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Full Length Research Paper

Occurrence and antibiotic resistance of enterococci in ready-to-eat food of animal origin

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The aim of this work was to determine which *Enterococcus* species could be isolated from food of animal origin and the significance of these enterococcal isolates according to their antibiotic resistance profiles. Ninety-two *Enterococcus* strains were isolated from retail food of animal origin in Olsztyn, Poland. They were classified as *Enterococcus faecalis* (44 strains), *Enterococcus faecium* (32 strains) or *Enterococcus* spp. (16 strains) by phenotypic method and confirmed by polymerase chain reaction (PCR). Susceptibility of these enterococcal strains to 15 selected antibiotics (ampicillin, penicillin G, gentamicin, streptomycin, vancomycin, teicoplanin, norfloxacin, levofloxacin, ciprofloxacin, tetracycline, tigecycline, rifampicin, nitrofurantoin, linezolid, fosfomycin, chloramphenicol and quinupristin/dalfopristin) was determined using the disk diffusion method according to the recommendations of Clinical and Laboratory Standards Institute (CLSI 2010, formerly NCCLS). All the investigated strains were sensitive to levofloxacin, ciprofloxacin and vancomycin. Antimicrobial susceptibility tests showed resistance phenotypes to a range of antibiotics widely administrated in humans such as tetracycline, nitrofurantoin and quinupristin/dalfopristin.

Key words: *Enterococcus* spp., antibiotic resistance, food, animal origin, food safety.

INTRODUCTION

The presence of antimicrobial resistant bacteria in foodstuffs of animal origin is becoming a matter of concern as these bacteria can be transmitted to humans through food supply. Therefore, protection of food supplies includes microbiological quality and safety of commodities available for public consumption. While such concerns most frequently address pathogenic microorganisms, that present immediate risks to human health, there is growing interest in commensal components of the microbiota associated with food (Hayes et al., 2003; Martin et al., 2005; Perez-Pulido et al., 2006; Ruiz-Moyano et al., 2009).

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Enterococcus ssp. constitutes part of the natural gut microflora in mammals. For many years, they have been considered harmless to man. However, *Enterococcus* spp. bacteria are important nosocomial pathogens with a remarkable capacity of expressing resistance to several antimicrobial agents. In the last decade, enterococci have become the second most frequently reported cause of surgical wound infections or nosocomial urinary tract infections and the third most frequently reported cause of bacteremia (Shaked et al., 2006). Moreover, treatment of enterococcal infections has become more difficult due to the increasing prevalence of antibiotic resistant enterococci (Ben-Omar et al., 2004).

The increasing incidence of antibiotic resistant *Enterococcus* spp. is due to the increase use of antibiotics both in the human health care system and in agriculture as animal growth promoters (Aarestrup, 2000;

Mannu et al., 2003). The emergence of resistance to antimicrobial agents during therapy threatens the successful treatment of infections and risks the further spread of resistant organisms to other patients and to the community. In fact, there is a correlation between antibiotic use and subsequent increase in resistance (Butaye et al., 2000).

Enterococci are widespread in a variety of food products (Barbosa et al., 2009; Belicova et al., 2007; Komprda et al., 2010; Giard et al., 2001). Researchers have suggested that enterococci can transfer antibiotic resistance genes through food. It has been shown that the same antibiotic resistance genes were found in bacteria isolated from unpasteurized cheese and in bacteria isolated from human patients (Mannu et al., 2003; Ogier et al., 2008). Moreover, most of the human clinical *Enterococcus* spp. isolates are species that normally colonize humans (Shaked et al., 2006). These observations support the hypothesis that *Enterococcus* spp. found on food either directly colonize humans or exchange antibiotic resistance genes with species that colonize humans. However, there is still no demonstrated correlation between ingestion of food products containing enterococci and infections.

