Virulence factors and antibiotic resistance in enterococci isolated from food-stuffs

K. Trivedi[1], S. Cupakova[1], R. Karpiskova[1,2]

[1]Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic
[2]National Institute of Public Health, Prague, Department of Food Safety and Nutrition Laboratories, Brno, Czech Republic

ABSTRACT: A collection of 250 enterococci isolated from various food-stuffs were used to investigate seven virulence determinants and the microbial susceptibility of eight antibiotics. Species-specific PCR revealed the presence of *E. faecalis* (127 isolates), *E. faecium* (77 isolates), *E. casseliflavus* (21 isolates), *E. mundtii* (19 isolates) and *E. durans* (six isolates). Multiplex PCR for virulence factors showed that from a total 250 isolates, 221 (88.4%) carried one or more virulence-encoding genes. β-Haemolytic activity was also evident in enterococcal species other than *E. faecalis* and *E. faecium*. Species other than *E. faecalis* and *E. faecium* isolated from food are also seen to harbour the potential for virulence. Antimicrobial susceptibility testing using the disk diffusion method showed that of the total 250 isolates, 114 (46%) were resistant to cephalothin and 94 (38%) to ofloxacin. Lower antibiotic resistance was seen with ampicillin, chloramphenicol, gentamicin and teicoplanin. None of the isolates was found to be resistant to vancomycin. The results of this study show that food can play an important role in the spread of enterococci with virulence potential through the food chain to the human population.

Keywords: *Enterococcus* species; food; virulence genes; antibiotic resistance

Enterococci are commonly found as part of the natural microflora of the gastrointestinal tract in humans and animals. They have been proposed as a part of defined starter cultures in some ripened cheeses and fermented salami and are used in the production of some meat and dairy products, due to their important role in flavour development and fermentation. Therefore, enterococci can be detected not only in raw materials but also in RTE (ready to eat) foods, of animal (e.g., milk, cheeses, meat and fish products) and plant origin (e.g., vegetable, fruits; Giard et al., 2001; De Castro et al., 2002; Abriouel et al., 2008).

Despite the use of enterococci in the food industry, their effects on human biology vary widely. While some strains are used as probiotics, others are recognized as serious nosocomial pathogens causing bacteraemia, urinary tract infections, and endocarditis (Kucerova et al., 2009). These bacteria are also implicated in surgical complications in neonatals, central nervous system disorders and other infections (Abriouel et al., 2008). In the last decade, enterococci have become the second most frequently reported cause of surgical wound infections and nosocomial urinary tract infections and the third most frequently reported cause of bacteraemia in humans (Kolar et al., 2002).

Enterococci may carry various genes directly or indirectly contributing to virulence (Shankar et al. 1999; Eaton and Gasson 2001; Franz et al., 2001; Kayser, 2003; Abriouel et al., 2008). Genes encoding virulence factors such as aggregation substances, endocarditis antigen, gelatinase, enterococcal surface protein, hyaluronidase or adhesion collagen

protein have been described in enterococci isolated from foodstuffs. The *efa*A gene is presumed to be involved in the adhesion of enterococci to biotic and abiotic surfaces or evasion of the immune response (Lowe et al., 1995; Shankar et al., 1999; Perez-Pulido et al., 2006). The *gel*E gene which encodes gelatinase is described to worsen the severity of endocarditis (Vnakerckhoven et al., 2004). The enterococcal surface protein *esp* gene is associated with increased virulence, colonization and persistence in the urinary tract along with biofilm formation (Shankar et al., 1999) while aggregation substances encoded by *asa*1 are responsible for increased bacterial adhesion to renal tubular cells and heart endocardial cells (Vankerckhoven et al., 2004). Enterococci are generally not considered as food-borne pathogens, although they can be associated with food-borne infections because of their frequent presence in foods and are capable of disseminating virulence or resistance genes through the food chain (Kojima et al., 2010).

To date, a major focus of most studies on enterococci has been on two main species, *E. faecalis* and *E. faecium*. Nevertheless, other enterococcal species have, from time to time, been reported to cause human infections (Smedo et al., 2003). A specific cause for concern regarding enterococci is their resistance to a wide variety of antibiotics, especially vancomycin (Franz et al., 2001; Kayser, 2003; Martin-Platero et al., 2009). In addition, these bacteria are able to acquire resistance determinants through gene transfer mediated by plasmids and transposons. The use of antimicrobials in animal feed as growth promoters has created large reservoirs of transferable antibiotic resistance genes in several ecosystems, and consequently a possible route of transmission of resistant *Enterococcus* spp. via food chain is feasible (Riboldi et al., 2009).

