Journal of Food and Nutrition Research, 2018, Vol. 6, No. 10, 626-637
Available online at http://pubs.sciepub.com/jfnr/6/10/3

DOI:10.12691/jfnr-6-10-3

Prevalence of Antibiotic Resistance in Enterococci: A 14 Year Survey

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Received August 14, 2018; Revised October 12, 2018; Accepted October 26, 2018

Abstract Enterococci are leading causes of nosocomial bacteremia, surgical wound and urinary tract infections. They are ubiquitous bacteria commonly occurring in foods, and in recent years there has been increased attention towards multidrug strains incidence, since they may cause the failure of therapeutic treatments. Therefore, we analyzed the occurrence of *Enterococcus* species isolated from raw meat (beef, chicken and pork), cheese, and ready-to-eat salads, and the change of the antibiotic resistance in *Enterococcus faecium* and *E. faecalis* strains in a 14 year survey. Among the 589 *Enterococcus* strains, *E. faecium* and *E. faecalis* represented the most numerous species in all types of food examined (42.8% and 38.7% respectively). Antibiotic resistance and number of Multi Drug Resistant strains have increased, reaching very high levels from 2002 to 2015. In the last two years, *E. faecalis* isolates sometimes reached percentages of resistance higher than 40% against tetracycline, vancomycin, linezolid, erythromycin, and ampicillin. Antibiotic resistance in *E. faecium* was lower than in *E. faecalis* for almost all antimicrobials tested. The highest percentage of resistance in 2014-2015 was registered for erythromycin (42.5%), followed by tetracycline (30%), ciprofloxacin, and linezolid (both 27.5%). The number of resistant phenotypes also increased during this survey in both species to more than 20 in 2014-2015. Despite the fact that *Enterococcus* spp. do not represent a problem for immunocompetent individuals, surveillance of antibiotic resistance in this kind of micro-organism continues to be important because, as shown in our results, antibiotic resistance has sharply increased in recent years.

Keywords: *Enterococcus, raw meat, cheese, salads, Multi Drug Resistance, survey*

Cite This Article: Carmela Calonico, Giovanna Pesavento, Vania Delfino, Sara Forni, and Antonella Lo Nostro, "Prevalence of Antibiotic Resistance in Enterococci: A 14 Year Survey." *Journal of Food and Nutrition Research*, vol. 6, no. 10 (2018): 626-637. doi: 10.12691/jfnr-6-10-3.

1. Introduction

Enterococci are ubiquitous bacteria commonly occurring in foods, especially those of animal origin, such as meat and milk [1], and those of soil origin [2,3]. Due to their primary habitat, identified in the gastrointestinal tract of animals, and due to contamination during slaughtering and manipulation, they can reach and hence contaminate water, environment, soil, and most foods. They are useful as fecal contaminant indicators owing to their ability to survive in very hostile environments [4]. They may also play a desirable role in the fermentation and ripening of certain foods of animal origin, such as cheese and fermented meat, because they are added during the production process of some foods, both to extend their shelf life and to improve their organoleptic properties and flavour [5,6]. As many authors reported, *Enterococcus* spp. are normally found in most foods, such as those of animal origin (raw meat, cheese) and vegetables, with predominance of *E. faecalis* and *E. faecium* [3,7-14], which are involved in severe human infections worldwide [15,16], especially in hospital acquired ones [17]. In recent years there has been increased attention towards multidrug strains incidence [18,19], since they 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 [20], with *E. faecalis* bearing the principal responsibility for at least 70-80% of the cases [21]. Researchers suggested that Enterococci can be spread from animals and vegetables to man both through direct contact or through their consumption, leading to a spread of antibiotic resistance genes in human beings [22,23], furthermore they can transfer resistance genes to their own or other species [24,25,26,27], with the possibility of causing endogenous infections even in colonized humans [28]. The acquisition of resistance determinants can occur through gene transference by plasmids and transposons [29,30] and include aminoglycosides, chloramphenicol, β-lactams, macrolides, quinolones, tetracycline, vancomycin and teicoplanin [31,32], as well as linezolid [18,33]. These new antibiotic resistances, together with the intrinsic ones to cephalosporins, and to low level aminoglycosides, polymixins, lincomycin, clindamycin, and often quinolones [34], can make the treatment of enterococcal infections very difficult. Antimicrobial resistant Enterococci are spread from hospitals to environment mostly through water and feces, secondly, from humans and other sources, increasing their prevalence in environment, humans and animals, and becoming a potential risk for human health [13]. Since *Enterococcus* species show the same pattern as other bacteria in increasing their antibiotic resistance, especially nosocomial strains [18,19], we think that a continuous monitoring of antibiotic resistance in Enterococci is necessary, both for clinical and foodborne strains, to verify if any changes occur over the years. For this purpose, we analyzed the occurrence of *Enterococcus* species isolated from raw meat (beef, chicken and pork), cheese, and ready-to-eat salads (RTES), and the change of the antibiotic resistance in *Enterococcus faecium* and *E. faecalis* strains during this 14 year survey.

2. Materials and Methods

2.1. Bacterial Strains

Enterococcus spp. were collected from Italian beef, poultry and pork, cheese, and RTES during a 14 year survey conducted in Italy from 2002 to 2015. Enterococci were isolated from food samples as previously described [35] in Slanetz and Bartley agar (Thermo Fisher Diagnostics, Milan, ITA) and incubated for 24±2 h at 37±1°C. Species identification was obtained through rapID STR (Thermo Fisher Diagnostics), according to the manufacturer's instructions.

2.2. Susceptibility Testing

Antimicrobial susceptibility of the *Enterococcus* spp. strains were determined by the Kirby- Bauer disk diffusion method [36,37] on Mueller Hinton agar (Thermo Fisher Diagnostics). The plates were incubated at 35±1°C for 18±2h (for glycopeptides 24h) according to EUCAST disk diffusion method [37].

