Antimicrobial Resistance in *Enterococcus* spp. of animal origin

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Abstract

Enterococci are natural inhabitants of the intestinal tract in humans and many animals, including food-producing and companion animals. They can easily contaminate the food and the environment, entering the food chain. Moreover, *Enterococcus* is an important opportunistic pathogen, especially the *E. faecalis* and *E. faecium* species, causing a wide variety of infections. This microorganism not only contains intrinsic resistance mechanisms to several antimicrobial agents, but also has the capacity to acquire new mechanisms of antimicrobial resistance. In this review we will analyze the diversity of enterococcal species and their distribution in the intestinal tract of animals. Moreover, resistance mechanisms for different classes of antimicrobials of clinical relevance will be reviewed as well as the epidemiology of multidrug resistant enterococci in the animal field, with special attention to beta-lactams, glycopeptides and linezolid. The emergence of new antimicrobial resistance genes in enterococci of animal origin, as is the case of *optrA* or *cfr*, will be highlighted. The molecular epidemiology and the population structure of *E. faecalis* and *E. faecium* isolates in farm and companion animals will be presented. Moreover, the type of plasmids that carry the antimicrobial resistance genes in enterococci of animal origin will be reviewed.

1.- INTRODUCTION

Enterococcus species are natural inhabitants of the intestinal tract in humans and animals, and due to their ubiquity in human and animal feces and their persistence in the environment, enterococci are considered as indicators of fecal contamination in water (1). Moreover, enterococci serve as important key indicator bacteria for several human and veterinary resistance surveillance systems.

During evisceration process at slaughterhouses, fecal enterococci can contaminate food products of animal origin. As a matter of fact, some studies reported that over 90% of food samples of animal orgin are contaminated with enterococci at the slaughterhouses, mostly with *Enterococcus faecalis,* followed by *Enterococcus faecium* (1, 2). In addition, enterococci are opportunistic pathogens, which become one of the main causes of nosocomial and community acquired human infections, including septicemia, endocarditis, and urinary tract infections, among others (3).

The genus *Enterococcus* presently contains over 50 species, and *E. faecalis* and *E. faecium* are the predominant isolated species accounting for more thant 80% of the isolates. In addition, these two species are considered as the third- to-forth most prevalent nosocomial pathogens worldwide (4). Others, such as *E. hirae*, *E. avium*, *E. durans*, *E. gallinarum*, *E. casseliflavus* or *E. raffinosus,* are rare causes of human clinical infections and thought to be more opportunistic in nature than those caused by *E. faecium* and *E. faecalis* (5-10). *E. faecalis* and *E. faecium* are also the most representative enterococcal species detected in the human intestine, being occasionally detected other species, like *E. durans* and *E. avium* (11). The most commonly encountered enterococcal species in the gut of animals are *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans*, being other species also detected sporadically, or in particular age groups (such as *E. cecorum* in older poultry) (11, 12). Several members of the genus *Enterococcus* can cause bovine mastitis, endocarditis,

septicemia and amyloid encephalopathy with sudden death in chickens (13), and diarrhea in dogs, cats, pigs and rats (12). In the last decade, *E. cecorum* has also emerged as an important poultry pathogen, associated with arthritis and osteomyelitis (14-15).

The intrinsic resistance to several antimicrobial agents compromised the choice of therapeutic options to treat enterococcal infections. Those intrinsic resistances confer resistance to semisynthetic penicillins (low level), aminoglycosides (low level), vancomycin (*E. gallinarum*, *E. casseliflavus* and *E. flavescens* species), or polymyxins and streptogramins (*E. faecalis*) (11). Moreover, enterococci frequently acquire antimicrobial resistance genes through plasmids and/or transposons. The antibiotic resistances in *Enterococcus* spp. have been reviewed previously (3, 16-18), which focus on specific agents (as vancomycin [19-22] or aminoglycosides [23]) or sources (livestock/food [24-26]). The zoonotic transmission potential of antimicrobial resistant enterococci has also been reviewed [27]. In the present chapter, we update the available knowledge on the prevalence and molecular mechanisms of antimicrobial resistance in enterococcal isolates from a wide range of animals (livestock, pets and wildlife) and animal-derived food, with particular emphasis on beta-lactams, vancomycin and linezolid. Furthermore, we outline the major clonal lineages and plasmids responsible for antimicrobial resistance in *Enterococcus* from farm and companion animals.

2. DIVERSITY OF ENTEROCOCCAL SPECIES IN ANIMAL INTESTINAL TRACT.

Enterococci are ubiquitous bacteria in the gastrointestinal tract of humans and a wide range of animals (mammals, reptiles, birds, and some invertebrates). In addition, they are also commonly found in vegetables, water, soil and food derived from animals (including fermented and dairy products) (11). Enterococci are classified as acid lactic bacteria,

highly adaptable to different environmental conditions. They survive over a wide range of temperature (10-45ºC), and pH (4.8-9.6), and are able to grow at high salt concentration (up 6.5% NaCl). Most of them can hydrolyze esculin in the presence of 40% bile salts, a characteristic used for phenotypic identification processes (11). These and other properties explain the utilization of enterococci in diverse roles and, for instance, they have been used as probiotics, starter cultures, bio-preservatives or indicators of fecal contamination of water and sanitary quality of food (28-30).

Genomic analysis revealed that members of the genus *Enterococcus* have a low G+C content, ranging from 34.29% to 44.75% (31). For a long time, *Enterococcus* species were considered as Streptococci of Lancefield group D. In 1984, application of nucleic hybridization and 16S rRNA sequencing led to a reclassification of *Streptococcus faecium* and *Streptococcus faecalis* in the genus *Enterococcus* (32). Currently, this genus includes around 50 species (33). Many of them were discovered in the present century, mostly recovered from non-human sources, such as plants (*E. plantarum, E. ureilyticus*), water (*E. quebecensis*, *E. rivorum*, *E. ureasiticus*), animals (*E. canis*, *E. phoeniculicola*, *E. devriesei*) and food products (*E. thailandicus; E. italicus*) (34-42).

A recent genomic study, which compared the concatenated nucleotide sequences of the core genes of 37 enterococci belonging to a variety of species, divided these strains into 6 branches: (i) *E. faecium* branch (containing *E. faecium*, *E. mundtii*, *E. durans*, *E. hirae*, *E. ratti*, *E. villorum*, *E. thailandicus*, *E. phoeniculicol*), (ii) *E. faecalis* branch (*E. faecalis*, *E. termitis*, *E. quebecensis*, *E. moraviensis*, *E. caccae*, *E. hemoperoxidus*, *E. silesiacus*), (iii) *E. dispar* branch (*E. dispar*, *E. canintestini*, *E. asini*), (iv) *E. casseliflavus* branch (*E. casseliflavus*, *E. gallinarum*, *E. aquimarinus*, *E. saccharolyticus*, *E. italicus*, *E. sulfureus*, *E. cecorum* and *E. columbae*), (v) *E. pallens* branch (*E. pallens*, *E. hermanniensis*, *E. devriesei*, *E. gilvus*, *E. malodoratus*, *E. avium*, *E. raffinosus*) and, (vi) *E. canis* branch,

which contained only one strain (31). Results showed that most strains from human and other mammals were clustered into *E. faecium*, *E. faecalis*, *E. dispar* and *E. pallens* branches, whereas the majority of the bird isolates belonged to *E. casseliflavus* branch.

In 1963, Mundt and colleagues carried out a relevant survey of the occurrence of enterococci among animals living in the wild environment (43). They obtained enterococci from the feces of 71% of the studied mammals, 86% of the reptiles and 32% of the birds. In addition, patterns of food and animal species dependence were observed. In general, enterococci were only isolated sporadically in samples recovered from herbivorous mammals. However, they were abundant in rodents, bats, and larger animals with omnivorous or carnivorous diet (43). But, as demonstrated in several other reports, the differences in the proportions of enterococci in each niche, as well as the species distributions, not only vary according to the diet, also to seasonal changes, individual characteristics (gender, age), and geographic location (11, 44).

In general, *E. faecium*, *E. faecalis*, *E. hirae* and *E. durans* are the most prevalent enterococcal species in the gastrointestinal tract of humans and other mammals (11). *E. cecorum* is also a relevant member of the normal enterococcal microbiota in the gut of farm and pet animals (cattle, pigs, dogs, cats) and birds (poultry and pigeons) (45-47). However, in chickens, a significant age-dependent increase in gut colonization has been reported for this species. In fact, *E. cecorum* has found to be a dominant part of the enterococcal gastrointestinal microbiota in mature chickens (48). Some other species, such as *E. gallinarum* and *E. avium*, which were first described in chickens, have not been frequently detected among enterococcal gut population in poultry (49, 50).

In cattle and swine, the proportions of the enterococcal species varies across studies. *E. faecium*, *E. durans*, *E. hirae* and *E. faecalis* were unanimously found in different surveys (46, 50-52). In some works, *E. faecalis* was the predominant enterococcal species in the

gut of bovine and swine (46, 53). In others, *E. hirae* and *E. faecium* were described as the more abundant bacteria in both livestock species (44, 51, 52). As observed, variations between geographical regions might explain these differences in the composition of the enterococcal populations (44). *E. casseliflavus*, *E. gallinarum*, *E. avium* and *E. cecorum* have also been reported as part of the bovine and swine microbiota, but they were present in lower proportions (46, 50, 51). Additionally, some minoritary species, such as *E. villorum* and *E. thailandicus*, have been sporadically detected in feces from cattle and pigs (52, 54, 55).