Studies of microorganisms present in many foods have indicated that enterococci play a dual role in some kind of food. On one hand, enterococci may be virulent to humans and express antibiotic-resistance genes. On the other hand, enterococci play an important role in the ripening of cheeses, contributing to their typical taste and flavor through proteolysis, lipolysis, and citrate metabolism (Manolopoulou et al., 2003). Enterococci are also present in other fermented foods, such as sausages (Franz et al., 2003; Giraffa, 2002) and olives (Ben-Omar et al. 2004). The contribution of enterococci to the organoleptic properties of fermented food products and their ability to produce bacteriocins active against spoilage or pathogenic bacteria, in particular *Listeria*, are important characteristics for their applications in food technology (Foulquié-Moreno et al., 2006; Franz et al., 2003; Sarantinopoulous et al., 2002; Valenzuela et al., 2009). However, the selection of enterococcal species for use in food processing is a difficult task, due to their potential risk to human health.

Enterococcus can survive in different environmental conditions, such as temperature ranging from 10 to 45°C, pH ranging from 2.7 to 10, or NaCl concentration up 6.5%. They can also survive heating at a temperature of 63.5°C for 30 min. The thermal resistance of enterococci explains their presence in cheeses produced from pasteurized milk. Enterococci may colonize raw foods (e.g. milk and meat) and multiply in these materials during fermentation because of their ability to survive to adverse environmental conditions such extreme pH, temperatures and salinity (Gardin et al. 2001).The presence of *Enterococcus* spp. in the gastrointestinal tract of animals may lead to contamination of meat at the time of slaughtering. Besides raw meats, they are also associated with processed meats. Heating of processed meats during production may confer a selective advantage to enterococci because these bacteria are among the most thermotolerant of the non-sporulating bacteria. After surviving the heat-processing step, enterococci have been implicated in spoilage of cured meat products, such as canned hams and chub-packed luncheon meats (Magnus et al., 1986, 1988). This is especially true where recontamination with competing bacteria is prevented, when products are heated after packaging in cans or in impermeable plastic films. The heat resistance of *Enterococcus* spp. in these products is influenced by components such as salt, nitrite, and meat tissue (Franz et al., 1999). In fermented sausages, viable enterococci (especially *E. faecium*) can be found in relatively high numbers. Contaminated poultry, pork and beef can contain 10^2 to 10^4 cfu g^{-1} of viable enterococci (Teuber et al., 1996). During fermentation, the contaminating enterococci may survive and multiply. These bacteria withstand normal conditions of food production and become an important part of ready-to-eat products (Ben-Omar et al., 2004; Bhardwaj et al., 2008; Majhenic et al., 2005). In German and Italian fermented sausages, Marchesino et al. (1992) reported concentrations of *Enterococcus* spp. from 10^3 to 10^5 cfu g^{-1} at the end of the ripening process.

The aim of the present work was to characterize *Enterococcus* spp. recovered from selected ready-to-eat food of animal origin, focusing on prevalence, phenotypic characteristics and resistance to antibiotics commonly used in human therapies. Our results contribute to evaluation of the microbiological risks of foods intended for human consumption, especially those from animal sources.

MATERIALS AND METHODS

Samples collection

Samples were obtained in a retail market in Olsztyn, Poland. A total of 122 samples were collected: 75 dairy products (cheeses) and 37 ready-to-eat meat products (sausages, hams, etc.). After transfer to the laboratory, food samples (10 g) were homogenized in 90 ml buffered peptone water (Merck, Germany) and incubated at 37°C for 16 h. Slanetz-Bartley agar was used for initial isolation of enterococci. Phenotypic criteria were used for strain isolation and colony picking of presumed enterococcal strains.

Bacteria isolates

Presumptive identification of isolates was carried out with the following tests: observation of colony characteristics and cell morphology, Gram staining, catalase and oxidase production, growth at 10 and 45°C, growth in the presence of 6.5% NaCl, growth at pH 9.6 and finally growth and esculin hydrolysis on bile-esculin agar (Merck, Germany). The genus *Enterococcus* was confirmed using the catalase and pyrrolidonyl arylamidase tests (Oxoid, United Kingdom). The species was determined by