Enterococcus faecalis ATCC 29212 and *Enterococcus faecium* ATCC 19434 were used as reference strains.

Disks containing the following antibiotics (all from Thermo Fisher Diagnostics) were placed at 3 cm interval: amoxicillin/clavulanic acid - 30 µg (1:2), ampicillin - 2 µg, chloramphenicol - 30 µg, ciprofloxacin - 5 µg, erythromycin - 15 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 [38] and, where not possible, according to CLSI [39] indications.

2.2. Statistical Analysis

The standard descriptive statistics of the contamination (percentages) and comparison tests were made using Stata/SE 8.0 (StataCorp, College Station, TX, USA). The frequencies were compared using the chi-squared test with a significant level of p-value < 0.05.

3. Results and Discussion

3.1. Prevalence of *Enterococcus* Species

As many authors reported, among the 589 *Enterococcus* strains, *E. faecium* and *E. faecalis* represented the majority in all types of food examined (42.8% and 38.7% respectively), followed by *E. durans* (12.7%) and other species (Table 1). In particular, *E. faecalis* was more frequent in raw meat, while *E. faecium* was found more often in cheese and salads.

Prevalence of *Enterococcus* species in beef was different from that found in the German study by Klein et al. [11], in which contamination was 89.8% *E. faecalis*, 2.8% *E. gallinarum* and *E. durans* and 1.8% *E. faecium*, and from the Hayes et al. [9] study in the U.S., in which 17.0% of beef was contaminated by *E. faecalis*, 65% by *E. faecium* and 14% by *E. hirae*. In poultry meat, *E. faecalis* was more frequent (44.7%) than *E. faecium* (30.7%) similarly to what Aarestrup et al. [40] found, but in contrast with other studies, such as Ali et al. [7], who reported 17.2% and 66.2% respectively, or Kilonzo-Nthenge et al. [41], who did not isolate *E. faecalis*, but *E. faecium* (27.3%), *E. gallinarum* (6.0%), *E. casseliflavus* (2.1%), and *E. durans* (1.4%). Our samples of pig meat had high percentages of *E. faecalis* (45.0%) and *E. faecium* (41.6%), similar to the ones reported by Jahan et al. [10] and Li et al. [12] who isolated *E. faecalis* in percentages of 51.7 and 36.9% and *E. faecium* of 44.8 and 53.6%, respectively. In several studies on cheese we observed highly variable percentages of *Enterococcus* species, probably due to the fact that these bacteria are part of the microflora of cheese. In Brazilian cheese [8], the most prevalent species were *E. faecium* (58.3%) and *E. faecalis* (27.8%), followed by *E. casseliflavus* (11.1%) and *E. gallinarum* (2.7%); in an Italian study [42] *E. faecium* was identified in 61.3% of the samples, percentage similar to what we detected, while *E. faecalis* was found only in 6.66% in contrast to the 17.9% in this report. Our samples of mixed lettuce had high percentage of *E. faecium* (65.6%), according to Torre et al. [3] who detected 59.1% of *E. faecium*, 18.1% of *E. faecalis*, 19% of *E. durans* and 3.8% of *E. avium*; *E. faecalis* was detected in 23.5% of the salad samples, similar to what reported by Ronconi et al. [2] who detected 32.6% of *E. faecium*, 21.7% of *E. faecalis* followed by *E. gallinarum* (13.04%), *E. casseliflavus* and *E. mundtii* (7.60%), *E. hirae*, (6.52%) and *E. durans* (4.35%).

Table 1. Number of positive samples and distribution of *Enterococcus* species in the products analyzed

Species	beef No. (%)	poultry No. (%)	pig No. (%)	cheese No. (%)	salads No. (%)	Total No. (%)
E. faecium	46 (34.6)	46 (30.7)	62 (41.6)	75 (61.0)	23 (65.6)	252 (42.8)
E. faecalis	64 (48.1)	67 (44.7)	67 (45.0)	22 (17.9)	8 (23.5)	228 (38.7)
E. durans	15 (11.3)	23 (15.3)	14 (9.4)	22 (17.9)	1 (2.94)	75 (12.7)
E. avium	8 (6.02)	8 (5.33)	5 (3.36)	4 (3.25)	0	25 (4.24)
E. gallinarum	0	4 (2.67)	1 (0.67)	0	2 (5.88)	7 (1.19)
E. raffinosus	0	1 (0.67)	0	0	0	1 (0.17)
E. casseliflavus	0	1 (0.67)	0	0	0	1 (0.17)
Total	133	150	149	123	34	589

All results, those cited and ours, are dissimilar (p<0.05), showing a not-regular distribution in time of *Enterococcus* species in animals and vegetables analyzed; this probably depends on environmental conditions in breeding and cultivation, including the quality of water used for watering animals and vegetables.

3.2. Total Antibiotic Resistance on *E. faecalis* and *E. faecium* Strains

Antibiotic resistance was determined only for *E. faecium* and *E. faecalis* strains, due to their importance in human infections. Of the 252 *E. faecium* identified, 13.1% were resistant to at least three antibiotics (Multi Drug Resistant, MDR), 28.6% were resistant to erythromycin, 17.9% to tetracycline, 12.7% to penicillin G and 12.3% to ciprofloxacin. Of the 228 *E. faecalis* identified, 23.7% were MDR, 54.4% to tetracycline, 24.1% to erythromycin, 19.7% to vancomycin, 13.6% to linezolid, 10.5% to chloramphenicol, followed by other antimicrobials (Table 2). In *E. faecalis* a significantly higher prevalence of resistance to ampicillin, chloramphenicol, linezolid, tetracycline and vancomycin was detected than in *E. faecium* (p<0.05), while in *E. faecium* a greater prevalence of resistance to ciprofloxacin, erythromycin and penicillin G was found than in *E. faecalis* (p<0.05). Due to the fact that most of our strains were sensible to ampicillin and penicillin, we do not agree with the generalization that considers Enterococci intrinsically resistant to ß- lactams [34].

