Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Dissertação

Antimicrobial susceptibility and molecular characterization of resistance mechanisms of Enterococcus spp. from the intestinal microbiota of food producing animals

Joana Gião Santana Oliveira e Silva

Orientador(es) | E L Duarte

Ana Filomena Romeira de Jesus Amaro

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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

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Os respetivos resumos estão anexados no apêndice da presente tese.

Caracterização da suscetibilidade antimicrobiana e dos mecanismos moleculares de resistência de *Enterococcus* spp. isolados do microbioma intestinal de animais de produção

RESUMO

O trato intestinal dos mamíferos constitui um reservatório natural de *Enterococcus*, bactérias oportunistas, ubiquitárias, frequentemente associadas a infeções nosocomiais multirresistentes em humanos, sendo *Enterococcus faecium* e *Enterococcus faecalis* as espécies mais importantes.

Neste estudo foram identificadas as espécies de *Enterococcus* predominantes no conteúdo cecal de bovinos e suínos através de técnicas moleculares, tendo sido avaliados os perfis de suscetibilidade antimicrobiana das estirpes de *E. faecium* e *E. faecalis* pelos métodos de agar difusão e microdiluição, e identificados determinantes de resistência por PCR e *Whole Genome Sequencing.*

Os suínos constituem um reservatório de estirpes multirresistentes de *E. faecium* e *E. faecalis*. *Enteroccocus* spp. resistentes a antibióticos de importância crítica, designadamente daptomicina e linezolid, foram identificados em intestino de bovinos e suínos, tendo sido detectados os determinantes de resistência ao linezolid (*optrA* e *poxtA*). Estes resultados enfatizam a importância de monitorizar a resistência antimicrobiana em bactérias de origem animal.

Palavras-chave: *Enterococcus;* Resistência Antimicrobiana; Animais de produção; Multirresistência

ABSTRACT

The intestinal tract of mammals is a natural reservoir of *Enterococcus*, opportunistic and ubiquitous bacteria, frequently associated with multidrug resistant nosocomial infections in humans, *Enterococcus faecium* and *Enterococcus faecalis* being the most important species.

In the present study, the prevalent species of *Enterococcus* in the cecum of cattle and pigs were identified through molecular techniques, the antimicrobial susceptibility profiles of *E. faecium* and *E. faecalis* strains were assessed by agar diffusion and microdilution methods, and resistant determinants were identified through PCR and Whole Genome Sequencing.

Pigs are a reservoir of multidrug resistant *E. faecium* and *E. faecalis* strains. *Enterococcus* spp. resistant to critically important antibiotics, namely daptomycin and linezolid, were found colonizing bovine and swine gut, and the corresponding linezolid resistance determinants were identified (*optrA* and *poxtA*). These results highlight the importance of monitoring antimicrobial resistance mechanisms in bacteria from animals.

Key words: *Enterococcus*; Antimicrobial Resistance; Food producing animals; Multidrug resistance