examining haemolysis behavior, pigment production, motility and utilization of carbon sources (ribose, arabinose, raffinose, mannitol, melibiose, lactose, sucrose, arginine, sorbitol, trehalose). Isolates were identified by phenotypic methods (Alves et al., 2004) as *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus* spp. PCR assay was used to confirm species identification. Analysis was performed using specific primers: *E. faecalis* – Forward 5'-ATCAAGTACAGTTAGTCTTTATTAG-3', reverse 5' ACGATTCAAAGCTAACTGAATCAGT- 3' and *E. faecium* – Forward 5'- TTGAGGCAGACCAGATTGACGG- 3', reverse 5'- TATGACAGCGACTCCGATTCC- 3. 1 µl of DNA (50 ng) was added to a mixture containing 2.5 µl of 10× PCR buffer, 0.5 µl each of 10 mM deoxynucleoside triphosphate, 2.0 µl 25 mM $MgCl_2$, 0.25 µl 5 U of Taq polymerase, and 0.5 µl of each 10 pmol primer (Fermentas, 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 (1.5%) electrophoresis were considered positive for the species identification.

Antibiotics resistance tests

Antimicrobial susceptibility was determined using the disk diffusion method. Fifteen antibiotics commonly used in the treatment of clinical infection or in agricultural procedures were tested. Their names and respective disk content were as follows: ampicillin 10 µg (AMP), penicillin G 10 IU (P), gentamicin 120 µg (CN), streptomycin 300 µg (S), vancomycin 30 µg (VA), teicoplanin 30 µg (TEC), norfloxacin 10 µg (NOR), levofloxacin 5 µg (LEV), ciprofloxacin 5 µg (CIP), tetracycline 30 µg (TE), tigecycline 15 µg (TGC), rifampicin 5 µg (RD), nitrofurantoin 300 µg (F), linezolid 30 µg (LZD), fosfomycin 200 µg (FOT), chloramphenicol 30 µg (CL) and quinupristin/dalfopristin (for *E. faecium*) 15 µg (QD). Cartridges with commercially prepared paper disks containing the appropriate antibiotic dosage were purchased from Oxoid (United Kingdom). Disk diffusion assays were performed on Mueller-Hinton Agar (Oxoid, United Kingdom). Overnight culture of enterococcal isolates was spotted on the surface of Mueller-Hinton agar. Antibiotic discs were then placed on the plates and incubated at 37°C. Zone diameters were recorded after 24 h incubation. Strains were classified as resistant and susceptible according to the criteria from CLSI (2010). *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality control organisms.

RESULTS

Among the animal food samples tested, a total of 92 isolates from 122 different samples were identified as presumptive enterococci. 82.1% of the food samples analyzed were positive for enterococci, with higher prevalence in cheeses (89.9%) compared to meat products (69.8%). Sixty-two strains were isolated from dairy products (cheeses) and thirty isolates were obtained from ready-to-eat meat products. Of these, 44 isolates were identified as the *E. faecium*, 32 as *E. faecalis* and 16 strains as *Enterococcus* spp. (Table1).

All enterococcal food isolates (n = 92) were examined for their susceptibility to 15 antibiotics. The data obtained from the disc diffusion testing are summarized in Table 2. All of the strains were sensitive to levofloxacin, ciprofloxacin and vancomycin. The overall percentages of antimicrobial resistant isolates were: 28.3% to tetracycline, 21.7% to fosfomycin, 20.7% to tigecycline, 19.6% to teicoplanin, 8.7% to streptomycin, 7.6% to gentamycin and norfloxacin, 6.5% to rifampicin, 5.4% to quinupristin/dalfopristin, 4.3% to nitrofurantoin, 3.3% to chloramphenicol and 1.1% to ampicillin, penicillin and linezolid. Resistance to tetracycline was more frequently observed among *E. faecalis* (43.2%) than *E. faecium* (7.0%).

There were minimal differences in antimicrobial resistance profiles between cheese and meat *Enterococcus* isolates (Figures 1 and 2). The greatest differences in antibiotic resistance incidence between cheese and meat isolates, respectively, the incidence of antibiotic resistance between cheeses and meat *Enterococcus* isolates were found for tetracycline (14.7 vs. 20.0%), tigecycline (9.4 vs. 14.7%), fosfomycin (10.7 vs. 16.0%) and rifampicin (1.3 vs. 5.3%). Only one *E. faecalis* strain isolated from cheese was resistant to both ampicillin (AMPR) and penicillin pattern.