The presence of virulence genes and antibiotic resistance in foods is currently a matter of concern because these bacteria may be involved in the transmission of virulence genes and resistance determinants via the food chain. There exists no major discussion on virulence distribution beyond *E. faecalis* and *E. faecium*; thus, the presence of virulence genes from other enterococcal isolates in food is an important gap that should be addressed. The aim of this study was to monitor the distribution of virulence factors and the antibiotic resistance of various enterococci species isolated from food-stuffs.

MATERIAL AND METHODS

Enterococci isolates

Enterococci originating from the strain collection of the Department of Milk Hygiene and Technology (University of Veterinary and Pharmaceutical Sciences, Brno) isolated from foods-stuffs in 2008–2010 and stored at −75 °C in 20% glycerol medium were used in this study. Out of 250 enterococci isolates, 100 originated from milk and dairy products, 75 were isolated from ready-to-eat meat products and 75 from fruits and vegetables. All isolates were resuscitated in Brain Heart Infusion Broth (Hi Media, India) at 37 °C for 24 h and then inoculated onto Blood Sheep Agar plates (Oxoid, UK) and incubated at 37 °C for 24 h.

DNA extraction

Target DNA was extracted using the boiling procedure (Perez-Hernandez et al., 2002) with a slight change in the procedure. Strains were grown overnight at 37 °C on Blood Sheep Agar plates (Oxoid, UK). Three to five colonies of each sample were taken from the surface of the agar plates and resuspended in 1 ml of sterile distilled water. The cell suspension was centrifuged at 11 000 g for 10 min. The water was then discarded from the suspension and 200 μl of 5% chelex (Bio-Rad, U.S.A.) was added. The cell suspension with chelex was heated for 15 min at 95.5 °C and then centrifuged again at 13 000 g for 3 min. The supernatant containing DNA was stored at −20 °C until used as template for PCR.

Species identification of enterococci

Enterococcus species identification was carried out using the PCR method based on the detection of species-specific sections of the *sod*A gene encoding the enzyme manganese-dependent superoxide dismutase (Jackson et al., 2004). PCR's were performed in a Peltier Thermal Cycler PTC-200 (MJ Research, USA) using the Qiagen Multiplex PCR kit (Qiagen, Germany) in the total volume of 25 μl. PCR products were detected by electrophoresis in 2% (w/v) agarose gels in 0.5 × TBE buffer at 120 V for 60 min and visualised with ethidium bromide staining.

PCR for detection of virulence and *van*A and *van*B genes in enterococci

Three different multiplex PCR's were carried out for the detection of seven different virulence factors, and two vancomycin resistance-expressing genes. The primers used and the PCR conditions were identical to those reported by Martin-Platero et al. (2009). The polymerase was from the Qiagen Multiplex PCR kit (Qiagen, Germany) and primers were purchased from generibiotech.cz.

Phenotype method for haemolysin and gelatinase production

Enterococci for haemolysin and gelatinase activity assays were cultured on Slantetz-Bartley medium (Hi Media, India) and incubated at 37 °C for 24 h.

Haemolysin activity was determined on Columbia Blood Agar base (Oxoid, UK) containing 5% defibrinated horse blood after 24 h of incubation at 37 °C. Zones of clearing indicated β-haemolysin production (Abriouel et al., 2008).

Gelatinase production was evaluated using Nutrient Gelatine (Oxoid) broth tubes incubated at 37 °C for 48 h. The tubes were refrigerated after incubation to read for liquefaction. Growth on the surface of the medium was considered to be positive due to liquefaction of the medium.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of enterococcal isolates was tested using the disk diffusion method on Mueller-Hinton agar (Oxoid, UK) in accordance with CLSI (formerly NCCLS; CLSI 2006a). The tested antimicrobial substances and interpretation criteria are listed in Table 1. *Staphylococcus aureus* ATCC 25923 was used as a control strain (Czech Collection of Microorganisms, Czech Republic).

RESULTS AND DISCUSSION

Enterococci isolates, species identification

Five different species of enterococci were identified from the food-stuffs containing raw and pasteurized milk samples, cheeses of different varieties, ready to eat meat products, various fruits and vegetables (Table 2). *E. faecalis* (127 isolates) followed by *E. faecium* (77 isolates) were the dominant species in foodstuffs of all origins. The other detected strains were *E. casseliflavus* (21 isolates), *E. mundtii* (19 isolates) and *E. durans* (six isolates). In this study, *E. casseliflavus* was found to be dominant after *E. faecalis* and *E. faecium* in products of dairy origin. The results are in contradiction to Morandi et al., (2006) who described *E. durans* to be the dominant species after *E. faecalis* and *E. faecium* from dairy products. In the case of fruits and vegetables, *E. mundtii* was the dominant species after *E. faecalis* and *E. faecium*. These results are in contradiction to those of Perez-Pulido et al., (2006) who showed a higher incidence of *E. faecium* and *E. casseliflavus* in fermented capers (spiny plant growing wild in desert-like regions located around the Mediterranean). In our study, *E. durans* was not detected from foodstuffs of fruits and vegetable origin at all.