The enterococcal microbiota of the intestinal tract of dogs and cats showed a predominance of *E. faecalis* and *E. faecium*, followed by *E. hirae* (56-59). *E. avium* has been commonly isolated in canines and also, although in less proportion, in felines' feces (56, 57). Other species, such as *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. cecorum* and *E. raffinosus*, have been occasionally reported (56, 58, 59). In addition, some newly characterized species were isolated from anal swabs and chronic otitis externa (*E. canis)* and fecal samples (*E. canintestini*) of dogs (34, 60).

Enterococci are also normal residents of the gut of a wide range of free-living animals. In pigeons, the predominant species is *E. columbae* and, to a lesser extent, *E. cecorum*. However, *E. faecium* and *E. faecalis* are rare in these birds (61). Other study reported a high prevalence of enterococci among three different species of coraciiform birds (74%), with a dominance of *E. faecalis*, followed by *E. casseliflavus* (62). In Portugal, *E. faecium* was the most frequently encountered species in buzzard fecal samples (63), and *E. faecium*, *E. durans* and *E. gallinarum* in feces of a variety of wild birds (64). Enterococcal gut microbiota has also been analyzed in wild marine species. *E. faecium* was identified as the most abundant species in echinoderms collected from Azorean waters. Minor species, such as *E. hirae*, *E. faecalis* and *E. gallinarum*, were also detected (65). In a recent study

from Southern Brazil, different wild marine animals were analyzed using real-time quantitative PCR to identify and quantify enterococci in feces. These bacteria were found in all the studied animal species, with a dominance of *E. faecalis* and *E. mundtii* in most of the marine mammals, *E. faecalis* in green turtles, Magellanic penguins and albatross, and *E. hirae* and *E. gallinarum* in white-backet stilt (66). Enterococci are also a relevant part of the facultative anaerobic microbiota of the gastrointestinal tract of large wild mammals (wolf, wild-boar, deer…) and rodents (67-69).

Administration of antibiotics in both human and animal medicine may shift the gut microbial community, allowing drug-resistant strains (e.g. vancomycin-resistant enterococci) to proliferate dramatically. As many enterococcal infections are caused by normal inhabitants of the gastrointestinal tract that become opportunistic pathogens, the selection of antibiotic-resistant strains raises the risk of developing difficult-to-treat infections. The following sections give an overview of the mechanisms and prevalence of antimicrobial resistance in enterococci in the animal setting.

3.- ANTIMICROBIAL RESISTANCE IN ENTEROCOCCI OF ANIMALS AND FOOD OF ANIMAL ORIGIN

3.1.- Beta-lactam resistance

Enterococci are intrinsically resistant to cephalosporins and present a natural reduced susceptibility to penicillins, due to the expression of low affinity penicillin binding proteins (PBPs) that bind weakly to β-lactam antibiotics. For this reason, the minimum inhibitory concentrations (MICs) for penicillins are higher in enterococci than in streptococci or other Gram-positive organisms, that do not produce chromosomally-encoded low-affinity PBPs (17). *E. faecalis* isolates normally exhibit lower MIC values for penicillins than *E. faecium* (18).

All enterococci have at least five PBPs, and six putative PBP genes have been detected by genomic analysis in the *E. faecalis* and *E. faecium* species (class A: *ponA, pbpF, pbpZ*; class B: *pbp5, pbpA, pbpB*) (18). The expression of the species-specific chromosomally-located *pbp5* gene, which encodes PBP5, with low affinity binding for penicillins and cephalosporins, is associated to the intrinsic resistance for beta-lactams. In *E. faecium*, the *pbp5* gene is included within an operon, together with other two genes that are also implicated in cell wall synthesis (*psr* and *ftsW*) (18).

Acquired (enhanced) resistance for penicillins (penicillin or ampicillin) has been frequently detected among clinical *E. faecium* isolates, being rare in the species *E. faecalis*. High level ampicillin resistance in *E. faecium* (≥ 128 µg/ml) has been associated with the increased production of PBP5 (requiring a higher concentration of the agent to saturate the active site) or to specific amino acid changes in its sequence, that make the low affinity PBP5 even less susceptible to inhibition by penicillins (70, 71). The amino acid substitutions near the Ser-Thr-Phe-Lys, Ser-Asp-Ala and Lys-Thr-Gly motifs, which are part of the active-site cavity, seems to be the most significant ones (16).

Combinations of specific amino acid changes in the C-terminal transpeptidase domain of PBP5 (specially the substitution Met-485-Ala/Thr, but also the changes Ala-499-Ile/Thr, Glu-629-Val or Pro-667-Ser), and the insertion of serine or aspartic acid after position 466, have been associated to ampicillin resistance in *E. faecium* isolates (72-76). It has been found that single substitutions at positions 485, 499, 629 and 466-insertion have only slight influence in ampicillin MIC, but when combined, the effect increases. Mutations in genes encoding other species-specific proteins that participate in the cell wall synthesis may also slightly increase the MIC value (76).

Two distinct allelic forms have been identified when the whole sequence of *pbp5* gene is considered, which differ in 5% of the sequence, yielding two types of PBP5 (PBP5-S and

PBP5-R) with changes in 21 amino acid residues. The type PBP5-S is usually detected in community-associated ampicillin-susceptible *E. faecium* isolates (MIC usually ≤ 2 µg/ml), and the type PBP5-R usually detected in hospital-associated ampicillin-resistant isolates (MIC usually ≥ 16 µg/ml) (77, 78). A hybrid-like type of PBP5 (PBP5-S/R), with a sequence between the other two types, has been observed in some isolates with a MIC for ampicillin around 4 µg/ml (77, 78).

Considering the population structure of *E. faecium*, two main lineages have been postulated in humans: 1) Subclade A1: hospital-associated, enriched in mobile genetic elements, usually implicated in human infections and, in most cases, ampicillin-resistant (MIC ≥ 16 µg/ml) with the consensus allele *pbp5*-R; and 2) Clade B: community-associated, detected in isolates of healthy humans (not implicated in infections), generally ampicillin-susceptible (MIC ≤ 2 µg/ml), harboring the consensus allele *pbp*5-S. The subclade A2 includes *E. faecium* isolates mostly of the animal setting, exhibits a wide range of ampicillin MIC values, (0.5-128 µg/ml), and generally carries the hybrid-like *pbp*5 allele (*pbp*5-S/R) (72, 78, 79). In addition to amino acid sequence alteration in PBP5, elevated levels of this protein are also observed in higly-ampicillin-resistant isolates of clade A (subclade A1 and part of A2), but not in the ampicillin-susceptible isolates of subclade A2 and clade B, suggesting a differential regulation process in each clade. The upstream region of *pbp*5 seems to have a role in the level of expression of the gene (72).

In *E. faecalis*, acquired ampicillin resistance is unusual, but is generally mediated by mutations in *pbp*4 (27, 80). Selected strains of *E. faecalis* produce a plasmid-mediated beta-lactamase that is similar to the enzyme produced by *S. aureus* (17, 81), encoded by the *blaZ* gene, although some polymorphisms in this gene have also been detected in some isolates. This beta-lactamase is expressed in a constitutive way in *E. faecalis*, in

contrast to the inducible production in *S. aureus.* The enzyme is produced in low amount in *E. faecalis*, and for this reason, the strain can appear as ampicillin susceptible when the MIC is tested *in vitro*. In any case, this mechanism of resistance is very infrequent in *E. faecalis.* Very unusual beta-lactamase producer *E. faecium* strains have also been reported (82). Chromosomal beta-lactamase-encoding genes conferring ampicillin resistance have also been detected in *E. faecium* isolates (83).

It has been previously reported the *in vitro* transferability of *pbp5* in *E. faecium* isolates (84), what suggests a mechanism by which high-level ampicillin resistance conferred by mutated *pbp5* alleles could be disseminated among clinical isolates. Moreover, Novais *et al.* (85) demonstrated the *in vitro* ampicillin-resistance transference by conjugation in 28% of the *E. faecium* isolates from a pig farm environment, although the genetic basis of this transference was not determined. Co-diversification of *E. faecium* core genome and *pbp*5 has been recently analyzed showing evidences of *pbp*5 horizontal transfer (86).

Different studies have evaluated the prevalence of penicillin or ampicillin resistance in enterococci from food producing animals, pets or wild animals, as well as in those from food of animal origin. In relation with *E. faecium*, the prevalence of resistance is variable depending on the countries and the type of animals. In this sense, no resistant *E. faecium* isolates were detected in a surveillance study performed in cattle population at slaughter in Australia (87), but a rate of 30% of resistance was detected in isolates of poultry in Portugal (88). In relation with pets, the following ampicillin resistance rates were reported among *E. faecium* isolates: 63%/37% in dogs/cats in the USA, and 3% in pets in Portugal (58, 88). Moreover, ampicillin resistant *E. faecium* isolates were detected in 23% of the dogs screened in a cross-sectional study in the United Kingdom and in 76% of the dogs analyzed in a longitudinal study in Denmark (89). Most of these resistant isolates belonged to the hospital-adapted clonal complex CC17. Frequencies of ampicillin

resistance in the range of 4.5-7.7% have been detected in *E. faecium* isolates recovered from different wild animals (wild boar, Iberian wolf or Gilthead seabream) (74, 90, 91), but no resistant isolates were detected in Iberian Lynx (92).

