Enterocins are bacteriocins produced by *Enterococcus* spp. They are mainly Class II bacteriocins and are distinguished by their activity against *Listeria* spp. The encoding of multiple enterocin genes in the genomes of enterococci is not unusual and the production of more than one enterocins by an enterococcal strain is of technological and commercial interest. Several researchers have isolated enterococci producing enterocins (Foulquié-Moreno *et al.* 2006; Chanos & Williams 2011). As enterococci

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are commonly present in many food systems and their technological and probiotic benefits are so well known, their production of bacteriocins (enterocins) has become a subject of great interest since they are found to be active against various pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* (Sabia *et al.* 2007). Bacteriocin producing enterococci can be intended only for such food products, in which their negative interference with other lactic acid bacteria used for the production of this food product is not expected. The ambivalence of enterococci consists in both the positive and negative roles. Some strains are used as probiotics whereas others are also recognised as serious nosocomial pathogens causing bacteraemia, urinary tract infections, and endocarditis (Kučerová *et al.* 2009a).

However, enterococci have been involved in food spoilage (Franz *et al.* 1999), in food intoxication (Gardini *et al.* 2002), and in spreading the antibiotic resistance through the food chain (Giraffa 2003). Food intoxication associated with a higher consumption of biogenic amines such as tyramine and histamine has been widely studied in recent years. The diet rich in tyramine can increase blood pressure and cause migraines, and that imbalance in the level of tyramine is thought to underlie altered brain function in many pathological conditions, including dystonias, Parkinson's disease, schizophrenia, drug addiction, and mood disorders (Marcobal *et al.* 2006). Several putative virulence factors have been described in enterococci such as aggregation substances, gelatinase, cytolysin, enterococcal surface protein, hyaluronidase, accessory colonisation factor, and endocarditis antigen (Martín-Platero *et al.* 2009). Until now, a major focus in most of the studies has been on two main species of enterococci, *Enterococcus faecalis* and *E. faecium*. These are most prominently occurring species in the entire habitat. Also, *E. faeacalis* and *E. faecium* have been found to be responsible for the presence of higher numbers of virulent genes causing serious nosocomial infections (Jett *et al.* 1998). Thus, the focus on the enterococcal species with less virulent traits and better bacteriocin producing capacity should be taken into consideration for the food processing application.

The aim of the study was to detect the bacteriocin producing enterococci isolated from various foodstuffs and to characterise them for the general risks associated with their occurrence in foods.

MATERIAL AND METHODS

Enterococci. In total 228 enterococci strains were isolated from various foodstuffs and stored at –75°C in 20% glycerol medium in the strain collection of the Department of Milk Hygiene and Technology (University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic). The strains were resuscitated on the Slanetz Bartley medium (HiMedia, Mumbai, India) at 37°C for 24 h and characterised on the species level according to (Jackson *et al.* 2004). Detailed characteristic of the strains is given in Table 1.

Collection strains. MRSA S15 (Department of Milk Hygiene and Technology, Brno, Czech Republic), *Listeria monocytogenes* LM CCM 4699, and *Staphylococcus aureus* SA CCM 4223 (Czech Culture Collection Centre, Masaryk University, Brno, Czech Republic) were used for testing the antibacterial activity of bacteriocins. Lyophilised strains were resuscitated according to the CCM procedure in Nutrient broth aerobically at 37°C for 24 hours. After incubation, the cultures were streaked on Nutrient agar and stored in refrigerator until the use.

Screening of enterococci for antibacterial activity. The antibacterial activity of the enterococci strains against selected Gram-positive bacteria was tested by double layer technique. Culture suspensions of 2 McFarland in physiological solution were prepared from fresh overnight cultures of pure enterococcal strains. Drops of 5 µl volume of enterococci culture suspensions were spotted onto plates containing 10 ml of GM-17 agar i.e. M-17 agar (Oxoid, Hampshire, UK) plus 1% D-glucose (Penta, Prague, Czech Republic) medium dissolved in 0.1M pH 7.2 sodium phosphate buffer (GM-17-B). The plates were incubated at 37°C for 18–24 hours. After the growth of enterococci strains on GM-17 agar, the plates were overlaid with 1 ml of Brain Heart Infusion broth (BHI; HiMedia, Mumbai, India) inoculated with an overnight culture with the cell density approx. $1–5 \times 10^8$ CFU of the tested strains and incubated once again at 37°C for 18–24 h to allow the growth of the tested strain. The appearance of a clear inhibition zone around the strain spot indicated the production of an inhibitor substance active against the indicator strain (Martín-Platero *et al.* 2009).