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ABBREVIATIONS

AAC aminoglycoside acetyltransferase

aa-tRNA aminoacyl-transfer ribonucleic

ABC ATP-binding cassette

AMP Ampicillin

ANT aminoglycoside nucleotidyltransferases

ARE Antibiotic resistance

APH aminoglycoside phosphotransferase

ATP Adenosine Triphosphate

bp base pair

BSI bloodstream infection

CFU Colony Forming Units

CGE Center for Genomic Epidemiology

CIA Critically Important Antimicrobials

CIP Ciprofloxacin

CLO Chloramphenicol

CLSI Clinical & Laboratory Standards Institute

D-al-D-al D-alanyl-D-alanine

D-ala-D-lac D-alanyl-D-lactate

D-al-D-ser D-alanyl-D-serine

DAP Daptomycin

DNA Desoxyribonucleic acid

DNSE Daptomycin non-susceptible *Enterococcus*

EASSA European Antimicrobial Susceptibility Surveillance in Animals

ECOFF Epidemiological cut-off

EMA European Medicines Agency

ENA European Nucleotide Archive

ERY Erythomycin

ESVAC European Surveillance of Veterinary Antimicrobial Consumption

EU European Union

EUCAST European Committee on Antimicrobial Susceptibility Testing

fMet N-formylmethionine

GEN Gentamycin

LRE Linezolid Resistant Enterococci

LS$_A$P lincosamides/ streptogramin A/ pleuromutilins

LZD Linezolid

MDK Minimum Duration for Killing

MDR Multidrug resistant

MHB Mueller-Hinton broth

MIC Minimum Inhibitory concentration

MLS macrolides/ lincosamides/ streptogramins

MLS_B macrolides /lincosamides /streptogramin B

MLST multi-locus sequence typing

mRNA messenger RNA

MRSA Methicillin-resistant *Staphylococcus aureus*

NPET Nascent peptide exit tunnel

OIE World Health Organization for Animal Health

PBP Penicillin Binding Protein

PCR polymerase chain reaction

PFGE Pulsed-field Gel Electrophoresis

PhLOPS_A phenicols/ lincosamides/ pleuromutilins/ streptogramin A

PTC Peptidyl Transferase Center

QRDR Quinolone resistance-determining regions

RIF Regions of increased fluidity

RNA Ribonucleic acid

TEI Teicoplanin

T.E.S.T. Tigecycline Evaluation and Surveillance Trial

TET Tetracycline

TIG Tigecycline

VAN Vancomycin

tRNA transfer RNA

VCIA Veterinary Critically Important Antimicrobials

VRE Vancomycin Resistant *Enterococcus*

VVE Vancomycin Variable *Enterococcus*

WHO World Health Organization

WGS Whole Genome Sequencing

SECTION 1: INTRODUCTION

1.1. The genus Enterococcus

1.1.1. Brief characterization and important aspects

Formerly classified as group D streptococci, enterococci are Gram-positive, non-spore-forming, catalase-negative, facultative anaerobic cocci. They were granted a separate genus in 1984 after nucleic acid hybridization studies demonstrated that *Streptococcus faecalis* and *Streptococcus faecium* were genetically different from other members of the *Streptococcus* genus [1]. Placed in the *Enterococcaceae* family, the *Enterococcus* genus consists of over 50 species found to be common residents of the gastrointestinal (GI) tract of a variety of hosts – humans and other mammals, birds and invertebrates – and are ubiquitous in nature, frequently being found in soil, plants (including aquatic vegetation), water, fermented food, and dairy products [2,3].

Members of this genus exhibit an array of intrinsic properties that allow them to survive the innate defense mechanisms of the GI tracts of a wide diversity of hosts, remaining there as part of the microbiota, and persisting outside the gut for prolonged periods. Some of these characteristics include their ability to survive nutrient-deprived conditions and desiccation, growing in the presence of bile salts, in 6.5% sodium chloride, and a wide range of temperatures and pH [3,4,5, referenced in 6].

The capacity of enterococci to persevere in the environment and their ubiquity in the feces of humans and animals substantiated their use as bacterial indicators of fecal contamination of food and water for human consumption and, more recently, as indicators of hand hygiene [7]. However, evidence that enterococci are not exclusively of fecal origin (potentially being endogenous in sediments and soils or being able to replicate in water containing kelp) has led many to question their use as fecal pollution indicators [7,8].

Because they are such recurring intestinal microbiome members, enterococci probiotic cultures have been used as pharmaceutical preparations for human consumption or added to animal feeds. In humans, these probiotics are usually consumed to treat diarrhea and irritable bowel syndrome, to lower serum cholesterol, or promote immune regulation. In animals, probiotic cultures are included as feed additives to prevent or treat diarrhea, to stimulate the immune system, and as growth promoters. However, as the number of multidrug resistant (MDR) enterococci increased, their use as feed additives became controversial due to the risk of potentiating the transfer of virulence and antibiotic resistance determinants, giving rise to problematic enterococcal lineages in the GI tract [9].