A surveillance study was performed in the USA analyzing the prevalence of antimicrobial resistance in 21077 *Enterococcus* isolates obtained from retail meat samples in the USA between 2002-2014, through the National Antimicrobial Resistance Monitoring System (NARMS) (2). A low frequency of ampicillin resistance was detected among *E. faecium* isolates of ground beef and pork chops (4% and 2.7%, respectively), but higher percentages were detected in the case of retail chicken (26%), and even higher for ground turkey (62.6%). Bortolaia *et al.* (25) reviewed the data of ampicillin resistance reported in different European countries (Denmark, Sweden, The Netherlands, Slovenia) and the USA for *E. faecium* isolates recovered from poultry meat, comparing with human isolates in the same countries (93-95). Human isolates showed very high rates of ampicillin resistance in works of all countries (>80% but resistance in food isolates was significantly lower than in humans. It is of note the detection of 10% of ampicillin resistance in *E. faecium* of (imported) broiler meat in Denmark and >50% of resistance in isolates of turkey meat in the USA. No ampicillin resistant *E. faecalis* isolates (with very few exceptions) have been reported in animals or food of animal origin.

3.2 Glycopeptide resistance

3.2.1.- Mechanism of resistance

Vancomycin and teicoplanin are two important members of the glycopeptide family, used for the treatment of severe human infections. Avoparcin, another member of this family, has been extensively used in the past as growth promoter in food producing animals in many countries.

The mechanism of action of glycopeptides is the inhibition of the synthesis of the bacterial cell wall, by the link to the D-Ala-D-Ala terminus of the pentapeptide precursor of the peptidoglycan, preventing cross-linking of peptidoglycan chain and inhibiting cell wall synthesis. The main mechanism of glycopeptide resistance in enterococci implicates the alteration of the peptidoglycan synthesis pathway. In this sense, the terminus D-Ala-D-Ala of the pentapeptide to which vancomycin binds, is modified to D-Ala-D-Lac (causing high level vancomycin resistance, >64 µg/ml) or to D-Ala-D-Ser (low level vancomycin resistance, 4-32 µg/ml). These modified cell-wall precursors bind glycopeptides with reduced affinity (about 1,000-fold and 7-fold for D-Lac and D-Ser substitutions, respectively) (18, 22).

The first vancomycin resistant enterococci (VRE) with an acquired mechanism of resistance were detected three decades ago in clinical *E. faecium* isolates in France and United Kingdom (96, 97). Since then, VRE have been extensively described in hospitals worldwide, and especially frequent in the United States (USA) since the decade of the 90´s of last century, mostly in patients of intensive care units, and in a lower level in Europe since the 2000´s (21). According with surveillance data of the ECDC (EARS-Net), the EU/EEA population-weighted mean percentage of vancomycin resistance in *E. faecium* was of 11.8% in 2016, and national percentages ranged from 0% to 46.3%; the prevalence of vancomycin resistance in the case *of E. faecalis* was lower (98).

Vancomycin resistance is mediated by *van* operons, which encode the modified peptidoglycan precursors. To date, eight different *van* operons have been identified in enterococci mediating acquired vancomycin resistance (*vanA, vanB, vanD, vanE, vanG, vanL, vanM,* and *vanN*), and one additional operon in intrinsic vancomycin resistance (*vanC*) (18, 19, 99-102). Three variants have been described of *vanC* gene (*vanC1, vanC2 and vanC3*), intrinsic of the species *E. gallinarum, E. casseliflavus* and *E. flavescens,*

respectively. Moreover, different subtypes have been identified for *vanB* (*vanB1, vanB2* and *vanB3*), *vanD* (*D1* to *D5*) and *vanG* (*G1, G2*) (100, 103, 104). An additional variant, *vanF*, has also been described, but until now only in the environmental microorganism *Paenibacillus popilliae* (105).

The *vanA* and *vanB* are the most frequent genotypes among VRE with acquired resistance mechanisms of humans and animals, mostly among *E. faecalis* and *E. faecium* species. The genotypes *vanD, vanE, vanG, vanL, vanM* and *vanN* are very unusual in VRE isolates, and the species *E. faecalis* (*vanE/G/L*) and *E. faecium* (*vanD/M/N*) are the most common carriers (22).

The *vanA* operon is associated with the transposon Tn*1546,* and includes seven open reading frames transcribed under two different promoters (106). Regulation is mediated by a *vanS-vanR* (sensor-kinase-response regulator) two-component system, transcribed with a common promoter (107). The remaining genes are transcribed from a second promoter (22). The proteins encoded by *vanH* (dehydrogenase that converts pyruvate into lactate) and *vanA* (ligase that forms D-Ala-D-Lac dipeptide) modify the synthesis of peptidoglycan precursors; moreover the proteins encoded by both *vanX* (dipeptidadase that cleaves D-Ala-D-Ala) and *vanY* (D, D-carboxipeptidase), interrupt the formation of the D-Ala-D-Ala end of the pentapeptide, and *van*Z gene is related to teicoplanin resistance (22, 108). Different IS elements can be included into the *vanA* operon, rendering different variants (109).

The *vanB* operon has been associated to different transposons (Tn*1547,* Tn*1549* and Tn*5382*). The Tn*1549* is widely prevalent among *vanB*-type enterococci, in most of the cases located in the chromosome and less frequently on plasmids (22). The structure of the *vanB* operon is similar to the one of *vanA*, with two promoters and seven open reading frames, but with important differences, mostly in the two-component signaling regulatory

system (encoded by *vanR*$_B$ and *vanS*$_B$), and in the absence of an homolog of *vanZ* (substituted by *vanW*, of unknown function); consequently, *vanB*-enterococci show vancomycin resistance (high or low level) but teicoplanin susceptibility (22, 108).

The structure of the different *van* operons and their mechanisms of action have been extensively reviewed in previous studies (17-19, 21, 22, 108, 110).

Origin of vancomycin resistance. Partially pre-assembled glycopeptide resistance-associated gene clusters present in environmental organisms are suggested as the source of the vancomycin resistance genes in VRE (105, 111). The environmental organism *P. popilliae*, carrier of a *vanF* variant with a high similarity at the amino acid level to *vanA*, has been suggested as the potential origin of vancomycin resistance in enterococci. In a lesser extent, this role could also be attributed to glycopeptide-producing organisms (e.g. the vancomycin-producing organism *Amycolatopsis orientalis*), which require these genes to inhibit the action of produced glycopeptides (111). Nevertheless, the genes in these organisms are probably not the direct source of the enterococcal vancomycin resistance genes since they are similar, but not identical; in this sense, transference could have occurred from a common ancestral bacterium, or via one or more bacterial intermediaries. In addition, considering the differences in G+C content, as well as the sequence homology among different organisms, it is possible that the genes of the *van* cluster could have more than one origin (111).

3.2.2.- Historical aspects related to glycopeptide resistance

During the decade of the 1990s, VRE with the *vanA* genotype emerged in food producing animals, healthy humans, food products and environmental samples throughout Europe and other countries; this fact was linked to the use of the glycopeptide avoparcin since the mid-1970s, in sub-therapeutical concentrations, as animal growth promoter (22, 26,

112, 113). This hypothesis was tested in poultry flocks and pig herds receiving or not avoparcin, confirming the significant role of avoparcin in VRE selection in the animals (112, 113). This association was also corroborated in an animal model with young chickens receiving avoparcin supplementation (114). Avoparcin as growth promoter was banned in the European Union (EU) in 1997, and a clear decrease in VRE fecal carriage in food producing animals and healthy humans was observed (115), as well as in food-derived products. Nevertheless, VRE persisted in the animal setting many years after avoparcin ban (116, 117). A similar situation happened in Taiwan after the ban of avoparcin in 2000 that resulted in a clear decrease of VRE prevalence in chicken, although still persisted in this animal population (118). In relation with dogs, high rates of fecal VRE carriage was reported before avoparcin ban in the EU (119), although no VRE was detected in dogs in Spain after a decade of banning (120). The frequency of human infections by VRE in the EU was low during the period of high prevalence in animals, but an increase in the frequency of VRE-related human infections was evidenced since 1999 (22).

The situation in the United States and Canada was completely different comparing with EU. Avoparcin use has never been approved in animal production in those countries, and VRE was not reported in animals until the end of 2000 decade (20, 76, 121, 122). Nevertheless, in North America, VRE was very frequent causing human infections, especially in patients of the Intensive Care Units, what was attributed to the high use of vancomycin in humans (22, 123). The differences in VRE prevalence in humans and animals in the EU and USA before and after the avoparcin ban in the EU, introduce some doubts about the possible routes of transmission of VRE determinants between animals and humans (22, 124).

Different theories have been postulated to explain the persistence of VRE in food-producing animals after the avoparcin ban in the EU and in other countries, as is the coselection by the use of other antimicrobials (like erythromycin or tetracycline). In fact, it has been shown that *vanA* and *erm*(B) genes (this last one implicated in erythromycin resistance) are frequently located in the same transferable plasmids (113). Moreover, the *tcrB* gene, implicated in copper resistance, has been detected in pig *E. faecium* isolates in the same plasmid as the *vanA* and *erm*(B) genes (125). On the other hand, the presence of plasmid addition systems in the same plasmid that carries *vanA* gene could forces bacteria to retain the resistance (125).

3.2.3 VRE in food producing animals and food of animal origin

Table 1 and 2 summarize the papers that have been published related to the prevalence and mechanisms of vancomycin resistance in enterococci of food-producing animals, and food of animal origin, respectively, as well as the genetic lineages of the isolates (when available). Data have been organized by animal species (poultry, pigs or cattle, among others), and by the year the isolates were recovered. Many of the studies have been performed in different European countries, but also in many countries of America, Africa, or Asia, as well as in Australia and New Zealand.

