

Antibiotic resistance of *Enterococcus* Species Isolated from Raw Foods of Animal Origin in South West Part of Slovakia

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Abstract

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We determined the prevalence and antibiotic resistance of enterococci isolated from raw foods of animal origin. All samples were positive for enterococci. The lowest count of enterococci was found in pork (2.00 log CFU/cm²), while bryndza cheese contained the highest count (4.99 log CFU/g). Among the 349 *Enterococcus* isolates, 49% were *E. faecalis*, 29% *E. faecium*, and 13% *Enterococcus* spp. Tetracycline and gentamicin resistance was the most common. We found the highest tetracycline resistance levels (91%) in isolates from poultry samples. The isolates from the poultry samples also displayed multidrug resistance to all antibiotics tested. The most common vancomycin-resistant species in poultry and milk was *E. faecalis*. In contrast, the pork samples contained vancomycin-resistant *E. faecium* isolates. It is interesting to note that vancomycin resistance in the pork and poultry samples was found only in combination with either four (28%) or all five (14%) of the tested antibiotics. Our results suggest that raw products of animal origin are possible reservoirs of multi-antibiotic resistant enterococci in the food chain.

Keywords: pathogenic species; probiotic culture; fermentation; microbiological analyse; milk; meat

Enterococci are used as probiotics and in food fermentation processes, where they contribute to the flavour and aroma of cheeses. The ability to produce bacteriocins effective against pathogenic bacteria, especially *Listeria*, might be a powerful tool to protect the products from spoiling: for instance, raw milk cheeses, traditional cheeses with an improved aroma, or medium-pH fermented sausages (BOVER-CID *et al.* 2001; GARDINI *et al.* 2001; LAUKOVÁ *et al.* 2001; METAXOPOULOS *et al.* 2001; GIRAFFA 2002; FONTÁN *et al.* 2007; CHOHO *et al.* 2008). Because enterococci are so common in the gastrointestinal tract of many animals, the

contamination of raw milk or meat is often unavoidable (HUYS *et al.* 2004). An increasing number of enterococci isolated from food production possess the development resistance to various therapeutic antibiotics, including vancomycin and tetracyclines. Enterococci belong to the lactic acid bacteria (LAB) group and are widely distributed in nature, but they are not generally recognised as safe (GRAS) (TSAI *et al.* 2004; GOMES *et al.* 2008).

Raw milk and meat inadvertently contaminated with fecal matter carry antibiotic resistant lactic acid bacteria into the final food product, such as raw milk cheeses and fermented sausages (TEUBER

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et al. 1999). Lactic acid bacteria used as probiotics or in starter cultures are potential hosts of the antibiotic resistance genes, and can transfer these genes to pathogenic bacteria (MATHUR & SINGH 2005). The use of enterococci as probiotics and starter cultures in Europe is still controversial (MORENO *et al.* 2006; OGIER & SERROR 2008).

The transfer of the resistance genes to pathogenic species, for example between enterococci and staphylococci (TENOVER & McDONALD 2005), is a serious problem. Multidrug-resistant enterococci, particularly those which are vancomycin-resistant, are a major cause of concern of the medical community (KOLUMAN *et al.* 2009).

CASEWELL *et al.* (2003) reported that the withdrawal of antibiotic growth-promoters had increased veterinary use of therapeutic antibiotics, which are identical to those used in human medicine. This poses a potential hazard to human health in the form of antibiotic resistance in salmonellae, campylobacters, and zoonotic strains of *E. coli*.

The differences in antibiotic resistance patterns between enterococci recovered from different animal products may reflect the use of the approved antimicrobial agents in each food animal production class (HAYES *et al.* 2003). The scope of the present study was therefore to detect the presence of antibiotic-resistant enterococci from different raw foods of animal origin.

MATERIAL AND METHODS

Sampling. The specimens of raw pork, poultry, cows and ewe's milks, and traditional Slovak Bryndza cheese ($n = 260$) were analysed. The swine were processed in three different slaughterhouses under different sanitary and pre-slaughter conditions. The pork samples ($n = 75$) were obtained 24 h *post mortem*. Surface swabs of pork were taken from 25 cm² of the thigh (*musculus semimembranosus*). Conventional broiler chickens ($n = 53$) were processed in our private processing plant. For the microbiological analysis, the sampling of poultry was carried out 1 h *post mortem* (abdomen area of 25 cm²). The ewe's milk samples ($n = 61$) were collected by hand ($n = 24$) and machine ($n = 37$) milking, and came from several areas of Slovakia. The unpasteurised cow milk samples ($n = 32$) were collected from different farms. The samples were treated with Heeshen-preserving agent, then transferred to the laboratory in a cold box and

analysed within 24 h following the milking. Bryndza cheese ($n = 39$) was made by three different manufacturers and the samples were purchased in retail outlets.

