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Antimicrobial Resistance in *Enterococci*

Antonio Marin Garrido[1], **Antonio Gálvez**[2*] **and Rubén Pérez Pulido**[2]

[1]*Real Academia de Ciencias Veterinarias de Andalucía Oriental, Spain*

[2]*Área de Microbiología. Departamento de Ciencias de la Salud. Facultad de Ciencias Experimentales. Universidad de Jaén, Spain*

***Corresponding author:** Antonio Gálvez, Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, Campus Las Lagunillas, Spain, Tel: 34-953-212160; Fax: 34-953-212943; E-mail: agalvez@ujaen.es

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Abstract

Enterococci show intrinsic low resistance to a large number of antibiotics (β-lactams, lincosamines, aminoglycosides and trimetoprim-sulfametoxazol). In addition, *Enterococci* can acquire new resistance to antimicrobial agents. This can happen by mutation or acquisition of extrachromosomal DNA, as plasmids or transposons. Resistance to erythromycin, aminoglycosides and *tet*racycline are common. Resistance to glycopeptide antibiotics and to newer antimicrobial substances may turn opportunistic *enterococcal* infections into high-risk infections, specially for immunocompromised patients. *Enterococci* isolated at different steps in the food chain also show a remarkable incidence of antimicrobial resistance. Heavy metal resistance and biocide tolerance could be factors in the co-selection of antibiotic resistance in the absence of antibiotic selective pressure, such as at certain steps of the food chain.

Keywords: Antibiotics; Biocides; Heavy metals; Resistance; *Enterococci*

Introduction

Enterococci have emerged as important nosocomial pathogens over the past decade [1,2], ranking only second to staphylococci as a leading cause of nosocomial infections, accounting for ~12% of hospital-associated infections yearly in the U.S. [3]. They frequently possess several specific traits that enable them to survive in the hospital environment, colonize patients, and cause infections such as bacteraemia, peritonitis, endocarditis and urinary tract, wound, and device-related infections [4]. In particular, *Enterococcus faecalis* and *Enterococcus faecium* have emerged as multi-resistant nosocomial pathogens in immunocompromised and critically ill patients. Multi-resistant strains have acquired virulence genes resulting in hospital-adapted clones.

Enterococci are natural inhabitants of the intestinal tract of many warm-blooded animals. As a result, they are released in large amounts with faeces, and may become the predominant contaminant microbiota in many foods [5]. They may also play a desirable role in the fermentation and ripening of certain foods of animal origin, and are known to be present in vegetable fermented foods [6], but they also may have a negative impact by spreading antibiotic resistance through the food chain [7]. Antimicrobial agents are used in large amounts in the production of food animals for therapy and prophylaxis of bacterial infections and in feed to promote growth [8]. A link between the use of antibiotics in animal husbandry and the raise of antibiotic resistance has been demonstrated. Resistance of *Enterococci* in food animals is very similar to what has been described of *Enterococci* isolated from nosocomial infections (including resistances to aminoglycosides, lincosamides, macrolides, nitrofurans, penicillins, quinolones, streptogramins, *tet*racycline, and rarely vancomycin) [9]. An overview of resistance in zoonotic and commensal bacteria in Europe focusing on *Salmonella*, *Escherichia coli*, *Campylobacter sp.* and *Enterococcus* sp. during the period of 2005-2011 has been published recently [10]. With the exception of cephalosporins, linear regressions showed strong positive associations between the consumption of the four different antimicrobial classes. Antibiotic amounts used to produce 1kg of meat were in the range of 117.2 mg/kg to 3.7 mg/kg, depending on the country. Furthermore, large variations in proportions of resistant bacteria were reported by the different countries, suggesting differences in veterinary practice. There is a steady persistence over the years of a low percentage of *E. faecium* exhibiting resistance to glycopeptides in poultry, cattle and pig. Macrolide resistant *Enterococci* were found at higher proportions in pigs than in poultry in some countries, but other countries reported much lower resistances. Quinupristin/dalfopristin resistance *E. faecium* from broilers was reported at high level in some countries.

Enterococci exhibit a variety of mechanisms for intrinsic and acquired resistance to the major classes of antibiotics of clinical use, and are endowed with efficient genetic exchange mechanisms that facilitate dissemination of antibiotic resistance genes [11]. Resistance to other antimicrobials such as biocides or heavy metals and the possible co-resistance with antibiotics are now becoming matters of concern. The purpose of this review is to give an overview of this diversity in resistance mechanisms.