The occurrence of high-level resistance to aminoglycosides (HLR-A) in the enterococcal strains is listed according to their food source in Table 3. HLR-A was detected in 18.5% of the isolates (13.0% from cheeses and 5.4% from meat products). These figures include high-level resistance to the two aminoglycoside antibiotics that were tested, gentamycin and streptomycin. High-level resistance to gentamycin (HLR-GE) was detected in 7.6% of the isolates. The frequency of HLR-GE was higher among isolates from cheeses than among those recovered from meat products. High level resistance to streptomycin (HLR-S) was also more frequently detected from cheese isolates (5.4%) than from meat isolates (3.3%). Isolates resistant to both gentamycin and streptomycin were found only among the *E. faecalis* strains isolated from cheese.

Resistance to multiple antibiotics was observed. Of the 92 isolates tested, 27 isolates (29.34%) were resistant to two or more antibiotics. The most frequent multiple-resistance phenotypes observed were combined tetracycline and fosfomycin resistance – 10 isolates (10.87%), or combined tetracycline, fosfomycin and tigecycline resistance – 7 isolates (7.61%). When the distribution of the antibiotic resistance according to the species was consider, it was found that *E. faecalis* possessed a higher number of resistances than *E. faecium* (Table 2).

DISCUSSION

Most of the previous studies on resistance have concentrated on enterococci isolated from clinical samples and unprocessed food samples. The present study investigated the prevalence of resistant strains isolated from retailed ready-to-eat food. The most prevalent species in our study identified in food of animal origin was *E. faecalis* (47.8% of all isolates). Similar observations were recently reported by other European

Table 1. Distribution of enterococci isolated from food of animal origin in Olsztyn, Poland.

Species	Number (%) of isolates					
	Cheese (n=75)		Meat products (n=37)		Total products	
E.faecalis	32	(51,6)	12	(40,0)	44	(47,8)
E.faecium	19	(31,0)	13	(43,3)	32	(34,8)
***Enterococcus* spp.**	11	(17,8)	5	(16,7)	16	(17,4)
Total	62	(100)	30	(100)	92	(100)

Abbrevations: **n**-number of isolates.

Table 2. Antibiotic resistance of 92 enterococci isolated from ready-to-eat food of animal origin in Poland.

Antimicrobial agent			*E. faecalis* (n=44)		*E. faecium* (n=32)		*Enterococcus* spp. (n=16)	
Group	Symbol and concentration		R(%)	S(%)	R(%)	S(%)	R(%)	S(%)
β-lactams	AMP	10 µg	2,3	97,7	0	100	0	100
	P	10 IU	2,3	97,7	0	100	0	0
aminoglycosides	CN	120 µg	9,1	90,9	25	75	6,3	93,7
	S	300µg	9,1	90,9	9,4	90,6	6,3	93,7
peptides	VA	30 µg	0	100	0	100	0	100
	TEC	30 µg	22,7	77,3	15,6	84,4	18,8	81,3
quinolones	NOR	10 µg	2,3	97,7	12,5	87,5	12,5	87,5
	LEV	5 µg	0	100	0	100	0	100
	CIP	5 µg	0	100	0	100	0	100
tetracyclines	TE	30 µg	43,2	61,4	21,9	78,1	0	100
	TGC	15 µg	27,3	72,7	31,3	68,8	12,5	87,5
ansamicins	RD	5 µg	6,8	93,2	6,3	93,8	6,3	93,7
nitrofurans	F	300 µg	4,5	95,5	3,1	96,9	6,3	93,7
oxazolidinones	LZD	30 µg	2,3	97,7	0	100	0	100
fosfomycins	FOT	200 µg	34,1	65,9	28,1	71,9	12,5	87,5
amfenicols	C	30 µg	4,5	95,5	0	100	6,3	93,7
streptogramins	QD	15µg	-	-	34,4	65,6	31,3	68,7

Abbrevations: **R**-resistant, **S**-sensitive, **n**-number of isolates, **%**-percent of isolates resistant/sensitive, **AMP**-ampicillin, **P**-penicillin, **CN**-gentamicin, **S**- streptomycin, **VA**-vancomycin, **TEC**-teicoplanin, **NOR**-norfloxacin, **LEV**-levofloxacin, **CIP**-ciprofloxacin,TE- tetracycline, TGC-tigecycline, RD-rifampicin,F- nitrofurantoin, LZD-linezolid, FOT-fosfomycin, C-chloramphenicol, QD-quinupristin/dalfopristin (for *E. faecium*).

