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Prevalence and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat

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ABSTRACT

Food specimens were analyzed in order to research *Enterococcus* spp.: 636 samples of raw meat (227 beef, 238 poultry, and 171 pork), 278 samples of cheese (110 fresh soft cheese and 168 mozzarella cheese), 214 samples of ready-to-eat salads, and 187 samples of ham. 312 strains of *Enterococcus* spp samples were isolated, then identified and submitted to susceptibility tests against 11 antimicrobial agents. The predominant species were *Enterococcus faecalis* in raw meat and *Enterococcus faecium* in retail products. Low percentages of microorganisms were resistant to vancomycin (3.53%), teicoplanin (2.24%), linezolid (0.32%), and amoxicillin in combination with clavulanic acid (0.32%). A high percentage of resistance was noted in *E. faecalis* at high level gentamicin (21.9%) and tetracycline (60.6%). In general, strains of *E. faecalis* were more resistant than *E. faecium*.

Enterococci should be considered not only potential pathogens, but also a reservoir of genes encoding antibiotic resistance which can be transferred to other microorganisms. Continuous monitoring of their incidence and emerging resistance is important in order to identify foods which potentially represent a real risk to the population, and to ensure effective treatment of human enterococcal infections.

1. Introduction

Enterococci are ubiquitous bacteria in the normal intestinal microbiota of both humans and animals. They reach environments, such as soil, receiving fertilizers of animal origin and urban sewage, as a consequence of faecal contamination. Through these vehicles enterococci may reach and contaminate water and vegetables, and from there they may invade domestic and wild animal intestinal tracts. Therefore, enterococci may be present in vegetables, raw meat and cheese, and, due to cross contamination throughout all production phases, in every type of food, causing gastroenteritis in immunocompromised people (Giraffa, 2002).

In Germany, Peters et al. (2003) reported a presence of 72.0% *Enterococcus faecalis*, 13% *Enterococcus faecium*, and 6% *Enterococcus durans* and *Enterococcus hirae* in 155 samples of sausage, ham, minced meat, and cheese. In Italy, Busani et al. (2004) detected strong presence of *Enterococcus* spp. in poultry and pork, with a high prevalence of *E. faecium* followed by *E. faecalis*, *E. durans*, and *E. hirae*. In the USA, McGowan et al. (2006) found high percentages of poultry (95.4%), beef (72.7%), and pork (68.2%) samples positive for *Enterococcus* spp., and the most frequently identified species was *E. faecalis*. Some authors, such as Andrighetto et al. (2001), Serio et al. (2007), and Gomes et al. (2008), found that enterococci were frequently present in cheese as well.

As enterococci in food are not always due to faecal contamination, the legislation in force (Commission Regulation, 2007) sets no limit for enterococcal presence in food. In fact, in some kinds of food, such as cheese and fermented meats, enterococci are added during the production process, both to extend their shelf life and to improve their organoleptic properties (Centeno et al., 1996. Cocolin et al., 2007). Some strains of *E. faecalis* and *E. faecium* are used in "food technology" because of their ability to produce bacteriocins inhibiting multiplication of other pathogenic bacteria (such as *Listeria monocytogenes*) (Izquierdo et al., 2009), and to act as a starter in fermented products (Settanni and Moschetti, 2010). Using enterococci as starters and probiotics (Gaggìa et al., 2010) is a disputed issue given the increasing incidence of human enterococcal diseases and multi-resistant enterococcal strains. *Enterococcus* spp. is able to transfer antibiotic resistance genes to its own species, to other pathogens such as *Staphylococcus aureus* and *Listeria* spp. (Charpentier and Courvalin, 1999), and to non pathogenic bacteria, in human or animal intestinal tract, in the environment, or even in food (Courvalin, 1994; Walsh et al., 2001; Sparo et al., 2011), thus contributing to the dissemination and persistence of antimicrobial

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Table 1
Prevalence of *Enterococcus* species in various products.

Type of products	No. of samples	No. of positive samples (%)	Strains	*E. faecium* no (%)	*E. faecalis* no (%)	*E. durans* no (%)	*E. gallinarum* no (%)	*E. avium* no (%)
Raw meat								
Beef	227	70 (30.8)	70	25 (35.7)	31 (44.3)	11 (15.7)	0 (0)	3 (4.29)
Poultry	238	68 (28.6)	68	25 (36.7)	30 (44.1)	6 (8.82)	2 (2.94)	5 (7.35)
Pork (tot)	171	73 (42.7)	73	25 (34.2)	39 (53.4)	5 (6.85)	0 (0)	4 (5.48)
Pork (whole meat)	79	35 (44.3)	35	11 (31.4)	18 (51.4)	3 (8.57)	0 (0)	3 (8.57)
Pork (sausage)	92	38 (41.3)	38	14 (36.8)	21 (55.3)	2 (5.26)	0 (0)	1 (2.63)
Total	636	211(33.2)	211	75 (35.5)	100 (47.4)	22 (10.4)	2 (0.95)	12 (5.69)
Fisher exact test $p =$	0.008		0.967	0.632	0.388	0.221	0.707	
Retail products								
Fresh soft cheese	110	38 (34.5)	38	24 (63.1)	9 (23.7)	1 (2.63)	0 (0)	4 (10.5)
Mozzarella cheese	168	37 (22.0)	37	23 (62.2)	2 (5.40)	12 (32.4)	0 (0)	0 (0)
Ready salads	214	8 (3.74)	9	6 (6.67)	1 (1.11)	1 (1.11)	1 (1.11)	0 (0)
Ham	187	17 (9.09)	17	12 (70.6)	2 (11.8)	2 (11.8)	1 (5.89)	0 (0)
Total	679	100(14.7)	101	65 (64.3)	14 (13.9)	16 (15.8)	2 (1.98)	4 (3.96)
Fisher exact test $p =$	<0.0005		0.967	0.137	0.003	0.064	0.127	
Total	1315	311 (23.6)	312	140 (44.9)	114 (36.5)	38 (12.2)	4 (1.28)	16 (5.13)

resistance (Pesavento et al., 2010). In fact, *Enterococcus* species show the same pattern as other bacteria observed worldwide in increasing the frequency of antibiotic resistant isolates, especially in nosocomial strains (EARS, 2009; Deshpande et al., 2007).