Resistance to β-lactam antibiotics

The species of genus Enterococcus usually show a low intrinsic resistance to β-lactam antibiotics like penicillin, ampicillin, piperacilin and imipenem, which exert on them a bacteriostatic effect [12]. For example, *E. faecalis* is between 10 and 100 times less sensitive to penicillin that most of the *streptococci*, whereas *E. faecium* is at least 4 to 16 times less susceptible than *E. faecalis* . These antibiotics, like the glycopeptides, have bacteriostatic activity against these microorganisms, a reason why a synergistic bactericidal association is required in case of serious infections as endocarditis or meningitis. The combination of aminoglycosides and an agent that acts on the cell

wall, like β-lactam antibiotics or glycopeptides, achieves a high bactericidal activity, but this disappears when a high resistance to anyone of these components is developed.

The main mechanisms of resistance to β-lactam antibiotics imply the production of penicillin-binding proteins (PBPs) of low affinity (Table 1). For example, resistance to penicillin is directly proportional to the proportion of PBP5. On the contrary, strains producing β-lactamases are infrequent and, unlike other bacteria like the staphylococci, the production of β-lactamases in *Enterococci* is not inducible, but constitutive. The genes for the production of β-lactamases in *Enterococci* can be located on plasmids, or on the chromosome.

Drug resistance	**Family genes/mechanisms conferring resistance**	**References**
β-lactams and cephalosporins	Penicillin-binding proteins (PBPs): PBP4, PBP5	[63,64]
	bla genes	[65,66]
	LD-transpeptidase	[13]
Aminoglucosides	efmM	[15,67]
	aac(6')-Ii	[68]
	aph(3')-IIIa	[69]
	ant(4")-Ia	[70]
	aph(2")-Ia-Aac(6')Ie	[71]
	aph(2")-Ib	[72]
	aph(2")-Ic	[73]
	aph(2")-Id	[74]
	ant(6')-Ia	[14]
	ant(3")-Ia	[75]
Glycopeptides	VanA operon (vanA, H, X, Y, Z, R, S)	[76]
	VanB operon	[76]
	VanC operon	[76]
	VanD operon	[76]
	VanE operon	[76]
	VanG operon	[76]
	VanL operon	[76]
	VanM operon	[76]
	VanN operon	[76]
	lsa	[34]
	msrC	[77]
	vgaB	[32]
	vgaD	[78]
	vat(B)	[32]
Macrolides and lincosamides	vat(D) (satA)	[32]
	vat(E) (satG)	[32]
	vat(G)	[32]
	vat(H)	[78]
	vga(B)	[32]
	vgb(A)	[33]
	erm(A)	[79]
	erm(B)	[77]
	linB	[28]
	mef	[29]
Tetracicline	*tet*(M)	[38]
	tet(O)	[38]
	tet(S)	[38]
	tet(K)	[39]
	tet(L)	[39]
Rifampicin	rpoB H486Y	[18]
Oxazolidinones	cfr	[81]
Daptomicin	cls	[81]
	gdpD	[81]
	liaF	[81]
Quinolones	GyrA, ParC mutation of DNA gyrase	[43,44]
	qnr	[45]
	emeA	[46]
	efrAB	[47]
Copper	copYZAB operon	[60]
	tcrYAZB	[61,62]

Table 1: Drug resistance determinants in *Enterococci*

An additional mechanism of resistance to β-lactam antibiotics has been described in E. faecium, mediated by an LD-transpeptidase that is different from the penicillin-sensitive DD-transpeptidase. This second LD-transpeptidase is found in low concentrations (0.7%), but it is insensitive to β-lactams. Therefore, the mutations that entail predominantly to the LD-transpeptidase phenotypes allow the appearance of β-lactam resistant strains [13].

Resistance to aminoglycosides

The first description of a high level antibiotic resistance in *Enterococci* dealt with streptomycin (MIC > 2000 mg/ml). Further studies on the sensitivity of *Enterococci* to aminoglucosides revealed three mechanisms of resistance, that can be summarized as follows [14] (Table 1):

All *Enterococci* offer a moderate intrinsic resistance (MIC, 62-500 µg/ml) due to a low cellular permeability, that can be solved by addition of penicillin (that facilitates the entrance of the aminoglycosides to the cell).