Enterococci are estimated to typically comprise less than 1% of the adult human gut microbiome and are generally bacteria of relatively low virulence [6]. Although enterococci are considered commensal microorganisms, they are also important opportunistic pathogens. If favorable

environmental conditions arise, these bacteria proliferate at rates that surpass clearance, increasing their overall number in the intestine. Such is often the case in hospital settings, where the use of histamine H2-receptor antagonists (which increase gastric pH, facilitating bacterial overgrowth) and prolonged courses of antibiotic therapy (employing broad-spectrum antimicrobial agents to which enterococci are commonly resistant) are often necessary for treatment and prophylactic purposes [10]. If enterococcal populations can thrive, the risk of enterococcal dissemination into the bloodstream increases, along with the contamination of the environment surrounding the patients. Enterococci can also access the bloodstream via intravenous catheters, ascendant genitourinary infections, surgical site infections, transplantations, and abscesses [10,11]. Furthermore, through the formation of biofilm, enterococci can adhere to stents, urinary and intravenous catheters, resist phagocytosis, and impair the efficiency of antibiotic therapy [12]. Enterococci intrinsically possess numerous antibiotic resistance mechanisms and are known to efficiently recruit and exchange antibiotic resistance determinants, allowing these microorganisms to proliferate and dominate the intestinal flora under the selective pressure established by heavy antibiotic use, making them important agents in nosocomial infections worldwide [13].

1.1.2. Species distribution in humans and food producing animals

The intestinal tract of animals is an important reservoir of enterococci. The most often encountered enterococci species in animals and humans are *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* [6], although different factors may affect the intestinal microbiota's composition. The presence and distribution of diverse enterococci species in the host gut can vary significantly according to the host species, age, diet composition, gastrointestinal tract region, environmental stress [14, 15], and season [16].

Chickens are an example of a host species with age-dependent enterococci gut colonization. Devriese et al., 1991 described the intestinal flora of one-day-old chicks as consisting predominantly of *E. faecalis* and *E. faecium,* being rapidly overtaken by *E. faecium* after their first few weeks of life, and ultimately dominated by *Enterococcus cecorum* at about 12 weeks of age [14]. In the same study, *E. hirae* and *E. durans* were frequently isolated from the small intestine but were absent in the ceca, supporting that enterococcal species distribution also relies on the intestinal compartment. Although not often, *Enterococcus avium*, *Enterococcus gallinarum, Enterococcus casseliflavus,* and *Enterococcus mundtii* are also isolated in chicken [17].

There is also evidence suggesting age-dependent enterococcal colonization in cows [18]. Devriese et al., 1992 revealed that in suckling calves the most frequent *Enterococcus* species found were *E. faecalis*, *E. faecium* and *E. avium*. In ruminating young cattle, the enterococcal flora seemed to have a significant increase in *E. cecorum* but is absent in *E. faecium* and *E. avium,* and in adult dairy cows the overall enterococcal population decreases [18]. *Enterococcus hirae*, *E. faecium* [19,20], and *Enterococcus villorum* [20] were among the most recovered species from the feces of cattle in European and Canadian studies.

Regarding swine, *E. faecalis*, *E. faecium*, and *E. hirae* can often be isolated and other species, such as *E. durans, E. cecorum,* and *E. gallinarum*, are seldomly found [6,15].

E. faecalis and *E. faecium* are the most abundant *Enterococcus* species in the human GI tract, usually found in the oral cavity, small and large intestine, and more rarely in the stomach. Other enterococcal species can be present in the human gut, including *E. durans*, *E. avium*, and *E. hirae* [21]. Illustrations of how the diet composition can affect human enterococcal intestinal population have been described in a study comparing the feces of breastfed infants to formula-fed infants [22], and in a study performed on healthy humans after ingestion of Camembert cheese depleted of any *Enterococcus* species [23].