Compared to our previous investigation [35], we detected higher resistance to all antimicrobials, particularly to vancomycin (Table 2), except for teicoplanin and tetracycline. Our results are in agreement with the general increment of multi resistant strains in recent years [43], infact percentages of *E. faecium* and *E. faecalis* isolates resistant to vancomycin were respectively 4.76% and 19.7%, much higher than those reported in Europe by EFSA [43] in recent years.

3.3. Antibiotic Resistance in the Types of Products Analyzed

Enterococcal populations are different in animal species and vegetables, so we compared antibiotic resistance in *E. faecium* and *E. faecalis* in the different types of food examined (Table 2).

Animals may acquire antibiotic resistant bacteria from the first moments of their life. Obeng et al. [44] concluded that, in chickens, a para-vertical transmission from egg shells to chicks could exist during hatching as a contamination through feeds. In fact, they found antibiotic resistant Enterococci from chicks of 3 days and older and, almost always, they observed an increasing trend of antibiotic resistance as chicks aged. In this study, carried out in Australia, Enterococci from chickens of 100 days were 53% resistant to ampicillin, 97% to erythromycin, and 100% to tetracycline. Amaechi and Nwankwo [45] found percentages of antibiotic resistance in Enterococci from poultry higher than ours (Table 3): 21.8% to ciprofloxacin, 71.2% to erythromycin, and 76.7% to chloramphenicol. Very different antibiotic resistance percentages are probably due to different selections of bacteria caused by different legislation in various countries regarding the use of nontherapeutic antimicrobial growth promoters in animal feed [22,44,45].

As most authors reported [22,44,46], erythromycin and tetracycline resistance were highly distributed in *E. faecium* and *E. faecalis*, although in different percentages. The highest resistance levels among *E. faecium* and *E. faecalis* isolates were from poultry and were comparable to European data [43].

Table 2. Number and percentage of antibiotic resistance among all *E. faecium* and *E. faecalis* strains in the types of products analyzed

	***Enterococcus faecium* No. (%)**						***Enterococcus faecalis* No. (%)**					
	beef	*pig*	*poultry*	*cheese*	*salads*	*Total*	*beef*	*pig*	*poultry*	*cheese*	*salads*	*Total*
Amox/Clavul.	0	0	2 (4.35)	0	0	2 (0.79)	0	2 (2.99)	0	0	0	2 (0.88)
Ampicillin	4 (8.70)	0	9 (19.6)	2 (2.67)	0	15 (5.95)	13 (20.3)	4 (5.97)	6 (9.0)	4 (18.2)	0	27 (11.8)
Chloramphenicol	2 (4.35)	3 (4.84)	4 (8.70)	3 (4.00)	0	12 (4.76)	10 (15.6)	3 (4.48)	8 (11.9)	3 (13.6)	0	24 (10.5)
Ciprofloxacin	3 (6.52)	3 (4.84)	12 (26.1)	11 (14.7)	2 (8.70)	31 (12.3)	2 (3.13)	3 (4.48)	4 (6.00)	0	2 (25.0)	11 (4.82)
Erythromycin	6 (13.0)	13 (21.0)	27 (58.7)	23 (30.7)	3 (13.0)	72 (28.6)	8 (12.5)	10 (14.9)	34 (50.7)	3 (13.6)	0	55 (24.1)
Linezolid	0	0	5 (10.9)	8 (10.7)	1 (4.35)	14 (5.56)	2 (3.13)	8 (11.9)	16 (23.9)	3 (13.6)	2 (25.0)	31 (13.6)
Penicillin G	2 (4.35)	3 (4.84)	17 (37.0)	7 (9.33)	3 (13.0)	32 (12.7)	1 (1.56)	1 (1.49)	2 (3.00)	0	0	4 (1.75)
Teicoplanin	1 (2.17)	3 (4.84)	1 (2.17)	2 (2.67)	0	7 (2.78)	0	0	1 (1.49)	2 (9.09)	0	3 (1.32)
Tetracycline	6 (13.0)	9 (14.5)	27 (58.7)	2 (2.67)	1 (4.15)	45 (17.9)	32 (50.0)	31 (46.3)	55 (82.1)	2 (9.09)	4 (50.0)	124 (54.4)
Vancomycin	3 (6.52)	2 (3.23)	2 (4.35)	4 (5.33)	1 (4.35)	12 (4.76)	13 (20.3)	6 (8.96)	21 (31.3)	5 (22.7)	0	45 (19.7)

Table 3. Percentage of antibiotic resistance among all *Enterococcus spp.* strains in the types of products analyzed

	Beef No. (%)	*Pig* No. (%)	*Poultry* No. (%)	*Cheese* No. (%)	*Salads* No. (%)	*Total* No. (%)
Amox/Clavul.	1 (0.85)	2 (1.41)	4 (2.84)	0	0	7 (1.24)
Ampicillin	14 (11.9)	6 (4.20)	19 (13.5)	6 (4.92)	0	49 (8.70)
Chloramphenicol	13 (11.0)	7 (4.90)	13 (9.22)	6 (4.92)	0	39 (6.91)
Ciprofloxacin	6 (5.08)	6 (4.20)	19 (13.5)	13 (10.7)	5 (14.7)	49 (8.69)
Erythromycin	14 (11.9)	26 (18.2)	76 (53.9)	28 (22.9)	3 (8.82)	148(26.2)
Linezolid	3 (2.54)	8 (5.60)	24 (17.0)	12 (9.84)	3 (8.82)	50 (8.87)
Penicillin G	3 (2.54)	6 (4.20)	26 (18.4)	8 (6.56)	3 (8.82)	46 (8.16)
Teicoplanin	1 (0.85)	3 (2.10)	2 (1.42)	6 (4.92)	0	12 (2.13)
Tetracycline	38 (32.2)	45 (31.5)	102(72.3)	5 (4.10)	5 (14.7)	198 (35.1)
Vancomycin	15 (12.7)	8 (5.60)	25 (17.7)	12 (9.84)	1 (2.94)	63 (11.2)

The percentages of antibiotic resistance in Enterococci from pigs isolated by Amaechi and Nwankwo [45] in Nigeria were 21.7% to ciprofloxacin, 71.8% to erythromycin and 85.8% to chloramphenicol. Our percentages were much lower, even compared to some antimicrobials cited in EFSA Summary Report [43], such as chloramphenicol, erythromycin and tetracycline for *E. faecalis* and ampicillin, erythromycin, linezolid and tetracycline for *E. faecium*.