High levels of resistance due to precise mutations that affect a protein of the 30S ribosomal subunit.

Resistance by modification of 16S rRNA. The rRNA methyltransferase, EfmM that uses S-adenosyl methionine as a methyl donor to methylate a specific residue on 16S rRNA [15].

High levels of resistance (MIC, 2.000 µg/ml), mediated by production of enzymes able to inactivate antibiotic molecules.

High-level resistance to aminoglycosides is most frequently mediated by aminoglycoside-modifying enzymes, such as phosphotransferases (APHs), acetyltransferases (AACs) and nucleotidyltransferases (ANTs). Importantly, these mechanisms of resistance abolish the synergistic bactericidal activity of aminoglycosides in combination with cell-wall–active agents that are important in the treatment of severe *enterococcal* infections, such as endocarditis.

Resistance to glycopeptides

At least eight types of acquired resistance to glycopeptides have been reported (Table 1) on the basis of phenotypic and genotypic criteria (VanA, VanB, VanD, VanE, VanG, VanL, VanM, and VanN), although VanC) is an intrinsic characteristic of *Enterococcus gallinarum* and *Enterococcus casseliflavus* [16-18]. Another *van* gene cluster, *vanF*, has been described in a biopesticide, *Paenibacillus popilliae*, but has not yet been found in *Enterococci*. Gene clusters conferring glycopeptide resistance are designated according to the name of the ligase gene, which encodes either a d-Ala:d-Lac (*vanA, vanB, vanD* and *vanM*) or a d-Ala:d-Ser (*vanC, vanE, vanG, vanL* and *vanN*) ligase for the synthesis of peptidoglycan precursors with low affinity for glycopeptides. The *vanA*, *vanB*, and *vanD* gene clusters contain genes for a two-component regulatory system (*vanR* and *vanS*), three resistance genes (*vanH*, encoding dehydrogenase; *vanA*, *vanB*, or *vanD*, encoding ligase; *vanX*, encoding dd-dipeptidase); an accessory gene (*vanY*); and the *vanZ* gene, which is present in the *vanA* gene cluster, whereas the *vanW* gene is found only in the *vanB* operon. Frequently, van genes are located in plasmids or transposons, which facilitates their dissemination by means of horizontal gene transfer [19]. The most prevalent resistance types are VanA and VanB. The reservoir of transferable VanA-type (and partly VanB-type) resistance in human medicine and other habitats is *E. faecium*.

The VanA phenotype

This phenotype represents the form of glycopeptide resistance more frequent in *Enterococci*. Generally, it is associated to a high vancomycin resistance (MIC ≥ 128 µg/ml). Most of the strains that show this phenotype are also resistant to teicoplanin (MIC ≥ 8 µg/ml). This type of resistance is induced by glycopeptides (vancomycin, teicoplanin, avoparcin and ristocetin) and by other different antibiotics, like bacitracin, polymyxin B or robenidin.

The prototype of VanA resistance is Tn1546, a transposon of 10851 bp related to Tn3. This element contains seven genes located immediately downstream of two open-reading frames (ORFs) associated with transposition. These genes are essential for the expression of the resistance phenotype.

vanA encodes for the D-ala-D-lac ligase (VanA), that synthesizes the terminal dipeptide D-ala-D-lac, with much lower affinity for vancomycin.

vanH encodes for one dehydrogenase (VanH), that produces D-lactate by reduction of pyruvate.

vanX encodes for one D, D-dipeptidase (VanX), which hydrolizes the dipeptide D-ala-D-ala, which is generated by the chromosomal ligase Ddl, and constitutes the end of the glycopeptide-sensitive pentapeptides. This way, the absence of any vancomycin target in the cell wall is ensured.

Upstream of the above-mentioned genes, there are two regulating genes, *vanR* and *vanS*, that encode for a two-component signal transduction system (VanR, VanS) in charge to detect the presence of glycopeptides and to induce the expression of resistance genes. VanS acts as a sensor to detect the presence of vancomycin or, more probably, some initial change caused by vancomycin on the cell wall. VanS transmits the signal to VanR, the response regulator, resulting in the activation of the synthesis of other proteins involved in resistance (VanH, VanA, VanX). In strains of VanA phenotype, vancomycin as well as teicoplanin can induce the resistance phenotype, although the exact signals have not been identified.