Most of the surveys on **food producing animals** reported *E. faecium* as the major species of the genus *Enterococcus* exhibiting acquired resistance to vancomycin, in most of the cases with the *vanA* genotype. However, *vanA*-containing *E. faecalis*, and in a lesser extent *E. durans* and *E. hirae* isolates, have also been quite frequently detected in food producing animals **(Table 1)** (27, 85, 87, 114, 121, 122, 125-165). Other enterococcal species have occasionally been reported as *vanA*-carriers, as is the case of *E. mundtii* in poultry in Hungary (130), *E. casseliflavus* in cattle in France (158), and in equine and swine in Italy (159). Available data indicates that *vanA* gene was, by far, the main

responsible for acquired VRE cases in food-producing animals worldwide, regardless the species. Nevertheless, the *vanB* gene (and specially the *vanB2* variant) was occasionally detected. The first detection of *vanB2* in animals was in a vancomycin-resistant *E. hirae* isolate recovered from a pig in Spain in 2008 (145); later on, *vanB*-positive *E. faecium* and *E. faecalis* isolates were detected in poultry in Czech Republic (132) and in *Enterococcus* spp. in pigs in South Africa (147). Moreover, *vanC1* was detected as an acquired gene in isolates of the species *E. faecium, E. faecalis* and *E. mundtii* in poultry in Australia (140). In most of the studies, VRE were detected when a selective protocol with media supplemented with vancomycin was used **(Table 1).** Resistance frequencies varied depending on the type of animals tested (poultry: 0-77%; pigs: 0-25.3%; and cattle: 0-0.5%), the year in which the study was performed, the country and the protocol used for VRE recovery **(see Table 1).** *vanA*-containing enterococci have also been detected in ostriches and mullet fish in Portugal (prevalence of resistance of 7.4% and 3.9%, respectively) (164). In eight of the revised papers in which VRE were detected in food-producing animals, the data of MLST was provided for *vanA*-positive *E. faecium* (most of isolates) or *E. faecalis* isolates. A wide variety of sequence types (ST) were identified among the *E. faecium* isolates from poultry and pigs (>30 different STs) (27, 85, 121, 122, 127, 129, 144, 156). Also, the lineage ST6 (CC2) was identified in *E. faecalis* of pig origin (85).

The *E. faecium* species carrier of *vanA* gene was the most frequent VRE detected in **food of animal origin**. Nevertheless, *vanA*-containing *E. faecalis*, *E. durans* and *E. hirae* isolates were, as well, frequently detected in these type of samples **(Table 2) (**2, 118, 128, 133, 162, 166-194). VRE with *vanB* gene was found in *E. faecium* isolates from veal and chicken in Spain (ST17-*vanB2*) (188), and in different types of food in Greece (*vanB2/3*) and Spain (*vanB*) (181, 190). It is interesting the identification of the unusual *van*N gene

in 5 *E. faecium* isolates of chicken meat origin in Japan, showing low level of vancomycin resistance (MIC 12 µg/ml) (177). Moreover, of relevance is the unusual detection of *vanA*-containing *E. cecorum* isolates in chicken samples from Japan (168), *vanA*-positive *E. gallinarum* in fishes from Egypt (193), or *vanC1*-positive *E. faecalis* isolates in sheep milk samples from Spain (192). The frequencies of detection of VRE with acquired resistance in food samples were variable **(Table 1).** In chicken and pork food samples analyzed in the period 1996-1999, the prevalence was in the range of 4.2-34% (**Table 2**), with a few exceptions (1.3%) (167). Very high frequencies were detected in different types of food in Korea (44%) (133), but no VRE were found in the studies performed in the USA (2, 171, 185). In some cases, isolates showing a phenotype usually associated to *vanB* genotype (high-level resistance to vancomycin, susceptibility to teicoplanin) were detected in *Enterococcus* strains harboring the *vanA* gene (118, 168, 173).

3.2.4. VRE in companion animals

Table 3 shows the detection of VRE with acquired mechanisms of resistance in companion animals. *vanA*-containing *E. faecium* has been the unique type of VRE with acquired resistance reported in dogs and cats (136, 145, 195-202). These isolates, recovered from fecal samples in the period 1996-2003, were found in USA, Spain and Portugal, with variable frequencies of detection (ranging from 2.8 to 22.7%) (136, 145, 195, 196). No VRE have been detected in studies performed in the following years (**Table 3**), not even in sick dogs (197, 200). Vancomycin-resistant *E. faecium* and *E. durans* isolates have been detected in fecal samples of equids obtained between 2007-2008 (prevalence 4.4%), in a study performed in Portugal (202).

3.2.5- VRE in free-living animals

Table 3 also shows the detection of VRE with acquired mechanisms of resistance in free-living animals, including different species of mammals and birds (136, 165, 203-226). Many studies have been performed in this type of animals, including various countries of Europe, America (USA, Canada and Brazil) and Africa (Tunisia and Tanzania). The most frequently detected mechanism of resistance was *vanA*, mainly among *E. faecium* isolates, followed by *E. faecalis* (*E. durans* and *E. hirae* were infrequently detected). Occasionally, enterococci were *vanB*-carriers: two small mammals (*Rattus rattus*) harbored *vanB2*-containing *E. faecalis* ST6 isolates in Spain (204), and *E. faecium vanB* was detected in wild game meat also in Spain (226). The frequencies of detection of *vanA*-containing enterococci in wild animals ranged from 0% to 13.5%, with the highest values detected in red foxes, seagulls and buzzards in Portugal (9-13.5%) (216, 220, 222). It is of interest the detection of *vanA*-containing *E. faecium* isolates ascribed to different sequence types included in the high-risk clonal complex CC17 (ST18, ST262, ST273, ST280, ST313, ST362, ST412, ST448, and ST555). These isolates were detected in corvids in USA and in mullet fish, gilthead seabream, seagulls, buzzards, partridges, red foxes and Iberian wolves in Portugal **(Table 3).**

3.3.- Resistance to linezolid

The wide spread of VRE in many countries make necessary to look for other therapeutic options, and linezolid is an important one. This oxazolidinone, introduced in 2000 in USA and in 2001 in the United Kingdom, is an important agent for the treatment not only of VRE, but also of other gram-positive bacteria, as is the case of methicillin-resistant *Staphylococcus aureus* (MRSA).

Linezolid resistance is still unusual among enterococci but is emerging in the last years in human and animal isolates (227). Mutations in the central loop of the domain V of the 23S rDNA is the most common mechanism of resistance in enterococci, being the amino

acid change G2576T the predominant one, although other changes have also been described (G2505A, U2500A, G2447U, C2534U or G2603U) (18). *E. faecalis* and *E. faecium* possess among four and six 23S rDNA alleles per genome, respectively, and depending on the number of mutated *versus* wild-type alleles per genome, correlate with the level of resistance of the isolates (227). In some cases, this mechanism appears along the course of treatment with oxazolidinones, and nosocomial transmission of linezolid-resistant enterococci has been reported (228). Linezolid-resistant *E. faecalis* and *E. gallinarum* isolates of swine origin were detected in China (MIC 8-16 µg/ml), and the nucleic acid change G2576T was identified in the 23S rDNA of these isolates (229). Mutations in the ribosomal proteins L3, L4 and L22, can confer decreased susceptibility to linezolid in enterococci and staphylococci (230).

In the last years, concern exists about the emergence of transferable resistance to linezolid, associated with the acquisition of the *cfr* gene, or with the recently described *optr*A gene. The *cfr* gene has been detected in enterococci of both human and animal origins (231) and encodes an rRNA methyltransferase that modifies the adenine residue at position 2503 in domain V of the 23S rRNA; it confers resistance to oxazolidinones, phenicols, lincosamides, pleuromutilins and streptogramin A (phenotype named as $PhLOPS_A$) (18). Among oxazolidinones, linezolid is mostly affected by *cfr* gene, showing telizolid, a new compound of this family, increased activity in *cfr*-positive enterococci, and so, isolates being susceptible for this agent. **Table 4** shows a summary of the data published until now in relation to linezolid resistance mechanisms in enterococci of animal and food origins, as well as in enterococci of environmental origin (229, 232-241).

The *cfr* gene was identified for the first time in enterococci in 2011, specifically in an *E. faecalis* isolate recovered in a dairy farm in China (232). Since then, the *cfr* gene has been detected in human clinical *E. faecalis* isolates (242), as well as in swine *E. casseliflavus*,

E. gallinarum and *E. faecalis* isolates in China or Brazil (233-235), and in a cattle *E. faecalis* isolate in China (234). A second variant of the *cfr* gene, named *cfr*(B), has been described in *E. faecium* isolates of human origin. This new plasmid-located variant, is more similar to a *cfr-like* gene of *Clostridium difficile* than to the *cfr* genes of staphylococci or other enterococcal species (243, 244), and has so far not been detected in enterococci of animal origin.