Beef samples were contaminated by Enterococci mostly resistant to tetracycline, ampicillin, vancomycin, erythromycin and chloramphenicol, although with lower levels compared to those of EFSA [47], which reported absence of resistance to vancomycin in Spain, whereas we found resistance both in *E. faecalis* and *E. faecium* isolates.

In cheese, we observed a higher prevalence (p<0.05) of resistant *E. faecalis* than *E. faecium* (Table 2) for ampicillin, chloramphenicol, linezolid, teicoplanin, tetracycline, and vancomycin, with percentages similar to those observed by Furlaneto-Maia et al. [8].

Despite the low number of strains isolated in salads compared to our previous investigation [35], we did note an increase in resistance against linezolid, one of the first-line therapeutic options for the treatment of vancomycin-resistant Enterococci (VRE) infections.

Ciprofloxacin and erythromycin resistance was spread in many *E. faecium* isolates from all analyzed types of food, whereas chloramphenicol, erythromycin, and tetracycline resistance was present in *E. faecalis* isolates from all the food groups, with the exception of salads.

3.4. Antibiotic Resistance Survey

To date, Enterococci are considered one of the most important opportunistic pathogens due to the high number of infections they cause, especially in immunocompromized patients, with severe underlying diseases [8]. The increasing levels of acquired resistance to antimicrobials is of special concern in public health because the therapeutic options are becoming ever more limited [48]. Furthermore, the transferring of antibiotic resistance genes through plasmids and transposons could occur between bacteria belonging to the same or different genus and in the intestinal tract of humans and animals [24]. Therefore the food chain is now considered one of the main routes of transmission of bacteria containing antimicrobial resistance genes between animals and humans [49]. With this in mind, we observed the variation of susceptibility of either *E. faecalis* or *E. faecium* over the years. Our results showed lower or similar resistance levels compared to other countries, mainly Austria and Switzerland [43]. As shown in Figure 1 and Figure 2, antibiotic resistance increased for almost all antimicrobials reaching very high levels from 2002 to 2015.

E. faecalis isolates in the last biennium reached percentages of resistance against tetracycline and vancomycin of 69.2% and 59.6%, respectively. Lower percentages were seen for linezolid (44.2%) erythromycin and ampicillin (both 40.4%).

Sensitivity to glycopeptides was different: we observed high sensitivity to teicoplanin, while vancomycin-resistant strains sharply increased in the past four years. Resistance to linezolid has always been low, but nearly doubled in the last two years, reaching worrisome percentages. Sensitivity to erythromycin and tetracycline also decreased, causing a reduction in efficacy of these antibiotics. We did not observe many strains resistant to chloramphenicol as the percentages were always lower than 26%.

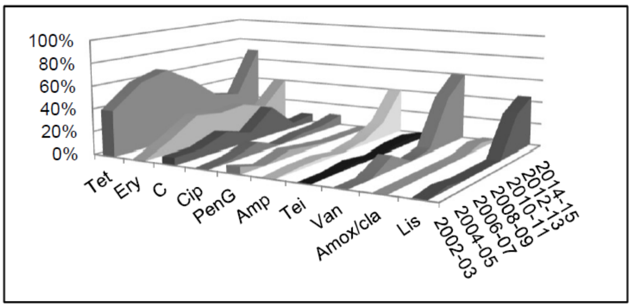

Figure 1. Temporal trends (2002-2015) in resistance among *E. faecalis*. Tet, tetracycline; Ery, erythromycin; C, chloramphenicol; Cip, ciprofloxacin; Pen G, penicillin G; Amp, ampicillin; Tei, teicoplanin; Van, vancomycin; Amox/cla, amoxicillin/clavulanic acid; Lis, linezolid

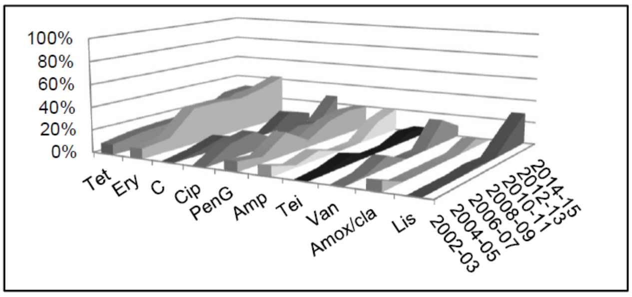

Figure 2. Temporal trends (2002-2015) in resistance among *E. faecium*. Tet, tetracycline; Ery, erythromycin; C, chloramphenicol; Cip, ciprofloxacin; Pen G, penicillin G; Amp, ampicillin; Tei, teicoplanin; Van, vancomycin; Amox/cla, amoxicillin/clavulanic acid; Lis, linezolid

Antibiotic resistance in *E. faecium* was lower than in *E. faecalis* for almost all antimicrobials: the highest percentage of resistance in 2014-2015 was seen for erythromycin (42.5%), followed by tetracycline (30%), ciprofloxacin, and linezolid (both 27.5%). We observed peaks of resistance occurring to almost all antibiotics in 2006-07. In contrast to the data shown in the EFSA report [43] for some countries, a general increase of resistance was also observed for *E. faecium*, especially in the last four years, with a more noticeable increase for some antimicrobials such as ciprofloxacin, erythromycin, tetracycline and linezolid.