Antibiotic resistance, and in particular multiresistance, is a dramatic public health problem since it may cause the failure of therapeutic treatment in case of enterococcal infections, especially in immunocompromised individuals, evolving into severe urinary tract diseases, bacteremias and endocarditis (Kayser, 2003). In the case of enterococcal infection, first-choice antibiotics are usually β-lactams and aminoglycosides. Second-choice antibiotics are glycopeptides and linezolid, against which enterococci are going to show high resistance level, thus causing an increased mortality rate of up to 83% (El Khoury and Fishman, 2003). Moreover, enterococci are naturally resistant to cephalosporins, low level aminoglycosides, polymixins, lincomycin, clindamycin, and often quinolones, and can acquire resistance to macrolides, tetracyclines, trimethoprim/sulfamethoxazole, chloramphenicol, and ampicillin (Barbosa et al., 2009). Over the last few decades, enterococci resistance to β-lactams, glycopeptides, and aminoglycosides (Grayson et al., 1991; Oster et al., 1990), as well as to linezolid (Deshpande et al., 2007; Scheetz et al., 2008), has been increasing.

To reduce the occurrence of vancomycin-resistant enterococci (VRE), avoparcin, a veterinary antibiotic used in poultry, has been banned in all European Union countries since 1995, and in fact the VRE incidence is slowly decreasing in some European countries (EFSA, 2011).

While in the past the antibiotic resistance occurrence mechanisms were studied focusing on pathogens, nowadays studies mainly consider the horizontal genetic transfer, in particular between animal and human commensal flora, environmental flora, and human pathogenic species. Given the fact that foods are vehicles of microorganisms transmitting antibiotic resistance, and today's scientific community is fully aware of the importance of monitoring antimicrobial resistance in the food chain, we have studied the antibiotic resistance of *Enterococcus* strains isolated from animal and vegetable raw food, in order to evaluate its impact in our country.

2. Materials and methods

2.1. Detection and identification of Enterococcus spp.

In order to isolate *Enterococcus* species, we analyzed 1315 food specimens purchased at 150 supermarkets in several cities in Tuscany (Italy): 636 samples of raw meat (227 beef, 238 poultry, and 171 pork), 278 samples of cow's milk cheese (110 fresh soft cheese and 168 mozzarella cheese), 214 samples of ready-to-eat salads, and 187 samples of ham (Table 1). All samples were obtained between January and October 2012.

A 25 g portion of each food sample was aseptically taken, placed in 225 ml of buffered peptone water (Thermo scientific – Oxoid), and homogenized through a Stomacher for 60 s at normal speed. A 0.5 ml portion of this primary enrichment was streaked to Slanetz and Bartley Agar (Thermo scientific - Oxoid) and incubated for 24 ± 2 h at 37 ± 1 °C.

Suspected colonies of *Enterococcus* spp. were those with a maximum diameter of 1 mm, pink or dark red, with a narrow whitish border. Three of the suspected colonies, per sample, were transferred to Tryptone Soya Agar (Thermo scientific - Oxoid) and incubated for 24 ± 2 h at 37 ± 1 °C, and characterized by Gram stain and catalase production. The *Enterococcus* species were identified through rapID STR (Thermo scientific - Oxoid) performed only on Gram positive and catalase negative cocci.

2.2. Susceptibility testing

All 933 isolates (311 positive samples multiplied by three strains) were tested by the standard disk diffusion method of Kirby–Bauer (Bauer et al., 1966; EUCAST, 2013a) on Mueller Hinton Agar (Thermo scientific - Oxoid) incubated at 35 ± 1 °C for 18 ± 2 h (for glycopeptides 24 h) according to EUCAST disk diffusion method (EUCAST, 2013a).

Reference strains were *E. faecalis* ATCC 29212 and *E. faecium* ATCC 19434.

Disks containing the following antibiotics (all from Thermo Scientific – Oxoid) were spotted with a 3 cm interval: amoxicillin/clavulanic acid 30 μg (1:2), ampicillin – 2 μg, ciprofloxacin – 5 μg, chloramphenicol – 30 μg, erythromycin – 120 μg, gentamicin – 30 μg, linezolid – 10 μg, penicillin G – 10 U.I., teicoplanin – 30 μg, tetracycline – 30 μg, vancomycin – 5 μg. Results were interpreted following EUCAST breakpoint tables (EUCAST, 2013b) and, where not possible, according to NCCLS (2007) indications.

Isolates of the same species with identical antibiotic resistance patterns isolated from the same sample were considered as the same strain.

2.3. Statistical analysis

The standard descriptive statistics of the contamination (percentages) and comparison test (Fisher's exact test) were made using Stata/SE 8.0 (StataCorp, College Station, TX, USA).