researchers. Andrighetto et al. (2001) found that most strains isolated from Italian cheeses were *E. faecalis*. Likewise, *E. faecalis* was the dominant species isolated from milk or Irish Cheddar-type cheese (Gelsomino et al., 2001). Klein (2003) concluded that *E. faecalis* is found more often in food and faeces from animal origin than *E. faecium*, possibly because these foods (especially cheeses) are manipulated by hands and thereby contaminated during the manufacturing process.

Enterococcus spp. are intrinsically resistant to a large wide of antimicrobials of therapeutic use. They also have the ability to acquire and transfer genetic resistance markers, a process mediated by genes present in plasmids or transposons that facilitate their dissemination (Martin et al., 2005). Enterococci are considered intrinsically resistance to β-lactams (Kak and Chow, 2002). Our results do not agree with this generalization since only one of our isolates was resistant to ampicillin and penicillin. Similar results were obtained by other authors (McGowan-Spicer et al., 2008; Valenzuela et al., 2008).

We observed resistance to several other antibiotics,

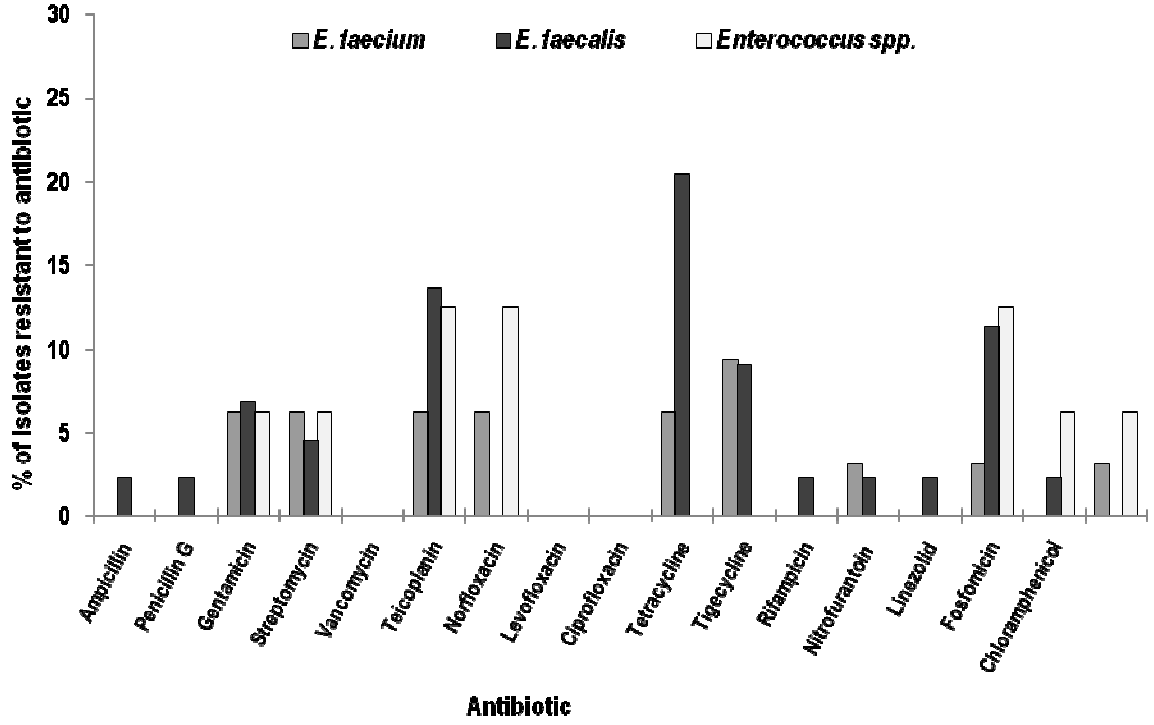

Figure 1. Distribution of antibiotic resistant *E. faecium*, *E.faecalis* and *Enterococcus* spp. strains isolated from cheeses.