Enterococcus faecalis and *E. faecium* are the species of *Enterococcus* most often involved in community-associated and hospital-acquired infections such as urinary tract infections, endocarditis, septicemia, pneumonia, peritonitis, and surgical wound infections [24]. *E. faecalis* is the main responsible for the infections while *E. faecium* is commonly associated with higher rates of antibiotic resistance and increased mortality upon infection [13,25,11]. *E. faecalis* bloodstream infection (BSI) is usually associated with a genitourinary source, whereas *E. faecium* BSI is often of gastrointestinal origin [11].

1.1.3. Enterococci from animals as a source of antibiotic resistance genes

Several studies have shown that *E. faecalis* from humans and pigs can exhibit similar resistance patterns, virulence gene profiles and multi-locus sequence typing (MLST)/ Pulsed-field gel electrophoresis (PFGE) types [26-31, referenced in 33]. These findings support that multidrug resistant *E. faecalis* strains from porcine origin can be considered a human hazard [33].

E. faecium isolates recovered from clinical outbreaks and community and animal associated *E. faecium* strains usually belong to different clonal complexes [34-37, referenced in 33]. This suggests that *E. faecium* strains from animals do not constitute a risk for humans directly [33]. However, it is known that enterococci of animal origin can colonize the intestine of humans for up to 30 days [38,39, referenced in 33], and the transfer of antimicrobial resistance genes between *E. faecium* of animal and human origin has been confirmed through experiments conducted in the intestine of gnotobiotic mice and healthy humans [40, 41, referenced in 33]. Considering these findings, it could be possible for *E. faecium* strains from animal origin to act as donors of antibiotic resistance genes to human-adapted enterococci strains or more pathogenic bacteria after ingestion of animal products.

1.2. The spread of antimicrobial resistance

From the time antibiotics were first introduced into the market, they have been used extensively in human and veterinary medicine, not only for the curative treatment of infectious diseases, but also for prophylactic (when administered before clinical signs of infectious disease manifest) and metaphylactic (if an entire group of animals is treated after the diagnosis of infectious disease in part of the group, to prevent the spread of the disease to at-risk animals) purposes, and as growth promoters [42]. The latter use has been deemed dangerous for its role in increasing antibiotic resistance and was officially banned in several countries including those in the EU [43].

Regardless of the purpose, every time an antibiotic is used, it introduces a selective pressure in the microbiome environment, favoring the survival and dominance of microorganisms that intrinsically possess or have acquired resistance to that antimicrobial agent. Antibiotic residues in manure, soil and sewage water can also exert this selective pressure on other bacterial communities. Some resistant bacteria may also be spread directly onto other individuals or contaminate and colonize different environments, including water, soil and plants, creating various reservoirs of antibiotic resistance (Figure 1) [43,44].
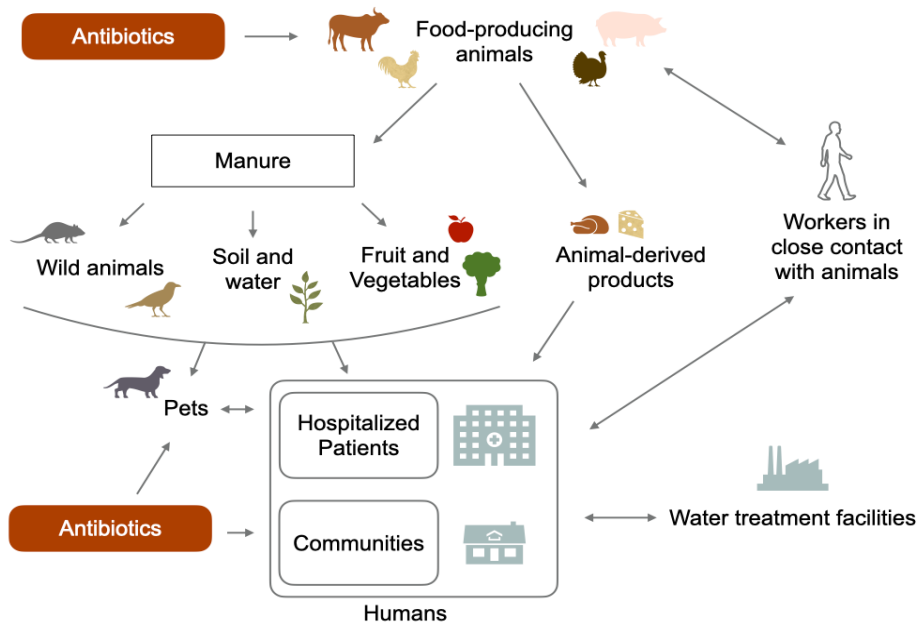
Figure 1. Schematic representation of the potential routes for the spreading of antibiotic resistance linking the communication networks between humans, animals and the environment.