The novel *optr*A gene confers transferable resistance to oxazolidinones (both linezolid and telizolid) and phenicoles (chloramphenicol and florfenicol) and has been detected in *E. faecalis* and *E. faecium* isolates of both human and animal origins (236). This gene encodes an ABC transporter and has been detected more frequently in *E. faecalis* than in *E. faecium* isolates, and also more frequently in isolates from food-producing animals (pigs and chicken), than in those of human origin (236). The *optr*A gene has been detected both in chromosomal as well as in plasmidic location in animal and human *E. faecalis* and *E. faecium* isolates. As shown in **Table 4,** *optr*A-positive enterococci have been detected in food producing animals (poultry, pigs and, occasionally, cattle) in Asiatic countries, mostly in *E. faecalis* and *E. faecium* belonging to many different sequence types, and sporadically in *E. gallinarum*. The prevalence of *optr*A-positive enterococci represents 10% and 5.7% of total *E. faecalis* and *E. faecium*, respectively, obtained from fecal samples of poultry and pigs in a study performed in China (236). In a recent study carried out in Korea, 11,659 *E. faecalis* and *E. faecium* isolates obtained from fecal and carcass samples of healthy cattle, pigs and chickens from farms and slaughter houses during 2003-2014, were tested for linezolid resistance, detecting a rate of resistance of 0.33%, mainly attributed to *optr*A carriage (238). The *optr*A gene has also been detected in sporadic isolates of *E. faecalis* and *E. faecium* (n=3) obtained in meat samples in Denmark (imported poultry, and veal), that represented <0.1% of total enterococci

recovered from these samples (239). In the American continent, *optr*A has been detected in three *E. faecalis* isolates of poultry meat origin, co-harboring *fex*A, *tet*(L) and *Isa*(A) resistance genes (240). Both *cfr* and *optr*A genes have been detected associated in VRE isolates of human origin (245), but not in animal isolates so far.

The *optr*A gene has also been detected in two *E. faecalis* isolates of the lineage ST86 recovered from urban wastewater in Tunisia, accounting for 1% of all chloramphenicol resistant enterococci tested (241); the *optr*A gene was located within a transferable mosaic plasmid, that also contained the *fex*A and *erm*(A) genes.

At least 12 and 5 polymorphic variants of the *optr*A gene have been detected among human and animal enterococci, respectively (237, 246-248). The wild OptrA type ($OptrA_{E349}$), and the variants Tyr176Asp + Lys3Glu-Gly393Asp or Thr481Pro or Thr112Lys or Gly393Asp, have been found among animal isolates (237, 246). Functional *cfr* and *optrA* genes have been identified in both enterococci and *S. aureus.* In most of the animal isolates, the *optr*A gene is located close to other genes, as is the case of *fex*A (implicated in phenicol resistance) and a novel *erm*(A)-*like* gene. This *erm*(A)-*like* gene encodes an rRNA methylase, which shows 85.2% amino acid identity to the Erm(A) protein of transposon Tn*554* of *S. aureus* (237).

Most of the *cfr*-positive enterococci of food producing animals (>90%) showed a MIC for linezolid of ≥8 µg/ml, but two *E. faecalis* isolates presented a MIC of 4 µg/ml. In relation with *optr*A-positive isolates of food-producing animals and food origin, they showed a linezolid MIC in the range 2->8 µg/ml, presenting 19% of the isolates MICs in the range 2-4 µg/ml (categorized as susceptible according to EUCAST breakpoints and susceptible-intermediate according to CLSI) **(Table 4).** It is interesting to remark that *cfr*- and *optr*A-positive enterococci could appear as linezolid-susceptible, probably leading to an underestimation of their real incidence.

Oxazolidinones are not used in food-producing animals. Nevertheless, the emergent detection in these animals of linezolid-resistant enterococci carrying the *optr*A gene in transferable plasmids, linked to resistance genes for antibiotics commonly used in animals (phenicols, tetracyclines, lincosamides and aminoglycosides), suggest its role in the co-selection of multiresistant bacteria, which pose a risk for public health.

Summarizing, transferable linezolid resistance genes, mostly *optr*A, have been detected in enterococci of food producing animals and food of animal origin in different countries of Europe, America and Asia, but up to date not in Africa. These mechanisms of resistance have not been detected so far, to our knowledge, in pets or in wild animals.

3.4.- Resistance to aminoglycosides

Enterococci are intrinsically resistant to clinically achievable concentrations of aminoglycosides due to their low cell wall permeability. In addition, some species as *E. faecium* [*aac(6')-Ii*], *E. durans* [*aac(6')-Id*] and *E. hirae* [*aac(6')-Ih*], intrinsically express a chromosomal-encoded acetyltransferase that confers resistance to tobramycin, kanamycin and amikacin (249). The chromosomally encoded methyltransferase EfmM has been exceptionally described in an *E. faecium* isolate (250) codifying resistance to kanamycin and tobramycin. Acquired resistances to aminoglycosides are detected in strains from both animals and humans and usually concern to high-level of resistance to gentamicin, kanamycin and streptomycin.

High-level resistance to gentamicin in enterococcal isolates from animal origin was first described in 1998 in Denmark (251) and in 2001 in United States (252). The acquired genetic mechanisms identified in animal isolates are identical to those described in human isolates. The most frequent ones are the bifunctional enzyme encoded by *aac*(6')-*Ie*-*aph*(2'')-*Ia* (conferring resistance to gentamicin, kanamycin, amikacin, netilmicin and

tobramycin) and the *aph*(3)-*III*a (conferring resistance to kanamycin and amikacin) (23, 253). High-level gentamicin resistance can also be due to the expression of the unusual *aph*(2")-*Ic*, *aph*(2")-*Id*, *aph*(2")-*Ie* and *aph*(2")-*Ib* genes (17, 23); the *aph*(2")-*Ic* seems to be more frequent in enterococci of animal origin and some farm animals could be a reservoir of this gene (252). High-level resistance to streptomycin is commonly caused by punctual ribosomal mutations, although acquisition of some modifying enzymes has been also described [*ant*(3'')-*Ia* and *ant*(6')-*Ia*]. **Table 5** shows a summary of papers (in the period 2013-2017) in which the rates of antimicrobial resistance (high-level gentamicin, and others as tetracycline, erythromycin or ciprofloxacin) is analyzed in enterococcal isolates from animals (65, 15, 87, 90, 92, 135, 141, 143, 147, 153, 154, 198, 205, 209, 254-276).

3.5.- Resistance to Tetracycline

This family integrates several antibacterial active compounds (277), although tetracycline, chlortetracycline, oxytetracycline, and doxycycline are the most used in veterinary. Despite the extensive review about the tetracyclines resistance mechanisms lead in 1996 by Roberts (278), a most recent update was published in 2005 (279). Almost 60 tetracycline resistance genes have been described, although the most frequent ones in *Enterococcus* are those implicated in ribosomal protection [*tet*(M), *tet*(O), *tet*(S)], efflux or enzymatic inactivation [*tet*(K), *tet*(L)]. In *Enterococcus*, as occurs in other gram positives microorganisms, the ribosomal protection protein mechanism encoded by the *tet*(M) gene is the most frequent, with independence of the origin of the strains. The transferability of the tetracycline resistance determinants in absence of plasmids has been described from the first studies (280), being the Tn*916*/Tn*1545* conjugative transposon family carrying the *tet*(M) gene the responsible, usually in combination with the *erm*(B) gene.

3.6.- Resistance to Macrolides/Lincosamines/Streptogramins

Numerous chemically diverse compounds are integrated into the macrolide family, with erythromycin as the most representative. Resistance to this antibiotic was immediately reported after their introduction in human clinical use in 1952; moreover, enterococci are intrinsically resistant to clindamycin and lincomycin. Tylosin, spiramycin and virginamycin were widely used in pigs and other animals before the EU limited their used. After the ban, the erythromycin resistance in *Enterococcus* strains from animals decreased spectacularly (281), demonstrating the link between the antibiotic consumption and the increase of the resistance rates, even in different environments.

Chromosomal intrinsic resistance to macrolides by *msr*(A) and to lincosamides by *linB* in *E. faecium* has been described (282, 283). Acquired resistance to macrolides can be codified by various genetic determinants (up to 92 have been described) (284), although the most common worldwide is *erm*(B), usually carried by Tn*917* that is widespread in human and animal isolates. Other relevant genes in the genus *Enterococcus* are the efflux genes *mef*(A) conferring resistance to macrolides, *vgb*(A) to virginiamycin, *lnu*(B) to lincosamide, *vat*(D) and *vat*(E) to streptogramins.

3.7.- Resistance to Quinolones

Fluoroquinolones have a reduced antimicrobial activity against enterococci, with levofloxacin and moxifloxacin as the most active compounds. Acquired resistance is the consequence of mutations in the *gyrA* and *parC* genes (286, 287) or the acquisition of the *qnr* genes (287). Efflux pumps as EmeA for *E. faecalis* (288), and NorA-*like* for *E. faecium* (289) have been also described, although their frequency is low. Resistance to ciprofloxacin is a conserved feature among the high-risk *E. faecium* CC17 clone linked to nosocomial outbreaks (290), and almost all isolates with resistance to glycopeptides.

Fluoroquinolones have never been used as growth promoters, although their use for veterinary therapy is common.

4.- MOLECULAR EPIDEMIOLOGY AND POPULATION STRUCTURE OF ENTEROCOCCI IN FARM AND COMPANION ANIMALS

Epidemiological studies in farm and companion animals were originally driven by the interest to establish a relationship between antibiotic resistant isolates from human and non-human hosts. At present, the resistance phenotypes of clinical relevance that may be linked to animals mainly comprise resistance to ampicillin, gentamicin, quinupristin-dalfopristin, vancomycin, and linezolid.

Molecular typing of enterococci strains has been performed by different methods that includes pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST), coregenome MLST (cgMLST), Bayesian analysis of population structure (BAPS) and whole genome sequencing (revised in 291).

The emergence of VRE in European foodborne animals and food of animal origin in early 1990s (128, 291, 292-296), as well as in feces of healthy volunteers or food handlers (297-299), encouraged surveillance studies in the community setting that led to suggest a relationship between the extensive use of animal growth promoters in veterinary (e.g. avoparcin and tilosin), the colonization pressure in animals, and the subsequent transmission to human hosts throughout the food chain (300-301).