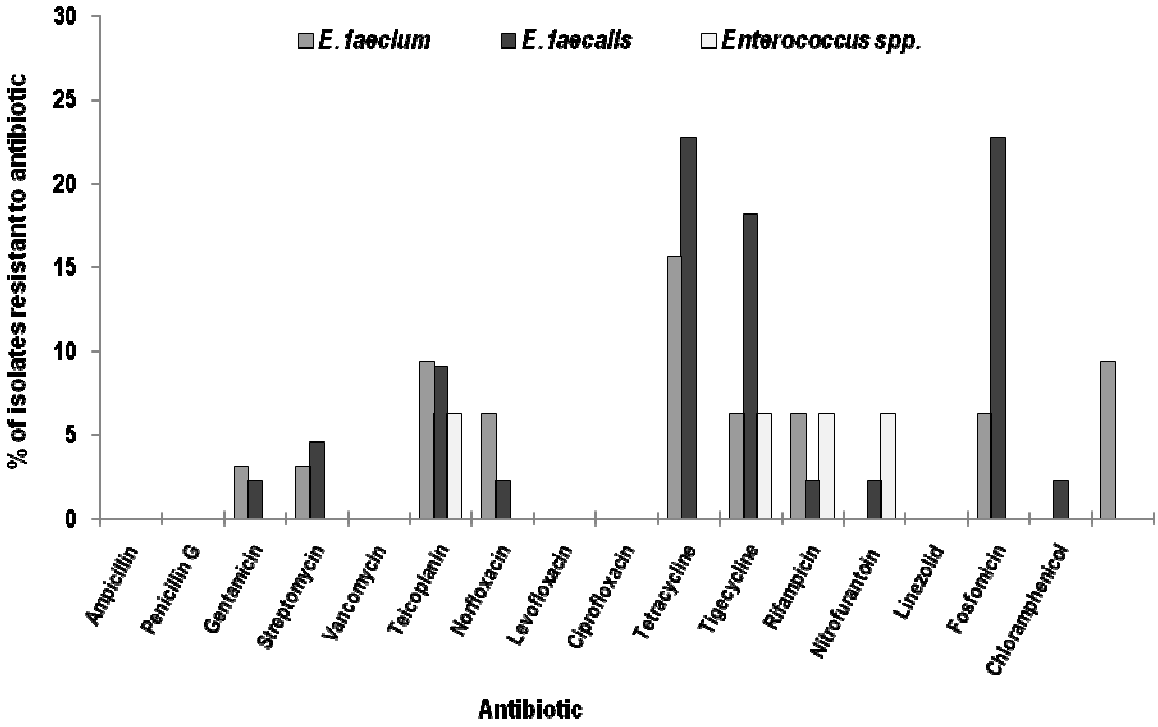

Figure 2. Distribution of antibiotic resistant *E. faecium*, *E.faecalis* and *Enterococcus* spp. strains isolated from ready-to-eat meat products.

Table 3. Occurrence of high level aminoglycoside resistance (HLR-A) among enterococci strain isolated from food of animal origin.

Species	Number (%) of resistance strain with phenotype HLR-A							
	Source	HLR-GE		HLR-ST		HLR-GE/ST	Total	
E.faecalis	cheese	3	(6,8)	2	(4,5)	1 (2,3)	6	(13,6)
(n=44)	meat	1	(2,3)	2	(4,5)	-	3	(6,8)
E.faecium	cheese	2	(6,3)	2	(6,3)	-	4	(12,5)
(n=32)	meat	1	(3,1)	1	(3,1)	-	2	(6,3)
***Enterococcus* spp.**	cheese	-		1	(6,3)	1 (6,3)	2	(12,5)
(n=16)	meat	-		-		-	-	
Total (n=92)		7	(7,6)	19	(7,1)	1 (0,4)	17	(18,5)

Abbrevations: **HLR-A** - high-level resistance to aminoglycosides (gentamycin and streptomycin); **HLR-GE** - high-level resistance to gentamycin; **HLR-ST** - high-level resistance to streptomycin; **HLR-GE/ST** - high-level resistance to both gentamycin and streptomycin.

including the aminoglycosides: streptomycin and gentamicin. Since gentamycin and streptomycin are not registered for use in animals in Poland, the prevalence of resistance was unexpected. Aminoglycosides are considered antimicrobials of choice to treat enterococcal infections (in combination with glycopeptides and/or β-lactams), so the possibility that resistance to these antibiotics could disseminate through the food chain is alarming. Our results provide a clear warning about high-level resistance to the aminoglycosides streptomycin and gentamicin among enterococci from food of animal origin, which has not previously been observed in Europe (Hayes et al., 2004; Busani et al., 2004; Junco et al., 2005; De Jong et al., 2009).