Preparation of culture supernatant. Strains with the proven antibacterial activity of whole cells were grown according to their optimal conditions of cultivation. The cultures were centrifuged at 6000 rpm

for 15 min; the cell-free supernatant was neutralised to pH 6.0 using NaOH (100 g/l solution) and heated at 90°C for 10 min to inactivate the remaining cells. The cell-free, neutralised supernatant (CFNS) was used in further bacteriocin assay by the above mentioned agar spot method (Kučerová *et al.* 2009b).

Method based on tyrdc gene detection (PCR). DNA extraction was performed by the boiling procedure (Peréz-Hernández *et al.* 2002) with a slight change in the process. Here, the cell suspension was prepared in 5% chelex (Bio Rad, Munich, Germany) instead of water. A multiplex PCR was designed to look for the production of tyramine by the detection of *tyrdc* gene. Primers used and the amplification program were identical to that of Trivedi *et al.* 2009.

Multiplex PCR for identification of virulence genes and vancomycin resistant genes. Three different multiplex PCR were carried out for the screening of seven different virulence factors *gel*E (Gelatinase), *hyl* (Hyaluronidase), *asa*1 (Aggregation substances), *esp* (Enterococcal surface protein), *cyl*A (Cytolysin), *efa*A (Endocarditis antigen), *ace* (Adhesion of collagen protein), and two vancomycin resistant (*van*A and *van*B) genes. The primers used and conditions for the PCR were according to Martín-Platero *et al.* (2009).

RESULTS AND DISCUSSION

Spectrum of enterococci species tested

Out of 228 enterococci strains, 150 (65.7%) were of dairy origin. The remaining 25 (10.96%) were of meat origin, 25 (10.96%) of fruits and vegetable

Table 1. Antibacterial effects of various enterococci strains isolated from various food-stuffs

Origin	Species	No. of strains (%)	No. of enterococcal strains producing antibacterial substance (%) against selected Gram-positive bacteria					
			SA CCM 4223		S15 MRSA		LM CCM 4699	
			without CFNS	with CFNS	without CFNS	with CFNS	without CFNS	with CFNS
Dairy	E. faecalis	103 (68.7)	79 (76.6)	39 (49.3)	67 (65.1)	47 (70.1)	12 (11.6)	3 (25)
	E. faecium	43 (28.7)	36 (83.7)	18 (50)	24 (55.8)	13 (54.1)	2 (4.6)	0 (0)
	E. casseliflavus	3 (2)	3 (100)	1 (33.3)	3 (100)	1 (33.3)	0 (0)	0 (0)
	E. durans	1 (0.7)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
	Total	150	119 (79.33)	58 (48.7)	94 (62.6)	61 (64.8)	14 (9.33)	3 (21.4)
Meat	*E. faecalis*	9 (36)	7 (77.7)	4 (57.1)	5 (55.5)	2 (20)	4 (44.4)	1 (25)
	E. faecium	12 (48)	7 (58.3)	3 (42.8)	3 (25)	1 (33.3)	5 (41.6)	2 (20)
	E. casseliflavus	1 (4)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
	E. mundtii	2 (8)	2 (50)	0 (0)	1 (50)	0 (0)	2 (100)	0 (0)
	E. hirae	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Total	25	17 (68)	7 (41.1)	10 (40)	3 (30)	11 (44)	3 (27.2)
Fruits and vegetables	*E. faecalis*	10 (40)	10 (100)	5 (40)	6 (60)	3 (50)	6 (60)	3 (33.3)
	E. faecium	9 (36)	6 (66.6)	2 (33.3)	4 (44.4)	1 (25)	4 (44.4)	2 (50)
	E. casseliflavus	3 (16)	2 (66.6)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)
	E. mundtii	3 (12)	2 (66.6)	0 (0)	1 (33.3)	0 (0)	0 (0)	0 (0)
	Total	25	20 (80)	8 (32)	11 (44)	4 (36.3)	10 (40)	5 (50)
Retail	*E. faecalis*	26 (92.8)	3 (11.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. faecium	1 (3.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. mundtii	1 (3.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Total	28	3 (10.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

origins, and 28 (12.2%) came from foods available in the commercial market. Five different species of enterococci were recovered from this analysis. Of these, *E. faecalis* followed by *E. faecium* were found to be dominating in all origins. The presence of *E. casseliflavus* and *E. durans* was also seen in the strains of dairy origin. The presence of *E. hirae* and *E. mundtii* along with *E. casseliflavus* was evident in the case of meat origin. The strains of fruit and vegetable origins revealed the presence of *E. mundtii* along with *E. casseliflavus* (Table 1).

Bacteriocin production

The production of antimicrobial substances against *Staphylococcus aureus*, MRSA, and *Listeria monocytogenes* was tested with all the enterococci strains isolated from various foodstuffs. Out of 228 samples, 150 (65.7%) of the strains were able to produce antimicrobial substances against the individual pathogens tested. The CFNS was prepared from the positive isolates and than tested for the potential bacteriocin production.