Downstream of the *vanRSHAX* cluster are two nonessential genes:

vanY encodes for a D-D-carboxypeptidase (*VanY*), that eliminates D-ala residues from the dipeptide end and complements the action of the dipeptidase.

vanZ encodes a peptide of unknown function (*VanZ*) that confers a low resistance to teicoplanin.
During the biosynthesis of peptidoglycan, crosslinking of the precursors is carried out by PBPs, predominating PBP5 in *Enterococci*. The substitution of D-ala-D-ala by D-ala-D-lac does not affect crosslinking, since PBP5 can be replaced by other PBPs produced by the same bacterium. This substitution causes a remarkable increase of the sensitivity of vancomycin resistant strains to β-lactam antibiotics, and explains the synergic effect of both types of antibiotics on such strains.

The VanB phenotype

Most of the VanB elements confer a variable degree of resistance to vancomycin (MIC, 4-1000 µg/ml), but most of the strains remain sensitive to teicoplanin in vitro, since this antibiotic cannot act as inducer. However, teicoplanin-resistant mutants have been described after in vivo treatment with vancomycin, and also in experimental animals treated with teicoplanin [20-22]. The disposition of the genes that code for this phenotype is different from VanA. The gene *vanYB* is located between the regulating genes and the *vanHBBXB* cluster. There is also an additional gene, *vanW* (of unknown function), that shows no homology with any of the VanA genes.

Three alele forms of vanB (vanB1, vanB2, vanB3) with homologous function to the vanA ligase have been described, corresponding to the phenotypes VanB1, VanB2, and VanB3. The presence of insertion sequences in this group is much lower than in the VanA phenotype, although ISEnfa200 has been described between *vanSB* and *vanYB*. The presence of three different subtypes suggests that they were acquired of independent form from unknown donors. However, it seems that most of the VanB-mediated resistance phenotype is acquired by the horizontal dissemination of *vanB2* genes by means of conjugative transposons of the type of Tn916.

Resistance to macrolides and lincosamides

Macrolide antibiotics are used in the treatment of infections in humans, being erythromycin the antibiotic of first choice in patients allergic to penicillins. Tylosin is an antibiotic pertaining to this group that was widely used in pigs [23]. Many studies have demonstrated a dissemination of macrolide resistance in staphylococci, streptococci and *Enterococci*. Resistance to macrolides is based on different mechanisms (Table 1):

Target modification by precise mutations.

Target modification by means of methylation of the 23S rRNA subunit, so that it prevents binding of macrolides (i.e., genes *ermA, ermB, ermC, ermTR*).

Hydrolysis of the lactone ring of the antibiotic molecule.

Efflux pumps, that remove antibiotic molecules from inside of the bacterial cell (i.e., genes *mefA, mefE, msrA, msrC, mreA*).

The more frequent macrolide resistance determinants are *erm* genes. These encode for one methyltransferase that acts on specific residues of the 23S rRNA subunit. This enzyme causes a N6-dimethylation of an adenine residue in the 23S rRNA subunit, inhibiting that way erythromycin binding [24]. The modification of the ribosomal target causes crossed resistance to macrolides, lincosamides and streptogramin B (MLSB), or to macrolides and lincosamides (MKS), or to macrolides, ketolides and streptogramin A and B (MKS). Several *erm* genes have been described, being *erm*(B) the predominant one in *Enterococci* [25-27]. Resistance to macrolides can be transferred from animals to humans, either by dissemination of the resistant bacteria or by horizontal transfer through movable genetic elements.

A second lincosamide resistance mechanism has been described in *E. faecium*, which is mediated by a lincosamide nucleotidyl transferase that catalyzes 3-(5´-adenilation) of lincomicin and clindamicin [28]. This enzyme, encoded by *linB*, is different from the lincosamide O-nucleotidyl transferases described in *S. aureus*. Finally, *Enterococcus spp.* may also contain export mechanisms for macrolide antibiotics. The genes responsible for this trait (*mef*) show a high mobility between diverse Gram-positive species [29].