Isolation and enumeration of enterococci. Each of the poultry and pork surface swabs was transferred to 10 ml 0.1% peptone water and homogenised by manual agitation. For the milk specimens, 10 ml of the sample were added to 90 ml of 0.1% peptone water. Similarly, 10 g of aseptically weighed Bryndza cheese sample was added to 90 ml of 0.1% peptone water and homogenised with Stomacher 400. The homogenate of each sample was serially diluted in 0.1% peptone water, and 1 ml of each dilution prepared was inoculated on Slanetz–Bartley agar (Biokar Diagnostic, Pantin Cedex, France). Enterococci were enumerated after 48 ± 2 h incubation at $37 \pm 1^\circ\text{C}$.

Species identification of *Enterococcus* spp. Typical colonies of enterococci were transferred to bile esculin azide agar (Biokar Diagnostic, Beauvais Cedex, France) and blood agar for species identification. Based on the positive growth (esculin hydrolysis and growth with hemolytic or nonhemolytic activity), the following tests were carried out for the presumptive identification of the isolates: the microscopic characteristic of the colonies (conformation, motility, cleanness of cultures), Gram staining, production of catalase and pyrolydonyl arylamidase (PYRAtest, Lachema, Brno, Czech Republic), and pigmentation. The Selected Gram-positive, catalase negative, and PYRAtest positive isolates were submitted to a growth test in the presence of 6.5% NaCl at pH 9.6. The isolates were identified at the species level using a biochemical EN-COCCUS test (Lachema, Brno, Czech Republic). The PCR method for the chosen species identification was performed using specific primers: *E. faecalis* – Forward 5'-ATCAAGTACAGTTAGTCTTTATTAG-3', reverse 5'-ACGAT-TCAAAGCTAACTGAATCAGT-3' and *E. faecium* – Forward 5'-TTGAGGCAGACCAGATTGACGG-3', reverse 5'-TATGACAGCGACTCGATTCC-3. One microliter of DNA (50 ng) was added to a mixture containing 2.5 µl of 10× PCR buffer, 0.5 µl each of 10mM deoxynucleoside triphosphate, 2.0 µl 25mM MgCl₂, 0.25 µl 5 U of Taq polymerase, and 0.5 µl of each 10 pmol primer (all Fermentas, St. Leon-Rot, Germany)). The thermal modes and cycles of the PCR assay were adjusted according to KARIYAMA *et al.* (2000). The isolates producing an amplicon band of the appropriate size

on agarose gel (2%) electrophoresis were considered positive for the species identification.

Antibiotic resistance. The antibiotic resistance of the isolates was determined by disc diffusion method according to the Clinical and Laboratory Standards Institute requirements (CLSI 1999). Inoculum was prepared from the overnight cultures incubated on Plate count agar (HiMedia Laboratories, Mumbai, India) at $37 \pm 1^\circ\text{C}$, and the suspension was adjusted to equal the 0.5 McFarland standard with Densi-La-Meter (Pliva Lachema, Brno, Czech Republic). Susceptibility to antimicrobial agents was tested using the following antimicrobial discs: Vancomycin (VAN) 30 μg per disc, Gentamicin (GEN) 10 μg /disc, Erythromycin (ERY) 15 μg /disc, Tetracycline (TET) 30 μg per disc, and Ampicillin (AMP) 10 μg /disc (Oxoid, Cambridge, UK). The isolates were classified as susceptible, intermediately resistant, or resistant according to the CLSI (1999) requirements.

RESULTS AND DISCUSSION

All samples were positive for enterococci. Lower levels of contamination were found in meat compared to milk or cheese. The bacterial counts for pork, poultry, milk, and Bryndza cheese are shown in Table 1.

No significant differences were found between pork coming from the three abattoirs ($P = 0.05721$). KNUDTSON and HARTMAN (1993) reported that pig carcasses from three different slaughtering plants contained mean counts of 10^4 – 10^8 enterococci per 100 cm^2 of the carcass surface. Higher enterococci counts (3.29 ± 0.35 log CFU/g) were reported by MAYR *et al.* (2003).