The horizontal transfer of resistance genes can occur through three mechanisms: transduction (mediated by bacteriophages), conjugation (involving plasmid or transposon exchange between bacteria) and transformation (by the bacterial inclusion of genetic material resulting from the lysis of another bacteria) [43, 44].

Because many bacteria infect both humans and animals, problematic strains of bacteria can potentially be transmitted from one to the other – directly or through their shared environments.

The One Health concept highlights the importance of this relationship and promotes a multi-sectorial, multidisciplinary, global approach to achieve better public and animal health outcomes. This concept has become essential in the modern setting, where globalization, the increasing demand for livestock products, and the travel of humans and animals across borders and continents have become crucial factors for spreading infectious agents throughout the world.

The use and abuse of antibacterial drugs in human and veterinary medicine together with the lack of new and effective antibiotic classes, has become a matter of concern worldwide [43]. In order to contain the spread of antibiotic resistance and promote the prudent use of antibiotics, the World Health Organization (WHO) published a list of critically important antimicrobials for human medicine (WHO CIA list) [45], which is regularly revised. The World Health Organization for Animal Health (OIE) and the European Medicines Agency (EMA) followed this lead, creating a list of Veterinary Critically Important Antimicrobial agents (VCIA) and a Categorization of the antibiotics used in animals, respectively [46]. Both the CIA and VCIA lists divide antibiotics into three categories, based on their importance in human or veterinary medicine: Critically Important (which can be subdivided into Highest Priority and High Priority in the CIA list), Highly Important and Important. Supplementary Table 1 contains the classification of important classes of antibiotics for the treatment of enterococcal infections according to the CIA and VCIA lists.

The categorization introduced by EMA (Table 1) has the purpose of serving as a tool to prepare treatment guidelines that promote the responsible use of antimicrobials in veterinary medicine and the associated veterinary medicinal products regulation (Regulation (EU) 2019/6) will become applicable on January 2022. It groups antimicrobial agents in four categories: Category A (Avoid), Category B (Restrict), Category C (Caution), and Category D (Prudence) [47].

Table 1. Categorization of antibiotics for use in animals according to the EMA, adapted from [47].

Category	Category description	Examples of antibiotics
A "Avoid"	• antibiotics not authorized as veterinary medicine in the EU • should not be used in food producing animals • may be given to companion animals under exceptional circumstances	Lipopeptides, Glycopeptides, Oxazolidinones, Glycylcyclines, Carbapenems
B "Restrict"	• antibiotics critically important in human medicine: use in animals should be restricted • should be considered only when antibiotics in Categories C or D are not effective • use should be based on antimicrobial susceptibility testing, wherever possible	Quinolones and Fluoroquinolones, 3rd- and 4th-generation Cephalosporins (except for combinations with β-lactamase inhibitors), Polymyxins
C "Caution"	• for antibiotics in this category there are alternatives in human medicine • for some veterinary indications, there are no alternatives belonging to Category D • should be considered only when no antibiotics in Category D could be clinically effective	Aminoglycosides (except spectinomycin), Aminopenicillins in combination with beta lactamase inhibitors, Macrolides, Amphenicols
D "Prudence"	• should be used as first line treatments, whenever possible • should be used prudently, only when medically needed	Tetracyclines, Spectinomycin, Aminopenicillins (without beta-lactamase inhibitors)

The inclusion of each antibiotic/antibiotic class into one of the four categories of the recent EMA categorization of antibiotics used in animals was based on the necessity for their use in animals and on the potential public health consequences from increased antimicrobial resistance to each of these agents if they are used in veterinary medicine [47].