In beef, we isolated *E. faecalis* resistant to tetracycline (Table 4) in all the years of sampling with a dramatic increase in the last two years, while penicillin G, teicoplanin, and amoxicillin/clavulanic acid resistance were not observed. Linezolid resistance was only observed in the last biennium, and vancomycin resistance has been fluctuating, as documented in human infections [50,51]. According to general results, resistance in *E. faecium* (Table 5) was lower than in *E. faecalis:* from 2006 onwards, tetracycline was ineffective on 10%-33.3% of strains. Vancomycin resistance was observed only in 2006-2007 and in the last biennium.

Poultry showed the constant presence of *E. faecalis* resistant to erythromycin and tetracycline from 2004 onwards at fluctuating rates, as in other European countries [43]. Rates of *E. faecium* resistant to penicillin G, tetracycline and erythromycin were similar to France and Austria. Resistance of *E. faecium* strains to other antibiotics occurred more frequently in the last two years, and particularly to linezolid, vancomycin and ampicillin.

In pork, we observed an increase of resistance in the last two biennia in *E. faecium* against most of the antimicrobials, except for ampicillin, ciprofloxacin, linezolid, and amoxicillin/clavulanic acid, which remained active against this bacteria with a sensitivity rate of 100% from 2010 onwards. *E. faecalis* resistance increased against ampicillin and decreased against vancomycin (from 75% to 42.9%), amoxicillin/clavulanic acid (from 25% to 14.3%) and linezolid (from 75% to 71.4%).

Resistance patterns of cheese strains varied over the years for both *E. faecalis* and *E. faecium.* The high percentages of vancomycin resistant *E. faecalis* and linezolid resistant *E. faecium* are alarming, confirming the necessity to use starter strains with antibiotic controlled resistance [5,52].

Table 4. Antibiotic resistance percentages in *E. faecalis* strains

No of isolates	2002-03 14	2004-05 42	2006-07 33	2008-09 31	2010-11 27	2012-13 20	2014-15 46
Beef							
Amox/cla	11.1	0	0	0	0	0	0
Amp	11.1	7.69	14.3	0	0	14.3	69.2
C	11.1	15.4	42.9	10.0	25.0	0	15.4
Cip	0	0	14.3	0	0	0	7.69
Ery	11.1	7.69	14.3	30.0	0	0	23.1
Lis	0	0	0	0	0	0	15.4
PenG	22.2	0	0	0	0	0	0
Tei	0	0	0	0	0	0	0
Tet	44.4	53.8	42.9	40.0	50.0	14.3	84.6
Van	0	0	42.9	0	0	57.1	38.5
Poultry							
Amox/cla	n.d.	0	0	0	0	0	0
Amp	n.d.	0	0	0	0	0	26.1
C	n.d.	0	22.2	0	42.9	16.7	8.70
Cip	n.d.	0	11.1	0	0	0	13.0
Ery	n.d.	13.3	55.6	50.0	71.4	50.0	69.6
Lis	n.d.	6.67	0	0	0	33.3	56.5
PenG	n.d.	0	22.2	0	0	0	0
Tei	n.d.	0	11.1	0	0	0	0
Tet	n.d.	86.7	77.8	83.3	57.1	66.7	91.3
Van	n.d.	0	11.1	0	0	16.7	82.6
Pig							
Amox/cla	0	0	0	0	0	25.0	14.3
Amp	0	0	0	0	0	25.0	42.9
C	0	0	0	13.3	8.33	0	0
Cip	0	10.0	7.14	0	8.33	0	0
Ery	0	10.0	21.4	20.0	8.33	0	28.6
Lis	0	0	0	0	0	75.0	71.4
PenG	0	0	0	6.67	0	0	0
Tei	0	0	0	0	0	0	0
Tet	40.0	40.0	78.6	66.7	25.0	0	14.3
Van	0	0	0	0	0	75.0	42.9
Cheese							
Amox/cla	n.d.	0	0	n.d.	0	0	0
Amp	n.d.	0	0	n.d.	25.0	0	33.3
C	n.d.	25.0	0	n.d.	50.0	0	0
Cip	n.d.	0	0	n.d.	0	0	0
Ery	n.d.	25.0	0	n.d.	25.0	0	0
Lis	n.d.	0	0	n.d.	0	33.3	33.3
PenG	n.d.	0	0	n.d.	0	0	0
Tei	n.d.	0	0	n.d.	25.0	0	0
Tet	n.d.	25.0	33.3	n.d.	0	0	0
Van	n.d.	0	0	n.d.	25.0	0	66.7

n.d. not determined (number of strains <5); Amp, ampicillin; Pen G, penicillin G; Tet, tetracycline; Ery, erythromycin; Van, vancomycin; Tei, teicoplanin; C, chloramphenicol; Cip, ciprofloxacin; Lis, linezolid; Amox/cla, amoxicillin/clavulanic acid.

Due to the limited number of strains isolated from RTES, we could study antibiotic resistance trend just for *E. faecium*. Since ready-to-eat salads are not rewashed or cooked before their consumption, a positive note is that we found few contaminating strains, like McGowan et al. [53], and they did not present much antibiotic resistance.