Similarly, there was no significant difference between the samples of ewe milk collected by hand or machine. In the INGHAM *et al.* (2000) study, the counts of enterococci in cow milk reached a comparable value (2.1–3.0 log CFU/ml). Higher counts of enterococci in cow milk (4.47 log CFU/ml) were reported by KAGKLI *et al.* (2007).

Of the 816 isolates, we identified 349 strains. The most common species were *E. faecalis* (49%) and *E. faecium* (29%) (Table 2). The microbiologically and biochemically positive strains not identified by EN-COCCUS test were designated as *Enterococcus* spp. In this study, *E. faecium* was the predominant species recovered from pork, which is supported by the findings of KNUDTSON and HARTMAN (1993) and FRANZ *et al.* (2003). *E. faecalis* was the predominant species in poultry, cow milk, and ewe milk. CITAC *et al.* (2006) reported similar results.

Traditional Slovak Bryndza cheese is prepared either from raw sheep milk, or as a mixture (1:1) of fermented sheep and cow cheeses made from pasteurised milk. Enterococci constitute a significant portion of its lactic acid bacteria (BELICOVÁ *et al.* 2007). Our results confirmed the prevalence of enterococci in Bryndza cheese; we observed equal numbers of *E. faecalis* and *E. faecium* in the Bryndza cheese samples. In a study by ORTIGOSA *et al.* (2008), *E. faecium* was the predominant species in cheeses made from pasteurised cow's and goat's milks. They also found *E. faecalis* and *E. faecium* species present at similar levels in the pasteurised ewe's milk cheeses.

Regardless of the origin, tetracycline and gentamicin resistance were the most frequent. The resistance to vancomycin, erythromycin, and ampicillin was detected primarily in poultry (Table 3).

The most common vancomycin-resistant species in poultry was *E. faecalis*. In contrast, the pork samples contained mainly vancomycin-resistant *E. faecium* (VRE) isolates. The most antibiotic resistant isolate from cow milk was *E. faecalis*.

GAMBAROTTO *et al.* (2001) found 100% VRE strains in poultry and 60% VRE strains in pork samples in France. LEMCKE and BÜLTE (2000) isolated 48% and 72.1% VRE from German pork and poultry. From the Dutch, French, and Hungarian poultry samples, they isolated 76.7%, 100%, and 100% VRE, respectively. KOLUMAN *et al.* (2009) found 8% VRE isolates in retail poultry samples. The results by HAYES *et al.* (2003) in the United States showed that the enterococci strains from poultry samples were not vancomycin-resistant.

Table 1. Count of enterococci in raw food samples (log CFU/ml)

	Meat		Milk		Bryndza cheese ($n = 39$)
	poultry ($n = 53$)	pork ($n = 75$)	cow ($n = 32$)	ewe ($n = 61$)	
Min	1.48	0.60	2.49	2.54	2.00
Max	5.79	6.48	4.48	6.04	7.80
\bar{x}	2.67 ± 0.7	2.00 ± 1.25	3.50 ± 0.18	4.82 ± 0.80	4.99 ± 1.65

Table 2. Occurrence of enterococci in raw foods of animal origin

<i>Enterococcus</i>	Meat			Milk		Bryndza cheese (<i>n</i> = 50)
	poultry (<i>n</i> = 88)	pork (<i>n</i> = 50)		cow (<i>n</i> = 101)	ewe (<i>n</i> = 60)	
<i>E. faecalis</i>	50 (57%)	5 (10%)		52 (51.5%)	42 (70%)	23 (46%)
<i>E. faecium</i>	12 (14%)	36 (72%)		11 (11%)	18 (30%)	23 (46%)
<i>E. casseliflavus</i>	6 (7%)			1 (1%)		
<i>E. gallinarum</i>	1 (1%)					
<i>E. durans/hirae</i>	6 (7%)			12 (12%)		
<i>E. mundtii</i>		4 (8%)		2 (2%)		
<i>E. spp.</i>	13 (14%)	5 (10%)		23 (22.5%)		4 (8%)

Tetracycline and erythromycin resistance in the poultry and pork samples is likely related to the wide use of these classes of antibiotics in husbandry activities. Our findings agree with those by ŠUSTÁČKOVÁ *et al.* (2004), who reported that the resistance to tetracycline and erythromycin demonstrates a link between the administration of antibiotics to farm animals and the occurrence of resistance in the bacteria isolated from raw poultry and pork.