Table 1. Survey of used antimicrobial agents and interpretation criteria

Antimicrobial agent[1]	Abbreviations	Disk content (μg)	Zone diameter of the resistant isolate (mm)	Interpretation criterium
Ampicillin	AMP	10	≤ 16	CLSI, 2006b
Cephalotin	CLT	30	≤ 14	NCCLS, 1999
Erythromycin	ERY	15	≤ 13	
Gentamicin	GEN	120	≤ 6	
Chloramphenicol	CMP	30	≤ 12	CLSI, 2006b
Ofloxacin	OFL	5	≤ 15	
Teicoplanin	TEI	30	≤ 10	
Vancomycin	VAN	30	≤ 14	

[1]Oxoid (UK) antibiotic disks were used; for hly-streptomycin BioRad (France) antibiotic disk was used

Table 2. Virulence factors encoding genes in enterococci strains by source and species

Source	Species	Strains tested	Phenotypic assay		Presence of virulence genes (%)						
			gelatinase activity (%)	β-haemolytic activity (%)	*gel*E	*hyl*	*asa*1	*esp*	*cyl*A	*ace*	*efa*A
Dairy (100 isolates)	*E. faecalis*	56	0	19 (34)	0	13 (23)	9 (16)	21 (37)	2 (4)	5 (9)	23 (41)
	E. faecium	30	0	5 (17)	1 (3)	12 (40)	5 (17)	27 (90)	1 (3)	4 (13)	9 (30)
	E. casseliflavus	12	0	3 (25)	0	3 (25)	3 (25)	4 (33)	0	1 (8)	3 (25)
	E.mundtii	1	0	1 (100)	0	1 (100)	0	1 (100)	0	0	1 (100)
	E. durans	1	0	1 (100)	0	1 (100)	0	1 (100)	0	0	1 (100)
Meat (75 isolates)	*E. faecalis*	41	1 (2)	13 (32)	1 (2)	6 (15)	5 (12)	4 (10)	1 (2)	4 (10)	6 (15)
	E. faecium	25	0	2 (8)	0	3 (12)	2 (8)	1 (4)	1 (4)	3 (12)	4 (16)
	E. casseliflavus	6	0	1 (8)	0	1 (8)	1 (8)	2 (33)	0	3 (50)	1 (8)
	E. mundtii	2	0	0	0	0	2 (100)	1 (50)	0	1 (50)	1 (50)
	E. durans	1	0	0	0	0	1 (100)	1 (100)	0	0	0
Fruit and vegetables (75 isolates)	*E. faecalis*	39	0	7 (18)	0	5 (13)	4 (10)	2 (5)	0	3 (8)	2 (5)
	E. faecium	23	0	1 (4)	0	3 (13)	3 (13)	2 (9)	1 (4)	5 (22)	2 (9)
	E casseliflavus	1	0	0	0	0	0	1 (100)	0	0	1 (100)
	E. mundtii	12	0	0	0	0	2 (17)	1 (8)	0	1 (8)	2 (17)
Total		250	1 (0.4)	53 (21)	2 (0.8)	48 (19)	37 (15)	69 (28)	6 (2)	30 (12)	56 (22)

Detection of virulence genes in enterococci

In total 221 (88%) of the strains were found to carry one or more virulence genes. Here, 56 (56%) of milk and dairy product isolates, 16 (21%) of meat products and 11 (15%) isolates of vegetable and fruit origin were found to carry these genes. These results are in agreement with Abriouel et al. (2008) who reported 5.92% positivity in strains of vegetable origin. Seven different virulence genes were detected in species other than *E. faecalis* and *E. faecium*. Detailed results are shown in Table 2. These findings differ from those of Vankerckhoven et al. (2004) and Martin-Platero et al. (2009) who described the presence of virulence traits in *E. faecalis* and *E. faecium* only.

The presence of *esp* genes 69 (28%), *efa*A genes 56 (22%), and *asa*1 genes 37 (15%) were found most frequently in all the tested enterococci species. On the other hand, the gelatinase gene was found only in two isolates in all tested species. Moreover, one of the gel positive isolates did not express gelatinase activity (Table 2). One silent gene (not showing phenotypic activity despite the presence of the *gel* gene) was found in *E. faecium* from meat origin in our study (Table 2). These silent genes were also described by Eaton and Gasson (2001) in their study.