Table 5. Antibiotic resistance percentages in *E. faecium* strains

No of isolates	2002-03 10	2004-05 53	2006-07 41	2008-09 64	2010-11 31	2012-13 11	2014-15 37
Beef							
Amox/cla	0	0	0	0	0	n.d.	0
Amp	0	0	10.0	0	0	n.d.	50.0
C	0	11.1	10.0	0	0	n.d.	0
Cip	0	0	20.0	11.1	0	n.d.	0
Ery	0	11.1	20.0	11.1	0	n.d.	16.7
Lis	0	0	0	0	0	n.d.	0
PenG	0	0	20.0	0	0	n.d.	0
Tei	0	0	10.0	0	0	n.d.	0
Tet	0	0	10.0	33.3	16.7	n.d.	16.7
Van	0	0	20.0	0	0	n.d.	16.7
Poultry							
Amox/cla	n.d.	0	0	0	0	n.d.	0
Amp	n.d.	0	14.3	0	0	n.d.	38.5
C	n.d.	11.1	28.6	11.1	0	n.d.	7.69
Cip	n.d.	0	14.3	0	16.7	n.d.	53.8
Ery	n.d.	44.4	85.7	44.4	33.3	n.d.	76.9
Lis	n.d.	11.1	0	11.1	0	n.d.	30.8
PenG	n.d.	22.2	14.3	22.2	66.7	n.d.	38.5
Tei	n.d.	0	0	0	0	n.d.	0
Tet	n.d.	55.6	71.4	55.6	33.3	n.d.	53.8
Van	n.d.	0	0	0	0	n.d.	15.4
Pig							
Amox/cla	0	0	0	0	0	0	0
Amp	0	0	0	0	0	0	0
C	0	0	0	0	0	25.0	40.0
Cip	0	6.67	14.3	4.55	0	0	0
Ery	0	0	14.3	22.7	66.7	25.0	80.0
Lis	0	0	0	0	0	0	0
PenG	0	0	0	4.55	0	0	40.0
Tei	0	0	0	0	0	25.0	40.0
Tet	0	0	0	9.09	0	75.0	80.0
Van	0	0	0	0	0	25.0	20.0
Cheese							
Amox/cla	n.d.	0	0	0	0	0	0
Amp	n.d.	0	8.33	0	0	14.3	0
C	n.d.	6.67	8.33	0	0	14.3	0
Cip	n.d.	20.0	25.0	0	15.4	0	28.6
Ery	n.d.	6.67	33.3	52.6	38.5	28.6	14.3
Lis	n.d.	0	0	0	0	28.6	71.4
PenG	n.d.	0	33.3	0	7.69	28.6	0
Tei	n.d.	0	16.7	0	0	0	0
Tet	n.d.	0	0	0	15.4	0	0
Van	n.d.	0	16.7	0	0	28.6	0
Ready Salads							
Amox/cla	n.d.	0	0	0	n.d.	n.d.	0
Amp	n.d.	0	0	0	n.d.	n.d.	0
C	n.d.	0	0	0	n.d.	n.d.	0
Cip	n.d.	20.0	0	0	n.d.	n.d.	0
Ery	n.d.	20.0	0	20.0	n.d.	n.d.	16.7
Lis	n.d.	0	0	0	n.d.	n.d.	0
PenG	n.d.	0	20.0	20.0	n.d.	n.d.	16.7
Tei	n.d.	0	0	0	n.d.	n.d.	0
Tet	n.d.	20.0	0	0	n.d.	n.d.	0
Van	n.d.	20.0	0	0	n.d.	n.d.	0

n.d. not determined (number of strains <5); Amp, ampicillin; Pen G, penicillin G; Tet, tetracycline; Ery, erythromycin; Van, vancomycin; Tei, teicoplanin; C, chloramphenicol; Cip, ciprofloxacin; Lis, linezolid; Amox/cla, amoxicillin/clavulanic acid.

From data presented in Table 4 and Table 5 and Figure 1 and Figure 2, we point out that in the first four years of the survey we did not isolate any VRE, except for one strain identified in 2004-2005 in RTES. From 2006 onwards, vancomycin resistance was observed in beef, poultry and cheese, and then in pig, with the highest percentages appearing in the last four years. The absence of VRE in the first years of the survey was probably due to the abolition of avoparcin (1997) as an antibiotic and growth promoter used in veterinary medicine [35], leading to an inferior spreading of these strains through the food chain, but most probably due to the limited use of this antibiotic as feed additive in Italy since, once acquired, antibiotic resistance traits tend to persist [54]. It might be possible that clinical strains of VRE are spreading in community and environment [13] because it has been demonstrated that glycopeptide-resistant Enterococci isolated from meat products (chicken and pork) are able to overcome the gastric barrier and actively multiply in the human intestine [55]. In 2013, in Europe [43], vancomycin resistant *E. faecium* and *E. faecalis* had percentages lower than 2% from all types of food samples, whereas in this study we found much higher percentages of VRE. Moreover, vancomycin-resistant *E. faecalis* in the 2014-15 biennium have increased dramatically, considering that the mean in all foods analyzed was about 60%, according to the results found by Guerrero-Ramos et al. [56]. The most vancomycin resistant *E. faecalis* were the ones isolated from poultry in the last biennium (82.6%).

Enterococci also showed a worrisome decreased sensitivity against linezolid: in the last two biennia *E. faecalis* reached resistance percentages of 71.4% in pig, 56.5% in poultry and 33.3% in cheese, whereas *E. faecium* of 71.4% in cheese and 30.8% in poultry.

Citak et al. [57] reported Enterococci resistant to penicillin, tetracycline, chloramphenicol, erythromycin, teicoplanin and vancomycin until 2005. Over the years, our strains also became resistant to ampicillin, ciprofloxacin and linezolid.

Despite EU Regulation No. 1831/2003 [58] on additives for use in animal nutrition, which stated that antibiotics could only be used when prescribed and not as growth promoters, we did not observe a decrease in resistance prevalence, in particular for erythromycin and tetracyclines, widely used worldwide as feed additives [59].

Overuse and misuse of antibiotics are largely regarded as main factors in promoting antibiotic resistance. As previously noted, Enterococci are intrinsically resistant to many commonly used antimicrobial agents and they have the ability to rapidly express resistance genes in response to selective pressure [55]. For this reason, environmental Enterococci are a source of antimicrobial resistance genes and they have an important role in the gene exchange between clinical and environmental bacteria [60].