The antibiotic combination quinupristin-dalfopristin (Q/D) was developed from a natural streptogramin obtained from *Streptomyces pristinaespiralis*. Both components bind to different sites of the 50S bacterial ribosome subunit and act synergistically by inhibition of the bacterial protein synthesis [30]. The minimum inhibitory concentration of Q/D is quite effective on sensitive *E. faecium*, with MICS between 0.5 and 3 µg/ml, while *E. faecalis* is intrinsically resistant to this type of antibiotics [31]. Acquired resistance to Q-D in *E. faecium* can be mediated by streptogramin acetyltransferase enzymes that acetylate streptogramin A (*vat*(B), *vat*(D), *vat*(E), or *vat*(G)), ATP-binding transporters encoded by vga(B) that presumably function to export the antibiotic from the cell [32], or hydrolases encoded by *vgb*(A) [33]. Streptogramin A and lincosamide resistance can also be mediated by an ABC transporter encoded by the gene lsa [34].

Resistance to *tet*racycline

Resistance to *tet*racycline is frequent in clinical and animal isolates of *Enterococci*. The presence of strains resistant to *tet*racycline has also been described in diverse foods of animal origin [35,36,37]. In *Enterococci*, *tet*racycline resistance is generally associated to the presence of the gene *tet*(M) which confers ribosomal protection, but other related genes affording ribosomal protection have also been described, like *tet*(O) and *tet*(S) [38] (Table 1). In clinical isolates, *tet*(M) is frequently associated to Tn916-type transposable elements, but it can also be found in conjugative plasmids and on the chromosome. The genes *tet*(K) and *tet*(L) encode *tet*racycline export pumps, the latter one being more frequent [39]. Other resistance genes encode proteins that bind to the ribosome and modify its conformation, preventing the union of *tet*racycline.

Resistance to rifampicin

Enterococci are frequently resistant to rifampicin, even though this antibiotic is not commonly used in *enterococcal* infections [40]. It is thought that rifampicin resistance arises from exposure of commensal microbiota to this antibiotic during treatment of other bacterial infections. Mutations in the RNA polymerase B subunit *rpoB* gene account for most of the observed resistance (Table 1). One particular mutation (*rpoB* H486Y) also confers an increased cephalosporin resistance, possibly by increasing transcription of genes involved in intrinsic resistance to this antibiotic [18].

Resistance to oxazolidinones

The representative antibiotic in this group is linezolid, which shows a high antimicrobial activity against Gram-positive bacteria (MIC, 4 µg/ml) [41]. Mutations in the 23S ribosomal subunit confer resistance to this antibiotic (MIC, 8 µg/ml). The levels of resistance depend on the numbers of alleles for rRNA genes mutated. Strains resistant to linezolid may also show co-resistance to other antibiotics such as vancomycin, ampicillin, macrolides, fluoroquinolones, chloramphenicol, rifampin, gentamicin, nitrofurantoin and trimethoprim/sulfomethoxazol [42].

Resistance to quinolones

Quinolones show a moderate activity against *Enterococci*. The use of fluoroquinolones in clinical applications has caused an increase of resistance in *Enterococci*, and mutations affecting the GyrA subunit of the DNA gyrase, and, more frequently, the ParC subunit of topoisomerase IV have been reported [43,44]. A second mechanism of quinolone resistance is mediated by proteins of the Qnr family, which protect DNA gyrase and topoisomerase IV from inhibition by quinolones [45]. A third mechanism of quinolone resistance is mediated by multidrug-resistance efflux pumps (Table 1), such as EmeA [46] and EfrAB [47].

Resistance to biocides

Among *Enterococci*, resistance to biocides and disinfectant has been studied to a much less extent compared to antibiotics [48-54]. In most reports, *Enterococci* have been found to be sensitive or moderately resistant to biocides such as benzalkonium chloride, triclosan or chlorhexidine. Most results are based on higher than wildtype tolerance levels, since there are no breakpoints established yet for biocide resistance in *Enterococci*. Suller and Russell [48] reported MICs of 4 to 6 mg/l for chlorhexidine, 3 to 4 mg/l for triclosan, 5 to 6 mg/l for cetylpyridinium chloride, and 5 to 6 mg/l for benzalkonium chloride in vancomycin-resistant *Enterococci* clinical strains and Beier et al. [51] reported that vancomycin-resistant *E. faecium* from human wastewater effluents had MICs of 8 mg/l or lower for triclosan and of 2