The first report of VRE in non-human hosts occurred in 1993 in the UK and documented the similarity between isolates of different origins (300). This study was followed by others, which confirmed the similarity of VRE strains from humans and farm animals exposed to avoparcin in different European countries (26, 292, 302-305). The potential

selection of antibiotic resistant enterococci by antibiotics led to the unilateral ban of avoparcin as animal growth promoter in Sweden in 1986, Denmark and Switzerland in 1995, and two years later in the rest of the European countries (Commission Directive 97/6/EC). By 1999, other antibiotics (as bacitracin, virginiamycin and tylosin) were also banned as growth promoters for healthy animals in Europe, and this was followed in 2006 for all antibiotics. In this way, Europe leaded the first intervention against VRE at global level. In contrast with western countries, the use of antimicrobials in livestock and poultry, as well as the standard policies on antimicrobial use, highly varies in each Asian country (revised in 306). In Korea, avoparcin was used in the management of poultry and swine from 1983 to 1997 but was banned thereafter to reduce the exposure of humans to VRE (133). After several years of avoparcin discontinuance in Korea, the prevalence of VRE in Korean livestock was investigated, and some studies reported that the VRE incidence rate in chicken samples was higher than that in pig samples (163, 307).

The ban led to a significant reduction of VRE colonization in animals, foods, and fecal samples of community-based persons of different countries. However, VRE was recovered in feces from animals and humans after years reflecting important effects of previous livestock practices in the population structure of enterococci in animals.

Most information came from the species *E. faecium* and *E. faecalis*, the predominant ones in the gastrointestinal tract of mammals besides *E. hirae*, *E. durans* and *E. cecorum* (11, 45, 46).

4.1.- Enterococcus faecium

PFGE remained the "gold standard" for molecular typing of *E. faecium* until the recent introduction of whole genome sequence (WGS)-based epidemiology (291, 308). By using PFGE, clonal dissemination of *E. faecium* strains with clinically relevant phenotypes

(ampicillin, gentamicin, quinupristin-dalfopristin and vancomycin) has been extensively documented between animals of the same or different farms and has also been suggested between animals and humans (309, 310). The data varies greatly among geographic areas and are normally associated with the use of antibiotics.

Ecological differentiation of *E. faecium* has been documented in epidemiological studies using AFLP, MLST and/or BAPS (311-314). AFLP analysis originally revealed different subpopulations (or ecotypes) corresponding to hospitalized patients, community-based persons, and farm animals including veal calves, poultry and swine (311, 315). Afterwards, MLST results using eBURST confirmed the split of *E. faecium* in host-specific subgroups, one from hospitalized patients [originally termed clonal complex 17 (CC17)], and others from domesticated animals (291, 316). More recently, BAPS analysis allowed the partitioning of 519 STs of 1720 *E. faecium* isolates into 13 non-overlapping groups. Again, BAPS groups were significantly associated with isolates from hospitalized patients (BAPS 3-3) and farm animals (BAPS 2–1 and 2–4) (313). More recently, single nucleotide polymorphism-based phylogenetic analysis of WGS data split *E. faecium* in isolates causing infections (clade A1), isolates from healthy humans (clade B) and isolates from healthy humans and animals (clade A2) (79). The clade A1 mostly comprises isolates from hospitalized humans associated with lineages 17 (including ST16 and ST17), 18 (ST18) and 78 (ST78 and ST192), although isolates from animals have been extensively reported (313, 89, 304). The ST78 isolates show putative evolutionary hallmarks with respect to pets (dogs and cats) and poultry isolates and diversified mainly through recombination and acquisition or loss of mobile genetic elements, which eventually led to adaptation to different ecological niches. Thus, ecological distinction is not absolute, and the main zoonotic risk linked to *E. faecium* isolates is represented by transfer of mobile genetic elements harboring antimicrobial resistance genes.

Poultry. *E. faecium* isolates resistant to macrolides, quinusptistin-dalfoprisitin or other streptogramins were extensively reported in poultry farms revealing high heterogeneity of PFGE types and STs, although some similar patterns were eventually detected in farms in Europe, USA and Asia (317-319). Clonal dissemination of VRE of the *E. faecium* species (VREfm) within poultry farms exposed to antibiotics before and after the ban of avoparcin (109, 302) were documented in European and Asian countries, with STs belonging to CC9 or CC96 as the predominant ones in Europe or Malaysia, respectively (320). A dramatic increase of VREfm in Sweden from 2000 to 2009 was due to the clonal expansion of the clone ST310, despite the absence of selection by antibiotics in this country, where the use of antibiotics as animal growth promoters was forbidden since 1986 (129). A Danish study showed the high rate of VREfm in Danish farms after 15-year ban of avoparcin, with different ST and the presence of a ST842 clone in 36 flocks analyzed corresponding to eight farms broadly distributed in the country (85). Recently, clonally unrelated *E. faecium* isolates resistant to linezolid emerged in farms from China (236, 237). Common PFGE profiles or STs between humans and broilers have also been documented (321-323), but the human health risk associated with the presence of *E. faecium* in poultry meat is under debate (25).

Swine. VREfm has been extensively documented in pig farms from European countries before and after the avoparcin ban (113, 324, 325). Clonal spread of VREfm was documented in Denmark, Norway, Finland (113), Switzerland (326), Portugal (304), and Spain (327), with predominance of STs belonging to the CC5 lineage (ST5, ST6, ST185). The persistence of VREfm in pig farms after the avoparcin ban was associated later with the use of tylosin, which facilitated the co-selection of strains resistant to both glycopeptides and macrolides due to the presence of both *vanA* and *erm*(B) genes in the same plasmid (113). VREfm was also detected in county fairs in Michigan from 2008 to

2010, which represents the first and unique report of VREfm in livestock in the USA to date (121, 122). In Asia, the occurrence varies with the countries and is sporadic in China (156). In all these studies, CC5 strains were also predominantly identified. A particular ST6 (CC5) clone was identified in farms of different EU countries and the USA, as well as in healthy volunteers and hospitalized patients, all carrying a Tn*1546* in *orf1* and a G-T point mutation in the position 8234 at *vanX* (304, 328). Besides tylosin, copper is frequently added to pig and cattle feeds, so co-location of heavy metal resistance determinants has been also demonstrated in Europe and the USA (329, 330). Copper resistance is often associated with resistance to macrolides (*erm*(B)), tetracyclines (*tet*(M)), and with glycopeptides (*vanA*). Although clonal dissemination has been reported (330), a great diversity has been documented in farms (331). Major human clones (early classified as CC17), CC9 and CC22 have also been documented in some studies (85, 332, 333).

Companion animals. A few studies have analyzed the fecal carriage of ampicillin resistant *E. faecium* (AREfm) and VREfm in companion animals. High rates of AREfm were observed among fecal samples of dogs collected in UK and Denmark in 2006 and 2008 (23% and 76%, respectively) (89, 334). Most of these isolates belonged to the major human clonal lineage CC17, which apparently suggested a possible transmission between hosts. Later, De Regt *et al.*, demonstrated some unique metabolic features in these CC17 canine isolates that would facilitate niche adaptation (335). A recent large Dutch countrywide population-based study reported a higher prevalence of fecal carriers of AREfm in dogs and cats than in healthy human population (25.6%, 5.1% and 1.5% respectively). This study concluded that isolates from pets were genetically distinct from those of humans based on the lack of co-occurrence and the cgMLST results (336). Prior antibiotic use and eating raw meat were considering a risk factor for acquiring AREfm in

all the available studies (197, 336). Clinical isolates from dogs and cats treated with amoxicillin belong to high clonal complex risks and were similar to those from humans (197, 337).

4.2.- *E. faecalis*

A plethora of molecular methods have been used to type this species including PFGE, AFLP, and MLST. In contrast to what happen for *E. faecium*, *E. faecalis* isolated from different sources/hosts cannot be grouped using MLST or AFLP. Different studies using MLST data revealed the presence of many different sequence types in different hosts including farm animals, companion animals and hospitalized patients (338, 339). Moreover, some sequence types are associated with a higher prevalence of antibiotic resistance, represented by ST2, ST8, ST9, ST16, ST40, and ST87 (303; 339, 340), all of them being overrepresented in humans. To date, ST16 is recovered from humans and farm animals, and is considered a zoonotic lineage (25), involved in the spread of resistance to all antibiotics used in animals including bacitracin, phenicols, oxazolidinones (341). Clonal outbreaks of *E. faecalis* ST82, a common cause of amyloid arthropathy in poultry, have been reported in farms of Denmark, United States, France and Germany (342).

Although the detection of more prevalent *E. faecalis* STs in distant geographical locations and different hosts suggest frequent horizontal gene transfer between different host populations (69, 211, 241, 339, 340, 343), some studies using comparative genomics discarded global transmission (344).

The incongruence in the topologies of the seven different MLST gene trees revealed this species was highly recombinogenic (291, 343). Subsequent analysis of the *E. faecalis* population structure based on MLST data using a Bayesian analysis of population structure (BAPS) also yield incongruent results, and confirmed the lack of host specific

groups or ecotypes (313, 314). This issue was also demonstrated by studies that characterized the phylogenetic diversity of *E. faecalis* using whole genomes (phylogenomics and cgMLST) of clinical, human commensal, and animal isolates, that observed the lack of distinct clustering of isolates according to the source (291, 345).