Of these 150, CFNS 107 (71.3%) were found to be active in the production of bacteriocin against one or more pathogenic strains tested. 83 (55.3%) were found to be active in the production of enterocins against *S. aureus* strain, 48 (32%) strains were found to be active against MRSA, and 24 (16%) strains were active against *L. monocytogenes*. *S. aureus* and MRSA were found to be more sensitive to antimicrobial substances than *L. monocytogenes*. Only 10 bacteriocins (6.7%) were found to be active against all three pathogens.

E. faecium along with other isolated species was found to be highly active in the production of enterocins. Our results in terms of *E. faecium* were in agreement with that of Chanos and Williams (2011).

Multiplex PCR for *tyrdc* gene, virulence factors and vancomycin resistant genes

The selected enterococci strains have been used for decades in the production of various fermented foods and cheeses. In spite of this they have not yet obtained the GRAS (Generally Recognize as Safe) status (Ogier & Serror 2008). This might be due to the involvement of these bacterial strains in nosocomial infections (Giraffa 2003) and other health risks associated with them.

All the potentially bacteriocin producing enterococci strains characterised in this study showed the presence of *tyrdc* gene and can be concerned as potential producers of tyramine.

One of the major concerns along with the production of biogenic amines is the potential pathogenicity of the enterococci. They are emerging pathogens involved in urinary-tract infections, bacteraemia, endocarditis, and multiple antibiotic resistance (Franz *et al.* 1999), their virulence and pathogenic mechanisms still being largely unknown (Creti *et al.* 2004). In our work, we have concentrated on the study of seven different virulence genes (*gel*E, *hyl*, *asa*1, *esp*, *cyl*A, *ace*, and *efa*A) from the 107 bacteriocin producing strains. PCR amplification of these genes yielded positive results with 51 (47.6%) samples of all the identified species. We were able to detect these genes in all the species which had been tested but their expression was not tested. The incidence of *gel*E was found to be the least in our study and the products from meat and retail did not show the presence of *gel*E. These results were in con-

Table 2. Virulence genes in bacteriocin producing enterococci strains isolated from food-stuffs

Species	No. of species producing bacteriocins	No. of strains producing virulence genes (%)	No. of virulence genes (%)						
			*gel*E	*hyl*	*asa*1	*esp*	*cyl*A	*ace*	*efa*A
E. faecalis	66	35 (53.03)	2 (5.7)	15 (42.8)	22 (62.8)	18 (51.4)	2 (5.7)	25 (71.4)	26 (74.2)
E. faecium	32	13 (40.6)	1 (7.6)	5 (38.4)	9 (69.2)	10 (76.9)	0 (0)	9 (69.2)	12 (92.3)
E. casseliflavus	4	2 (50)	0 (0)	0 (0)	2 (100)	2 (100)	0 (0)	2 (100)	2 (100)
E. mundtii	4	1 (25)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)
E. durans	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	107	51 (47.6)	3 (5.8)	20 (39.2)	34 (66.6)	31 (60.7)	2 (3.92)	37 (72.5)	41 (80.3)

tradiction to those of (Creti *et al.* 2004), who had referred to a higher incidence of *gel*E genes in *E. faecalis* strains. The incidence of *hyl*, *asa*1, *esp*, *ace*, and *efa*A was evident to be the highest in our study. Also, *E. faecalis* was found to be the most active in the production of the virulence genes while the presence of the virulence genes in other species was evident but to a lesser extent. Here, *E. durans*, *E. casseliflavus*, *E. mundtii* irrespective of the origin were found to be the least active in the production of the virulence genes. The highest numbers of positive results were harboured from *E. faecalis*. Detailed results are shown in Table 2.

The use of antimicrobials in animal feed as growth promoters have created large reservoirs of transferable antibiotic resistance genes in several ecosystems, and consequently a possible route of the transmission of resistant *Enterococcus* spp. via the food chain could be suggested (Riboldi *et al.* 2009). A specific cause for concern and contributing factor to pathogenesis of enterococci is their resistance to a wide variety of antibiotics, especially vancomycin (Franz *et al.* 2001). None of the food isolate in our study was found to carry the vancomycin resistant gene.

CONCLUSION

Nearly half of the enterococci tested showed inhibitory effects (potentially bacteriocins). All the enterococci tested in this study contained the gene encoding the production of tyramine. *E. faecalis* showed to be the most virulent species, but the genes encoding virulence were detected also in other species. This fact should be taken into consideration in view of the use of enterococci in the food processing. In the agreement with the GRAS definition, such strains should be free from the virulence genes, genes coding resistance to antimicrobials, and should belong to low tyramine producers.

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