The occurrence of the *cyl*A gene was also found quite rarely and *E. casseliflavus, E. durans* and *E. mundtii* never harboured the *cyl*A gene (Table 2). The frequency of the *cyl*A gene among enterococci is very variable and does not correlate with clinical or food isolates (Smedo et al., 2003).

Up until now, the *hyl* gene seemed to be specific for *E. faecium* (Vankerckhoven et al., 2004). However, in our study it was also detected in *E. casseliflavus*, *E. mundtii* and *E. durans* (Table 2). It can be concluded that enterococcal species other than *E. faecalis* and *E. faecium* can also code for virulence genes.

β-Haemolysin assay

In our phenotypic study β-haemolytic activity was higher in *E. faecalis* (29%) compared to *E. faecium* (10%). Similar results were reported by Eaton and Gasson (2001) in isolates obtained from foods. Activity was also observable in *E. mundtii* and *E. durans* of dairy origin and two *E. casseliflavus* strains of dairy and meat origin.

Table 3. Resistance to antimicrobial agents in enterococci strains by source and species

Source	Species	Strains tested	Vancomycin resistant gene (PCR)		Data for antibiotic resistant enterococci strains (%) using the disk diffusion method							
			*van*A	*van*B	VAN	AMP	CLT	OFL	ERY	CMP	GEN	TEI
Dairy (100 isolates)	*E. faecalis*	56	0	0	0	0	46 (82)	22 (39)	0	0	0	0
	E. faecium	30	0	0	0	0	18 (60)	9 (30)	2 (7)	3 (10)	0	0
	E. casseliflavus	12	0	0	0	0	0	8 (67)	0	0	0	0
	E. mundtii	1	0	0	0	0	1 (100)	0	0	0	0	0
	E. durans	1	0	0	0	0	1 (100)	0	0	0	0	0
Meat (75 isolates)	*E. faecalis*	41	0	0	0	1 (2)	12 (29)	13 (31)	2 (5)	1 (2)	1 (2)	1 (2)
	E. faecium	25	0	0	0	0	13 (52)	9 (36)	3 (12)	0	0	0
	E. casseliflavus	6	0	0	0	0	0	0	0	0	0	0
	E. mundtii	2	0	0	0	0	0	0	0	0	0	0
	E. durans	1	0	0	0	0	0	0	0	0	0	0
Fruits and vegetables (75 isolates)	*E. faecalis*	39	0	0	0	2 (5)	11 (28)	17 (44)	6 (15)	0	0	1 (2)
	E. faecium	23	0	0	0	2 (9)	10 (43)	14 (61)	9 (39)	1 (4)	1 (4)	0
	E. casseliflavus	1	0	0	0	0	0	0	0	0	0	0
	E. mundtii	12	0	0	0	0	2 (17)	2 (17)	1 (8)	0	0	0
Total		250	0	0	0	5 (2)	114 (46)	94 (38)	23 (9)	5 (2)	2 (0.8)	2 (0.8)

Detection of *van*A and *van*B genes

Not a single isolate of any food origin tested in the study was found to encode the *van*A or *van*B genes responsible for expression of vancomycin resistance in enterococci. Consistent with this observation, none of the isolates was found to be resistant to vancomycin.

Antibiotic sensitivity

Enterococci isolated from various food sources exhibited high resistance to cephalothin (46%), ofloxacin (38%) and erythromycin (10%). A lower incidence of resistance was seen with clinically relevant antibiotics (ampicillin, gentamicin and teicoplanin; Table 3). As already mentioned, no isolate out of the 250 tested in this study was found to be resistant to vancomycin (Table 3). None of the isolates was resistant to three or more antibiotics simultaneously.

CONCLUSION

To date, there are only a few reports available showing the presence of virulence genes in species of enterococci other than *E. faecalis* and *E. faecium*.

Our results demonstrate that genes coding for hyaluronidase, cytolysin, endocarditis antigen, aggregation substances, gelatinase, and enterococcal surface protein can also occur in food enterococcal isolates. Our results suggest that, fermented foods of both animal and plant origin and enterococci used as probiotics or starter cultures should come under tighter control.

REFERENCES

Abriouel H, Omar NB, Molinos AC, Lopez RL, Jose Grande M, Martinez-Viedma P, Ortega E, Canamero MM, Galvez A (2008): Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical 265 samples. International Journal of Food Microbiology 123, 38–49.