The increase of resistant Enterococci could be explained by the overuse of antibiotics in agriculture and livestock facilities, as reported in many studies [61,62,63,64]. Antibiotics used on farms, especially if administered in high doses or if added to the feed in sublethal concentrations, can reach the surrounding environment through animal excrements, water run-off and sewage. Consequently, the natural environment becomes a reservoir of antibiotic residues that enhances the spread of resistance genes in microbial communities [65]. This condition is supported by the persistence of particular antibiotic-resistant clones non-sensitive to banning interventions, as previously reported [61,66]. Moreover, resistant Enterococci can spread among different geographical areas through importation and exportation of livestocks. This is the reason why it would be desirable the transport of already slaughtered animals.

Although in the European Union antibiotics cannot be used for growth promotion and a veterinary prescription is always required, most countries, including Italy, still permit antibiotics to be used for routine disease prevention. In 2015 farm antibiotic use in Italy was well above the EU average, with a weight of active ingredient per unit of livestock of 322.0 mg/PCU, whereas the average for 25 European countries was 141 mg/PCU [67].

Resistant Enterococci can enter humans for example through direct contact between humans and animals or through transmission via food (ingestion of contaminated raw foods of animal and vegetal origin, consumption of crops grown by contaminated sludge used as fertilizer, drinking of water drawn from contaminated ground or surface water). Some studies showed that the same resistance gene are found in bacteria isolated from both food samples and patients [31,68], supporting the hypothesis that Enterococci from food have the capacity to spread their resistance genes to the human microbiome and to disseminate in the bacterial community [65,69].

Finally, another reason that could explain the emergence of resistant Enterococci would be the massive use of antibiotic in clinical contexts. Enterococci, as normal commensals of the human gastrointestinal tract, are exposed to antibiotics during medical treatment. Resistant Enterococci can colonize the gut, deplete the protective commensals (*Lactobacillus* spp. and *Bifidobacterium* spp.) and spread from patient to patient within the hospital setting [69]. In support of our results, it is interesting to note that Italy have one of the highest levels of antibiotic consumption for systemic use in the EU, i.e. about 30 defined daily doses per 1000 inhabitants per day [70].

3.5. Antibiotic Resistance Phenotypes

We observed that both *E. faecium* and *E. faecalis* multi drug resistance had fluctuating rates in the first years of the survey, reaching 20% in 2006-2007, and increasing from 2009 onwards (Figure 3).

The number of resistance phenotypes also increased during this survey in *E. faecium* and in *E. faecalis* from 1 and 3 in 2002-2003, to 20 and 24 in 2014-2015, respectively.

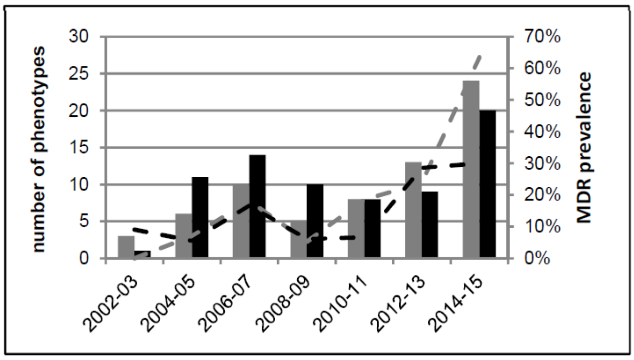

Figure 3. Time line of number of phenotypes (histograms) and MDR (lines) of *E. faecalis* (grey) and *E. faecium* (black)

Some antibiotic resistances, such as to tetracycline and tetracycline together with erythromycin, were present constantly over the years in both species, and particularly in *E. faecalis* (Table 6 and Table 7). Percentages of *E. faecalis* strains resistant only to tetracycline and of *E. faecium* strains resistant only to erythromycin were higher than those of erythromycin together with tetracycline, as Hidano et al. [71] observed.

Our results are not in agreement with the findings of De Leener et al. [72] in which trasposons Tn 1545 are more frequent in Enterococci from pork than from other foods. In our study, we observed higher resistance against tetracycline together with erythromycin in poultry (53.2%), probably due to the extended use of these antibiotics as growth promoters until 1973 [59]. No statistical difference was found between the prevalence of coupled tetracycline and erythromycin resistance in *E. faecium* and *E. faecalis* strains in the types of food analyzed. The fluctuating presence of resistant *E. faecalis* to both vancomycin and linezolid and of resistant *E. faecium* to linezolid is worrisome.

Table 6. Percentages of antibiotic resistance phenotypes of *E. faecalis*

E. faecalis	2002-03	2004-05	2006-07	2008-09	2010-11	2012-13	2014-15	Total
Amp							1.92	0.65
Amp+Tet							3.85	1.29
Amp+Tet+Ery							1.92	0.65
Amp+Tet+Ery+Van							3.85	1.29
Amp+Tet+Ery+Van+Lis							1.92	0.65
Amp+Tet+Ery+Clor							1.92	0.65
Amp+Tet+Van							3.85	1.29
Amp+Tet+Van+Lis							1.92	0.65
Amp+Tet+Van+Clor+Cip							1.92	0.65
Amp+Van+Clor			3.03					0.65
Amp+C		2.33						0.65
Amp+Ery+Lis						4.76	3.85	1.94
Amp+Ery+Van							1.92	0.65
Amp+Van					3.70	4.76		1.29
Amp+Van+Lis							3.85	1.29
Amp+Lis+Amox/Cla						4.76		0.65
Amp+Lis							1.92	0.65
PenG				2.63				0.65
PenG+Tet	4.76							0.65
PenG+Tet+Ery+Van+Tei+C+Cip			3.03					0.65
PenG+Tet+Ery			3.03					0.65
Tet	23.81	46.5	39.4	31.6	14.8	9.52	7.69	38.7
Tet+Ery+Cip		2.33	3.03					1.29
Tet+Ery+C		4.65	3.03	5.26	11.1	4.76	1.92	6.45
Tet+Ery		4.65	12.1	15.8	3.70	4.76		9.03
Tet+Ery+C+Cip					3.70			0.65
Tet+Ery+Van							5.77	1.94
Tet+Ery+Van+Liz							9.62	3.23
Tet+Ery+Van+Clor+Cip+Lis							1.92	0.65
Tet+Ery+Van+Cip+Lis							3.85	1.29
Tet+Van			3.03					0.65
Tet+Lis							1.92	0.65
Tet+Van+Lis						4.76	1.92	1.29
Tet+Cip+Lis						4.76	1.92	1.29
Ery+Van+C+Cip			3.03					0.65
Ery+C				2.63				0.65
Ery					3.70	4.76		1.29
Ery+Tei+Clor					3.70			0.65
C	4.76		3.03		7.41			2.58
Lis		2.33				4.76		1.29
Van						23.8	1.92	3.87
van+Lis						9.52	5.77	3.23
Tei						4.76		0.65