Further whole genome sequence studies are necessary to characterize and describe the role of animals in the evolution, genetic diversity and population structure of *E. faecalis.*

5.- PLASMIDS IN ENTEROCOCCI FROM FOODBORNE AND COMPANION ANIMALS

Horizontal gene transfer plays a relevant role in the dissemination of antibiotic resistance in non-human hosts, and plasmids play a central role in this dissemination. Classically plasmid categorization is based on the presence and diversity of replication (346), which were established by rep-initiator proteins (*rep*) scheme (347, 348) identified in Gram positive species to date. In **Figure 1** we show the plasmid content (percentage and diversity of *rep* sequences) of the 67 *E. faecium* and 47 *E. faecalis* genomes with animal origin obtained from the WGS database of the NCBI. The enterococcal genomes from public databases were classified according to their origin (**Table 1 suppl**), information obtained from the Pathosystems Resource Integration Center (PATRIC) database (349). The *rep* genes obtained by the PlasmidFinder bioinformatics tool (350) belong to plasmid families with theta (RepaA_N, Inc18, Rep3_small tetha) or rolling-circle replication (RCR) mechanisms (**Figure 1**).

Plasmids conferring resistance in enterococci to vancomycin, macrolides, tetracycline, aminoglycosides, and heavy metals (copper, cadmium, bacitracin zinc) have been detected in farms that were exposed to antimicrobials used as growth promoters (avoparcin, virginiamycin, tylosin, or bacitracin zinc), therapeutically (tetracyclines,

gentamicin, penicillins) or dietary supplements (e.g. copper). Antibiotic resistant plasmids have also been recovered from areas where selection was not apparent. Some emblematic examples are transferable *vanA* in commercial animal husbandry in Michigan farms, USA, where avoparcin has never been licensed for use in growth promotion (121, 122), or persistent *vanA*-Inc18 plasmids in Norwegian broiler flocks after the ban of some antibiotics. These studies suggest alternative routes of selection, introduction and spread of *vanA*-type vancomycin resistance, plasmid fitness and other phenomena (351).

5.1.- Plasmids conferring resistance to glycopeptides.

Tn*1546* (*vanA*), the predominant mechanism of glycopeptide resistance in enterococci, has been successfully disseminated among poultry and swine through plasmids of the Inc18 and RepA_N families, respectively (352, 353). In poultry, an 18-25kb fragment that includes the 10.85kb of Tn*1546* (*vanA*), is conserved in Inc18 plasmids detected in Norwegian broiler flocks for more than one-decade (from 1999 to years after the avoparcin ban) and in the pIP186, the first Inc18 (*vanA*) plasmid described in 1986 in a *E. faecium* clinical isolate (354, 355). The persistence of *vanA* plasmids in Norwegian poultry farms is attributed to the toxin–antitoxin system ω–ε–ζ originally described in pRE25, a plasmid of *E. faecalis* carrying resistance to different antibiotic families and prevalent in animal and foods (127, 354). Analysis of the Tn*1546* insertion sites and plasmid backbones made to suggest spread of the *vanA* transposon across different clonal lines in the broiler industry (125, 354-356). Bortolaia *et al.* recently associated the persistence of glycopeptide resistance in Danish poultry flocks after 15-year of avoparcin ban with a non-transferable 54kb plasmid in isolates that only confer resistance to glycopeptides (27). It is of note that broiler flocks raised in Denmark come from parent birds imported from Sweden, and the high occurrence of VREfm was also observed in Swedish broiler flocks until 2011 (129).

In swine, large plasmids belonging to the RepA_N family (150-190kb, rep_{pLG1}), which carry a truncated variant of Tn*1546* and *tcrB* (coding for resistance to copper), have been detected in a pandemic CC5 *E. faecium* clone circulating in swine farms of Spain, Portugal, Denmark, Switzerland and the USA for decades, and in other *E. faecium* lineages of pigs and humans, what would suggest transmission (304). These plasmids use to carry *erm*(B) gene (macrolide resistance) and, eventually, *trcB* (copper resistance) (see below).

Also, sporadic reports have documented the occurrence of strains carrying other *vanB* or *vanN* operons on plasmids in poultry meat (178, 188), game meat or wild game meat (226). Finally, vancomycin susceptible *E. faecalis* strains carrying *vanC1* on transferable elements (plasmids, transposons and integrons) have also been reported in cloacal swabs of broilers (357), and feces of diseased pigs from different farms (358). Transmission of species-specific *vanC1* and *vanC2/C3* genes could be currently underestimated given the high presence of *E. gallinarum* and *E. casseliflavus*, respectively, in foodborne animals (159, 359-360), and the scarcity of studies that screen *vanC* genes in other different species.

5.2.- Plasmids conferring resistance to macrolides, streptogramins and lincosamides.

They have been extensively recovered in enterococci from poultry and porcine farms where macrolides (spiramycin and tylosin) and streptogramins (virginiamycin) were used as growth promoters and pleuromutilins (tiamulin and valnemulin) to treat infections in these animals. Lincomycin, alone or in combination with spectinomycin, have been widely used to control respiratory and gastrointestinal bacterial pathogens in cattle, swine, poultry, dogs and cats, with pirlimycin only used to treat bovine mastitis cases. Clindamycin is a common therapeutic option for topical infections in dogs and cats.

Macrolides. The most widespread gene that confers resistance to macrolides in enterococci is *erm*(B), which is located in different transposons and plasmids in species of the *Enterococcus, Streptococcus, Staphylococcus* and *Clostridium* genera (346, 361). pRE25, a multidrug resistant plasmid originally recovered from a *E. faecalis* isolate of a sausage sample, is the paradigm of the Inc18 family and has greatly contributed to spread of *erm*(B) among animals and humans (346, 353, 362). The plasmid encodes resistance to 12 antimicrobials of five structural classes (macrolides, lincosamides, streptothricin, chloramphenicol, aminoglycosides) due to the presence of *erm*(B) (macrolide-lincosamide-streptogramin B), cat_{pIP501} (chloramphenicol) and Tn*5405* that comprises the genes *aadE-sat4-aphA3* (aminoglycoside-streptothricin) (363, 364). The genes carried by pRE25 are present in different animal pathogens, namely, *Streptococcus pyogenes, Streptococcus agalactiae, S. aureus, Bacillus subtilis, Campylobacter coli, Clostridium perfringens, and Clostridium difficile.* The *erm*(B) gene has also been found in small plasmids in poultry samples (365), and in large plasmids of food besides other genes as *msr*(C) and *lnu*(B), *tet*(L) and *tet*(W) (366). Location in chromosome is also frequent.

The gene *erm*(A) associated with Tn*554,* commonly found in staphylococci from swine, has also been found in streptococci and sporadic isolates of *E. faecalis* and *E. faecium* from pigs, suggesting transfer events (282, 367). More recently, a novel *erm*(A)-like gene that confer high level of resistance to erythromycin (MIC>128 µg/ml) has been detected in Inc18 plasmids with genes encoding resistance to phenicols and oxazolidinones (see below). This gene differs of the widespread *erm*(A) gene on Tn*554* and the *erm*(A) gene formerly called *ermTR*, predominant in staphylococci and streptococci, respectively (82-85% homology at amino acid level). This *erm*(A) enterococcal variant has a 116 bp deletion in the translational attenuator (237).

Streptogramins. Genes conferring resistance to streptogramins (acetyltransferases encoded by *satG/vatE* and *satA/vatA* genes and ABC transporters by *vgb*/*vgb*B), and macrolides (23rRNA methylases encoded by *erm*(B), *erm*(A), *erm*(C) genes), are observed in a diversity of plasmids and clonal backgrounds. In addition, *vat* genes are often co-transcribed and co-transferred along with *vga*, *vgaB*, *vgb*, *vgbB*, or *erm*(B) genes through transposable elements, some of them, previously observed in staphylococci (364, 368-373). Transferability of *vat* genes and streptogramin resistance in *E. faecium* strains through contaminated pork and chicken meat, raw manure, and surface/ground water has extensively been documented (374, 375).

Lincosamides. Resistance to this antibiotic family can be due to the presence of genes coding for ABC transporters or modifying enzymes, most of them located on plasmids and/or transposable elements. These elements have been extensively documented in staphylococci, and to a lesser extent in streptococci, *Clostridium* and other species of Gram-positive bacteria in animals.

ABC transporters that confer resistance to pleuromutilins, lincosamides, and streptogramin A antibiotics (PLS_A) include the genes *vga* and *vga*(A)v, *vga*(C), *vga*(E), *vga*(E)v, *eat*(A)v, *sal*(A), *lsa*(A), *lsa*(C), and *lsa*(E). They frequently appear within clusters in plasmids or transferable chromosomal regions previously reported in *S. aureus* (230). A 8,705 bp region flanked by IS*Efa8* and IS*1216*, and comprising genes coding for one or more antibiotics, namely *lnu*(B) (lincosamide), *lsa*(E) (PLS_A), *spw* (spectinomycin), *aadE* (streptomycin), and *erm*(B) (macrolide-lincosamide-streptogramin B), is common for plasmids of *S. aureus* (pV7037) and *E. faecium* (pY13, pXD4, pXD5) strains recovered from pigs (230, 376, 377). The pY13 plasmid further contains a copy of the genes *lnu*(B) (lincosamide), *aphA3* (kanamycin/neomycin) and a

second copy of *erm*(B), highlighting the redundancy of determinants in settings under high selective pressure.

Two genes coding for nucleotidyl transferases (*lnu*), which only confer resistance to lincosamides, have been described in *Enterococcus* from swine recovered in Chinese farms (229, 378). The *lnu*(G) is part of a 4738 bp functionally active transposon designated as Tn*6260*, firstly detected in an *E. faecalis* isolate of swine orgin; this element is similar to others of the Tn*554* family that includes different antibiotic resistant genes (378). The *lnu*(B) has been detected in porcine *E. faecium* isolates, and it has been found in a non-conjugative plasmid linked to *erm*(B), *lsa*(E), *spw*, *aadE*, and *aphA3* genes, which account for resistance to macrolides, lincosamides, streptogramins, pleuromutilins, streptomycin, spectinomycin, and kanamycin/neomycin (229).