We found that the enterococci isolated from pork and poultry meat were resistant to at least two antibiotics. It is interesting to note that vancomycin resistance in the pork and poultry samples was found only in combination with either four (28%) or all five (14%) of the tested antibiotics.

The most prevalent multi-resistance was observed in the poultry samples. BUSANI *et al.* (2004) isolated 80 strains of VRE from raw poultry and 30 isolates from raw pork (*n* = 235 isolates), and reported high levels of tetracycline and erythromycin resistance in all strains. More than 10% of these *E. faecium* isolates were multidrug resistant to 5 antibiotics. JOHNSTON and JAYKUS (2004) found 61% of *E. faecium* and 11% of *E. faecalis* isolates to show multidrug resistance to 17 different antibiotics (including vancomycin). GARCIA-

MIGURA *et al.* (2005) recorded 11% multi-resistant *E. faecium*, including vancomycin-, erythromycin-, and tetracycline-resistant isolates coming from English conventional poultry farms. MIRANDA *et al.* (2007) showed that the enterococci isolates from organic poultry were less resistant to ampicillin ($P = 0.0067$), erythromycin ($P = 0.0028$), and vancomycin ($P = 0.0241$) than the isolates from conventional poultry. Multidrug-resistant strains were found in both organic and conventional samples, but multidrug resistance was significantly higher in the strains from conventional poultry.

In our study, only 4% isolates from cow milk were resistant to erythromycin. Considerably higher levels of enterococci resistance to vancomycin (37%), tetracycline (45%), gentamicin (68%), erythromycin (86%), and ampicillin (42%) were reported by CİTAC *et al.* (2006).

Enterococci isolates from Bryndza cheese (86%) and ewe's milk (68%) had intermediate resistance or were susceptible to low levels of gentamicin (10 µg/disk). Enterococci are generally regarded as intrinsically resistant to low levels of gentamicin. LOPES *et al.* (2003) revealed that 30% of enterococci isolated from Portuguese dairy products were not intrinsically resistant to gentamicin. Gentamicin

Table 3. Percentage of antibiotic resistant (R), intermediate resistant (I) and susceptible (S) enterococci

Antibiotics	Meat						Milk						Bryndza cheese (<i>n</i> = 50)		
	poultry (<i>n</i> = 88)			pork (<i>n</i> = 50)			cow (<i>n</i> = 101)			ewe (<i>n</i> = 60)			R	I	S
	R	I	S	R	I	S	R	I	S	R	I	S			
Vancomycin	19	0	81	14	0	86	10	0	90	0	0	100	0	0	100
Erythromycin	44	17	39	10	64	26	4	13	83	2	13	85	6	34	60
Gentamicin	56	23	21	24	66	10	27	27	46	32	36	32	14	24	62
Tetracycline	91	8	1	34	54	12	19	39	42	32	2	66	0	16	84
Ampicillin	26	55	19	18	70	12	10	80	10	16	77	7	14	76	10

resistance should be monitored in dairy enterococci to prevent further development of resistance; currently, this does not seem to pose any problem. The low level of antibiotic resistance in the samples of ewe's milk is likely related to the geographical location of sampling. The ewe's milk was obtained from farms in the mountains of Slovakia, far from cities. There is no official data on the use of antibiotics, but it is not common for farmers in that region to add antibiotics to the animals' feed. BELICOVÁ *et al.* (2007) isolated 36% of *E. faecium* strains and 22% of *E. faecalis* strains resistant to erythromycin. They found that all enterococcal isolates from Bryndza cheese were susceptible to ampicillin, gentamicin, and vancomycin. ORTIGOSA *et al.* (2008) found only three VRE strains ($n = 333$) in goat's milk cheese made from pasteurised milk in Spain. They did not find VRE isolates in ewe's milk cheeses.

Our results suggest that raw products of animal origin are possible reservoirs of multi-antibiotic resistant enterococci in the food chain. Due to the number of isolates resistant to common antibiotics, it is necessary to reevaluate the use of therapeutic antibiotics in stock farms (especially poultry farms) at both the regional and international levels. Furthermore, the data on the prevalence and types of antibiotic resistance in *Enterococcus* species isolated from raw animal products should be continuously monitored. This data would serve as an indirect control of antibiotic use in animal husbandry. In combination with proper hygiene and manufacturing aspects, a careful study and use of antibiotics in farm animals may prevent further proliferation of antibiotic resistant bacteria in our foods.

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