CLSI (2006a): Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard. 9th Ed. Clinical and Laboratory Standards Institute document M2-A9. CLSI, Wayne, P.A. 37 pp.

CLSI (2006b): Performance Standards for Antimicrobial Disk Susceptibility Tests: 16th Informational Supplement: Clinical and Laboratory Standards Institute document M100-S16. CLSI, Wayne, P.A. 183 pp.

De Castro A, Montano A, Casado FJ, Sanchez AH, Rejano L (2002): Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter cultures for Spanish-green olive fermentation. Food Microbiology 19, 637–644.

Eaton TJ, Gasson M J (2001): Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Applied and Environmental Microbiology 67, 1628–1635.

Franz CMAP, Muscholl-Silberhorn AB, Yousif NMK, Vancanneyt M, Swings J Holzapfel WH (2001): Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. Applied and Environmental Microbiology 67, 4385–4389.

Giard JC, Laplace JM, Rince A, Pichereau V, Benachour A, Leboeuf C, Flahaut S, Auffray Y, Hartke A (2001): The stress proteome of *Enterococcus faecalis*. Electrophoresis 22, 2947–2954.

Jackson CH, Fedorka-Cray PJ, Barrett JB (2004): Use of genus and species-specific multiplex PCR for identification of enterococci. Journal of Clinical Microbiology 42, 3558–3565.

Kayser FH (2003): Safety aspects of enterococci from the medical point of view. International Journal of Food Microbiology 88, 255–262.

Kojima A, Morioka M, Minima M, Ishihara K, Asai T, Fujisawa T, Tamura Y, Takahashi T (2010): Classification and antimicrobial susceptibilities of *Enterococcus* species isolated from apparently healthy food-producing animals in Japan. Zoonoses and Public Health 57, 137–141.

Kolar M, Pantucek R, Bardon J, Vagnerova I, Typovska H, Valka I, Doskar J (2002): Occurence of antibiotic-resistant bacterial strains isolated in poultry. Veterinarni Medicina 47, 52–59.

Kucerova K, Svobodova H, Tuma S, Ondrackova I, Plockova M (2009): Production of biogenic amines by enterococci. Czech Journal of Food Sciences 27, S2-50–S2-55.

Lowe AM, Lambert PA, Smith AW (1995): Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with adhesions from some oral streptococci. Infection and Immunity 63, 703–706.

Martin-Platero AM, Valdivia E, Maqueda M, Martinez-Bueno M, (2009): Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. International Journal of Food Microbiology 132, 24–32.

Morandi S, Brasca M, Christian A, Lombardi A, Lodi R (2006): Technological and molecular-characterisation of enterococci isolated from north-west Italian dairy products. International Dairy Journal 16, 867–875.

NCCLS (1999): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard M31-A. NCCLS, Wayne, P.A. 57 pp.

Perez-Hernandez X, Mendez-Alvarez S, Claverie-Martin F (2002): A PCR assay for rapid detection of vancomycin-resistant enterococci. Diagnostic Microbiology and Infectious Disease 42, 273–277.

Perez-Pulido R, Abriouel H, Ben Omar N, Lucas R, Martinez-Canamero M, Galvez A (2006): Safety and potential risks of enterococci isolated from traditional fermented capers. Food and Chemical Toxicology 44, 2070–2077.

Riboldi GP, Frazzon J, Azevdo PA, Frazoon APG (2009): Antimicrobial resistance profile of *Enterococcus* spp. isolated from food in southern Brazil. Brazilian Journal of Microbiology 40, 125–128.

Shankar V, Baghdayan AS, Huycke MM, Lindahl G, Gilmore MS (1999): Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. Infection and Immunity 67, 193–200.

Smedo T, Almeida Santos M, Martins P, Silva Lopes MF, Figueiredo Marques JJ, Tenreiro R, Barreto Crespo MT (2003): Comparitive study using type strains and clinical food isolates to examine hemolytic acitivity and occurrence of the cyl operon in enterococci. Journal of Clinical Microbiology 41, 2569–2576.

Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H (2004): Development of Multiplex PCR for the detection of *asa*1, *gel*E, *cyl*A, *esp*, and *hyl* genes in Enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. Journal of Clinical Microbiology 42, 4473–4479.

Corresponding Author:

Mrs. Krina Trivedi, University of Veterinary and Pharmaceutical Sciences, Faculty of Veterinary Hygiene and Ecology, Department of Milk Hygiene and Technology, Palackeho 1–3, 612 42 Brno, Czech Republic
Tel. +420 774 542 767, E-mail: H07315@vfu.cz; trivedikrina@gmail.com