5.3.- Plasmids conferring resistance to phenicols and oxazolidinones

Genes coding for resistance to non-fluorinated phenicols (*cat*), non-fluorinated and fluorinated phenicols (*fexA*, *fexB*), and to both phenicols and oxazolidinones (*cfr*, *optrA*), have been detected in enterococcal species from animals, foods and humans.

The production of chloramphenicol acetyltransferase (or CAT) enzymes seems to be the main mechanism of resistance to chloramphenicol although the number of studies addressing the diversity and the genetic context of *cat* genes in *Enterococcus* is still scarce. The predominant *cat* variants are *cat*(A-7), associated with pRE25-like plasmids of the Inc18 family which are widely disseminated in food and farm animals, predominantly poultry (241); and *cat*(A-8), also known as cat_{pC223}, associated with pC223 plasmids originally detected in *S. aureus* that are now predominant in *E. faecalis* from swine. This gene eventually appears in tandem with *tet*(M) and *tet*(L) genes within the transposon Tn*6245* and relics of this transposon have been observed in plasmids that also

carry *fexA* and *oprtA* (237). Although isolates positive for the *cat*(A-9) gene have been recently identified in *E. faecalis* from swine, their genetic context has not been characterized (Ana Freitas, personal communication).

The florfenicol exporter gene *fexB* was initially detected in non-conjugative plasmids of *E. faecium, E. faecalis* and *E. hirae* isolates collected in swine farms heavily exposed to florfenicol in China (379). These plasmids share common regions with the backbone of Inc18 plasmid derivatives (e.g. pVEF4), widely disseminated in Norwegian poultry farms (355). The *fexB* gene is bracketed by IS*1216,* and would have been acquired by widespread pRE25-like plasmids, as occurred for other antimicrobial resistant genes flanked by this IS. The *fexB* gene has also been identified in enterococci from other farm animals (bovine) and aquacultures, although the plasmids are not still characterized (380, 241). A different epidemiological landscape occurs for the *fexA* gene, which is located on plasmids (241) and chromosome (236) of enterococcal animal isolates, often in tandem with the *optrA* gene (237, 241) or the *cfr* gene (235). The *fexA* gene is inserted in the emblematic Tn*554* of staphylococci, although in enterococci traces of this transposon might be absent as a consequence of different events of horizontal gene transfer (237).

Enterococcal plasmids carrying *optrA* have been detected in poultry, swine and humans. Despite differences in size (30-80 kb) and the backbone, all share similar regions upstream and downstream the *optrA* gene (236, 237, 241). It is of note the presence of a novel *erm*(A)-like gene that confer high level of resistance to erythromycin (237). The genetic context of *optrA* is flanked by copies of IS*1216* in the same or opposite direction, which determine the mobility.

Conjugative and non-conjugative plasmids carrying the *cfr* gene flanked by different IS (IS*1216*, IS*Enfa4*, IS*Enfa5*, IS*256*), have been described in animal isolates of different Gram-positive species, including enterococci. The non-conjugative pEF-01 (32.2 kb)

plasmid represents the first description of a *cfr*-plasmid in this bacterial genus and was identified in a fecal *E. faecalis* isolate of bovine origin collected in 2009 in a Chinese farm (232). This plasmid has three Rep proteins of the Inc18 and Rep3 plasmid families, and 9kb and 6kb regions which exhibit high similarity with the backbone of *vanA* Inc18 plasmids (pVEF1-2-3), widely isolated in poultry farms (232). Moreover, the *cfr* gene was flanked by IS*1216* that would facilitate recombination processes, and plasmid also contains the *fexA* gene, that provides resistance to phenicols. Conjugative plasmids carrying the *cfr* bracketed by IS*Enfa4* copies, were isolated from *E. thailandicus* and *E. faecalis* from swine Chinese farms. These are closely related to other emblematic Inc18 plasmid, the pAMb1, and contained *erm*(B) and *erm*(A) genes, conferring the MLS_B phenotype, and also the ω-ε-ζ toxin-antitoxin module, which may promote the persistence of plasmids by encoding a system that kills or prevents the growth of plasmid-free cell (55). This genetic context has also been detected in streptococci and staphylococci and pointed out of independent acquisition events for *cfr* gene. The *cfr* gene bracketed by two copies of IS*Enfa5*, has been documented in *E. gallinarum and E. casseliflavus* of swine orgin (235).

5.4.- Plasmids conferring resistance to bacitracin.

Bacitracin has been used as an animal growth promoter in China, and recent reports documented *E. faecalis* isolates with high-level resistance to this antibiotic (MIC ≥ 256 μg/ml), due to the presence of the *bcrABDR* cluster, composed by the *bcrABD operon* and its regulatory gene *bcrR*. The cluster either bracketed by two, one (or none) IS*Enfa1* copies is located on transferable plasmids (341) or chromosome. The structure IS*Enfa1-bcrABDR*-IS*Enfa1* may be circulating and been transferred to other species by IS-mediated recombination. A multiresistant 79 kb pheromone responsive plasmid carrying this IS*Enfa1-bcrABDR*-IS*Enfa1* platform as well as *optrA*, *fexA*, Tn*6425*

(*cat*$_{pC223}$-*tetM-tetL*), Tn*5405* (*aph-sat-str*) and genes for resistance to copper and cadmium seems to be disseminated in Chinese farms (341), frequently associated with ST16 *E. faecalis*. This *bcrABDR* cluster is also common in *E. cecorum*, a chicken commensal species (341).

5.5.- Plasmids conferring resistance to copper.

Transferable resistance to copper (*tcrB*) in enterococci has been detected in piglets, calves, poultry, and also in humans of European, Asian, Australian and American countries (148, 331, 368, 381-383). Plasmids carrying *tcrB* are identified in intensively copper-supplemented livestock species, but plasmids with additional linkage with erythromycin (*erm*(B)) and/or vancomycin resistance (*vanA*) genes has only been observed in heavily copper-exposed swine (often with different copper compounds) of European countries where avoparcin was used as growth enhancer in the 1990s (148, 329, 381, 383, 384). The plasmids were detected in different enterococcal species (*E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, *E. mundtii*, *E. hirae*), and conjugation has been experimentally demonstrated from *E. faecalis* to *E. faecium* (381). Copper fed to feedlot cattle at a growth promotion concentration (10× basal requirement) was associated with increased frequencies of *tcrB*-positive, macrolide-resistant-*erm*(B) and eventually, tetracycline resistant-*tet*(M) enterococci; on the other hand, copper susceptibilities was not increased in piglets in which the effect of in-feed tylosin or chlortetracycline was evaluated (382, 385). Co-transmission of *tcrB* and *erm*(B) genes between *E. hirae* from a sediment-derived livestock and *E. faecalis* has been experimentally demonstrated (386). A recent analysis of whole genome sequences of *E. faecalis* from copper-supplemented Danish pigs also documented the presence of a chromosomal cluster of genes involved in susceptibility to copper, including the *tcrYAZB* operon, in three of six isolates analyzed, all containing plasmids (387). A detailed characterization of this

chromosomal region was not provided, although other authors, who also identified redundancy of copper genes in chromosome, demonstrated its co-transferability with ampicillin resistance (331).

6.- CONCLUDING REMARKS

This review summarizes the current knowledge concerning the epidemiology and population structure of antibiotic resistant *Enterococcus* species from foodborne-, wild and companion animals. Members of this genus are normal components of the intestinal microbiota of animals and some species may also be aetiological agents of a wide variety of infections as *E. faecalis* ST16 (considered a zoonotic pathogen), or ST82 (etiological agent of the amyloid encephalopathy in chickens).

Enterococcus are frequent contaminants on foods (especially poultry meat), although the risk of transmission from animals to humans through the food chain is based on indirect evidence and thus, the bacterial load necessary to colonize human gut remains greatly unknown. Food and animal trade seem have contributed to the spread of certain pathogenic lineages (*E. faecalis* ST82 and ST16 lineages) or multidrug resistant strains. Other species adapted to animals seem to act as important reservoirs of adaptive traits (*E. cecorum*). However, transmission of antimicrobial resistance by horizontal gene transfer events represent the main risk of contaminated foods by enterococci. Genes encoding resistance to vancomycin, macrolides, phenicols, and linezolid have been extensively documented in animals, frequently in response to heavy selection by antimicrobials (antibiotics and heavy metals) used in prophylaxis or as growth promoters. Although the same genes and plasmids may be present also in humans and animals, particular plasmid variants are often documented in farms, suggesting certain host specificity and transmission at local level. Deep analysis of antimicrobial resistant genes reveals a wide diversity of alleles (e.g. *erm*(A), *optrA*, *cfr*), and also the frequent presence of IS (e.g.IS1216) that

highlight the risk of frequent and independent acquisition and selection events of antimicrobial resistance in farms. More studies are necessary to establish the risks of the emergence and transmission of antibiotic resistant enterococci from animals to humans.

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Figure 1. Plasmid gene content of 67 *E. faecium* and 47 *E. faecalis* genomes with animal origin from NCBI whole genome database. Plasmid data were obtained by PlasmidFinder bioinformatics tool. The genomes from database were classified by source extracting the isolate information from the Pathosystems Resource Integration Center (PATRIC) database (344). Reps, replicases.

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