Tetracyclines are broad-spectrum antibiotics frequently used in the treatment of humans and animals infections (Chopra and Roberts, 2001). The high prevalence of resistance to tetracycline is determined in this study (28.3% of isolated strains) may have resulted from the frequent use of this antibiotic in veterinary medicine in Poland. Tetracycline resistance has been previously reported at high frequency among enterococcal isolates from different sources (Aarestrup, 2000; Butaye et al., 2000; Pavia et al., 2000). A high incidence of tetracycline resistance (68%) was recently found among enterococci isolated from raw milk cheeses, where it was associated with chloramphenicol and erythromycin resistance (Templer and Baumgartner, 2007). In our study of the tetracycline resistant enterococci, 75% also showed resistance to tigecycline and 60% were also resistant to fosfomycin.

It is worrying that enterococci resistant to quinupristin/dalfopristin (Q/D) were observed, because these antibiotics are considered to be the last line of treatment against vancomycin-resistant *Enterococcus* spp. Streptogramin resistance in enterococci could be an example of cross-resistance. Synercid®, a semisynthetic streptogramin-derived antibiotic containing dalfopristin and quinupristin (Kehoe et al., 2003). Synercid® (Q/D) was approved for the treatment of vancomycin-resistant *E. faecium* (Hancock, 2005). Virginiamycin, an analogue of Synercid, has been used in animal production for over two decades and it is therefore possible that Q/D-resistant *E. faecium* have already emerged in the animal population, which could be disseminated among humans and thus impact treatment (Hancock, 2005; Hershberger et al., 2004).

The use of chloramphenicol for treatment of human infections is low. In animal husbandry, chloramphenicol use was banned in Europe in 1994. Our results showed that almost all the isolates (96.7%) were sensitive to chloramphenicol. Other authors (Riboldi et al., 2009) also reported low incidence of chloramphenicol resistant enterococci isolated from food.

Our results revealed that both *E. faecium* and *E. faecalis* are resistant to several antimicrobials used in human medicine such as tetracycline, nitrofurantoin and teicoplanin. Resistance to nitrofurantoin, an antibiotic used for treatment of genitourinary tract infections, was observed among cheese isolates. *Enterococcus* spp. strains were also found resistant to norfloxacin, a drug used to treat urinary tract infections.

The observation of susceptibility results of strains isolated from cheeses (Figure 1) was consistent with previous results from Franz et al. (2001). They studied 47 *E. faecalis* strains, isolated mostly from cheeses, that were all susceptible to vancomycin, but mostly resistant to chloramphenicol, streptomycin, tetracycline, erythromycin, ciprofloxacin, gentamicin, penicillin and ampicillin. The absence of vancomycin resistant enterococci in our study is consistent with previous observations (Hayes et al., 2003; Olawale et al., 2010). In contrast, vancomycin resistant enterococci are frequently isolated from raw meat products (Busani et al., 2004; Pavia et al., 2000) from European countries as a result of selection of resistant populations by the use of the glycopeptide avoparcin in food animal production environments (Aarestrup, 2000; Mannu et al., 2003).

The high prevalence of antibiotic resistance observed in our study suggests that bacteria in food of animal origin can be a significant reservoir of antibiotic-resistant bacteria. Similar conclusions have been suggested from

previous studies (De Young et al., 2009; Gifaffa, 2002; Franz et al., 2003; Hammerum et al., 2004). The emergence of *Enterococcus* sp. expressing antimicrobial resistance and its potential spread in food suggest a situation of public health risk for the community. Effective control strategies are required to reduce contamination of foodstuffs by these microorganisms to prevent potential spread of antibiotic resistance.

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