Amp, ampicillin; Pen G, penicillin G; Tet, tetracycline; Ery, erythromycin; Van, vancomycin; Tei, teicoplanin; C, chloramphenicol; Cip, ciprofloxacin; Lis, linezolid; Amox/cla, amoxicillin/clavulanic acid.

Table 7. Percentages of antibiotic resistance phenotypes of *E. faecium*

E. faecium	2002-03	2004-05	2006-07	2008-09	2010-11	2012-13	2014-15	Total
Amp							2.50	0.85
Amp+Tet							2.50	0.85
Amp+PenG			2.38					0.85
Amp+PenG+Van+Cip			2.38					0.85
Amp+Pen+Tet+Ery+Cip+Amox/Cla						7.14		0.85
Amp+Pen+Tet+Ery+Cip							5.00	1.71
Amp+Pen+Tet+Ery+Lis							2.50	0.85
Amp+Pen+Tet+Ery+Amox/Cla	9.09							0.85
Amp+Pen+Tet		1.85						0.85
Amp+Pen+Ery						7.14		0.85
Amp+Pen+Tet+Ery			2.38					0.85
Amp+Pen+Cip+Lis							2.50	0.85
Amp+Ery+Cip							2.50	0.85
Amp+Ery							2.50	0.85
Pen			2.38	1.54	3.33		2.50	3.42
Pen+Tet				1.54				0.85
Pen+Tet+Ery		1.85		1.54	3.33		2.50	3.42
Pen+Ery			4.76		6.67			3.42
Pen+Ery+Van+Tei+C+Cip			2.38					0.85
Pen+Ery+Van+Cip			2.38					0.85
Pen+Ery+Cip							2.50	0.85
Pen+Ery+Lis						7.14		0.85
Pen+Tet+Cip		1.85			3.33			1.71
Pen+Tet+Lis				1.54				0.85
Tet				6.15	6.67	7.14		5.98
Tet+Ery		5.56	4.76	1.54			2.50	5.98
Tet+Cip		1.85			3.33		2.50	2.56
Tet+Ery+C			2.38	1.54				1.71
Tet+Van+Tei+C+Cip			2.38					0.85
Tet+Ery+C+Cip			2.38					0.85
Tet+Ery+Tei+C						7.14	5.00	2.56
Tet+Ery+Van+C							2.50	0.85
Tet+Ery+Van+Cip+Lis							2.50	0.85
Tet+Ery+Cip				1.54				0.85
Ery		3.70	9.52	26.1	20.0	7.14	5.00	27.3
Ery+Lis							5.00	1.71
Van						7.14	2.50	1.71
Van+Cip		1.85						0.85
Tei		1.85	2.38					1.71
C+Cip		1.85						0.85
C		1.85				7.14		1.71
Cip		5.56	2.38	1.54	3.33			5.13
Lis						7.14	5.00	2.56
Cip+Lis							10.0	3.42

Amp, ampicillin; Pen G, penicillin G; Tet, tetracycline; Ery, erythromycin; Van, vancomycin; Tei, teicoplanin; C, chloramphenicol; Cip, ciprofloxacin; Lis, linezolid; Amox/cla, amoxicillin/clavulanic acid.

4. Conclusions

Over the past few decades, Enterococci have become one of our most challenging nosocomial problems. They can survive and live in adverse environmental conditions, and have therefore the ability to colonize different ecological niches and spread within the food chain through contaminated animals and foods.

Our study focused on *Enterococcus* strains collected from 2002 to 2015 and showed an increase of antibiotic resistance for almost all antimicrobials, especially tetracycline and vancomycin in *E. faecalis* and erythromycin, tetracycline, ciprofloxacin and linezolid in *E. faecium*. We suppose that the trend observed has not changed in the biennium 2016-2017, in particular because Italy has taken limited actions so far aimed at reducing farm antibiotic use and resistance.

Despite the fact that many Enterococci with one or more antibiotic resistances have been noted, we can conclude that the use of some antibiotics, such as ß-lactam, may be effective in most cases. As happens in many therapies for the treatment of enterococcal infections, the combined use of antibiotic molecules belonging to different classes can often be very useful, as well as the use of more recently discovered drugs, such as linezolid, to which 91.1% of the tested strains were sensitive.

Finally, although *Enterococcus* spp. do not represent a problem for immunocompetent individuals, there is a need to monitor their presence and reduce their opportunity to spread because, as shown by our results, antibiotic-resistance has sharply increased in recent years. It would be useful to identify new effective antibiotics for the treatment of enterococcal infections and to try to reduce the diffusion of resistant Enterococci, limiting or avoiding the use of strains which present specific antibiotic resistance genes in food production and as probiotics.

Furthermore, it's important to focus on reducing antibiotic use, improving surveillance and better understanding the interaction between enterococci, the hospital environment, and humans. Good hand hygiene and proper cleaning are also essential for patients to minimize the spread of enterococci in the clinical settings.

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