mg/l or lower for chlorhexidine. Aarestrup and Hasman [50] reported MICs of 2 to 16 mg/l for benzalkonimu chloride and of 0.5 to 8 mg/l for chlorhexidine in *Enterococci* (*E. faecalis* and *E. faecium*) of animal origin. Braga et al. [54] revealed similar MIC values of 8 mg/l for chlorhexidine and 4 mg/l for benzalkonium chloride in *Enterococci* from dust samples taken at pig breeding facilities. Furthermore, in a study on 500 Enterococcus spp. from food and food-processing industries, all isolates had MIC values for benzalkonium chloride < 30 mg/l and were considered to be susceptible to this biocide [49]. According to a recent study, *Enterococci* from different sources were quite heterogeneous in their response to chlorhexidine [55]. In some cases, as in foods from animal origin, up to 25% of isolates were inhibited at 2.5 mg/l chlorhexidine. However, many isolates required between 25 and 250 mg/l chlorhexidine for inhibition. Remarkably, up to 74.5 of isolates from clinical samples followed by 15.62% of isolates from vegetable foods (along with 4% of isolates from seafood and 2.1% of isolates from wild flowers) were not inhibited at 250 mg/l chlorhexidine, although they were inhibited at 2.5 g/l. Multiple tolerant isolates were infrequent, except for two *E. faecalis* isolates from clinical sources, which resquired 2.5 g/l chlorhexidine for inhibition or 250 mg/l of benzalkonium chloride or cetrimide and in one case also of triclosan.

For several bacterial species, including methicillin resistant *Staphylococcus aureus*, a link between resistance against antibiotics and reduced susceptibility for disinfectants has been described in the past [56,57], but inconsistent results have also been reported. In a recent study, *E. faecalis* and *E. faecium* from blood and feces of hospitalized humans, from feces of outpatients and livestock and from food were screened for their susceptibility to a quaternary ammonium compound (didecyldimethylammoniumchloride, DDAC) and to 28 antibiotics [58]. Strains for which DDAC had MICs > 1.4 mg/l ("non-wildtype¨) were most often found in milk and dairy products (14.6%), while their prevalence in livestock was generally low (0–4%). An association between reduced susceptibility to DDAC, high-level-aminoglycoside resistance and aminopenicillin resistance was seen in *E. faecium* from human feces, indication a link between antibiotic resistance and (moderate) tolerance to disinfectants in a constrained number of isolates. However, it was concluded that the main driving force for the spread of such co- or cross-tolerant strains would be the use of antibiotics, not of disinfectants [58].

Resistance to copper

Bacterial resistance to heavy metals is also a matter of concern, since resistance genes are often located on the same mobile elements as those conferring resistance to antibiotics, raising opportunities for cross-selection of antibiotic resistance. Copper sulfate is used in feed for slaughter pigs and broilers as growth promoter and is also used for disinfection of the claws of cattle [50,59]. In *Enterococci*, the *copYZAB* operon from *Enterococcus hirae* was the first copper homeostasis system described [60] (Table 1). In addition, a transferable and plasmid-located copper resistance gene, designated *tcrB* (transferable copper resistance homologous to *copB*) was also described [61]. The *tcrB* gene (found in *tcrYAZB* operon, which is similar to the *copYZAB* copper homeostasis operon) encodes a putative protein belonging to the CPx-type ATPase family of heavy metal transporters [62]. Bacteria carrying the plasmid-borne *tcrYAZB* operon can tolerate up to 28 mM copper sulfate, while those lacking this gene can only tolerate up to 8 mM [61]. The *tcrB* gene is genetically linked to genes encoding resistance to macrolides [*erm*(B)] and glycopeptides (*vanA*) in the plasmids originating from pig isolates [61].

Conclusions

Enterococci are versatile bacteria widely distributed in the environment. As natural inhabitants of the human and animal intestine, they are exposed to the selective pressure of antibiotic administration. Both clinical and intensive animal farm use of antibiotics selects for resistant bacteria at the same pace as new drugs are introduced into market. Due to the various genetic transfer mechanisms they may elicit, *Enterococci* may become important reservoirs for the spread of antibiotic resistance genes. Heavy metal resistance and biocide tolerance could be factors in the co-selection of antibiotic resistance in the absence of antibiotic selective pressure. Furthermore, as opportunistic pathogens, antibiotic resistant strains pose higher risk for terminal outcome of the more susceptible patients suffering *enterococcal* infections. Once acquired, antibiotic resistance traits tend to persist as shown for vancomycin resistance, which persists in *Enterococci* from farms long after the ban of avoparcing. With this premise, decreasing the levels of antibiotic resistance in commensal *Enterococci* may be a difficult task possibly requiring a long-term evolution of *enterococcal* populations in the absence of antibiotic selective pressure.

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