The EMA categorization, as well as the CIA and VCIA lists, will always be subject to change since resistant strains are constantly emerging and spreading, causing the loss of efficacy of certain antibiotics while new antibiotics capable of overcoming these resistance mechanisms are created and older, infrequently used antibiotics may regain their effectiveness. Thus, regulations regarding the use of antibiotics in veterinary medicine should continuously be revised and updated according to the data obtained in monitoring programmes for antibiotic resistance.

1.3. Major antibiotic classes and their modes of action

1.3.1 β-lactams

This class of bactericidal antibiotics is characterized by the presence of a β-lactam ring in its chemical structure. They work by inhibiting the synthesis of peptidoglycan, an essential cell wall constituent found in most bacteria, particularly in Gram-positive microorganisms (representing approximately 50% of the weight of the bacterial wall). With low toxicity and a broad spectrum of activity, β-lactams are among the most prescribed classes of antibiotics in the world.

They include penicillins, cephalosporins, monobactams, carbapenems and β-lactamase inhibitors [48, 49].

The most frequently used members of this class used to treat enterococci infections are either penicillin or aminopenicillins (with or without β-lactamase inhibitors), often in association with an aminoglycoside antibiotic.

<u>Mode of action</u>: In the final stage of the peptidoglycan biosynthesis, linear glycan strands are cross-linked to the mature peptidoglycan on the cell wall. Enzymes that possess DD-transpeptidase activity — otherwise known as penicillin-binding proteins (PBP's) — cleavage the peptide bonds between the two terminal D-alanine residues of the pentapeptide chains on these glycan strands, and the resulting molecule can then be bonded to the existing cross-linked peptidoglycan on the cell wall [50]. β-lactam antibiotics mimic the terminal structure of these pentapeptide chains and bind covalently to the PBP's, preventing them from catalyzing the transpeptidation of the peptidoglycan layer. The integrity of this layer is further compromised because bacterial enzymes continue to hydrolyze the cell wall, which ultimately results in bacterial rupture and cell death [49].

1.3.2. Glycopeptides

Glycopeptides are a class of bactericidal antibiotics composed of glycosylated cyclic peptides that, similarly to β-lactams, inhibit the final stage of the peptidoglycan biosynthetic pathway. They

are effective against most Gram-positive bacteria. However, the bulky nature of glycopeptides may hamper their movement through the porin channels of the outer membrane of Gram-negative microorganisms, precluding their use in infections caused by this group of bacteria. Vancomycin and teicoplanin were the first members of this class to be discovered, followed by the development of semi-synthetic lipoglycopeptide derivatives such as telavancin, dalbavancin, and oritavancin [51].

Vancomycin resistant enterococci (VRE) were first reported in the 1980s in Europe. This outbreak has been attributed to the use of avoparcin, a glycopeptide used as a growth promoter in food producing animals at the time [51].

Although resistance to glycopeptides has been reported worldwide in enterococci and staphylococci strains, they are still an essential antibiotic class used in the treatment of severe gram-positive infections caused by multi-resistant bacteria, including infections by methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* [49].

Mode of action: Throughout peptidoglycan assembly, a transglycosylase catalyzes the reaction by which disaccharide-pentapeptides —present in a membrane-bound cell wall precursor (lipid II) — are incorporated into linear glycan chains. These glycan chains can then proceed to the final cross-linking stage of peptidoglycan biosynthesis. However, glycopeptides bind to the D-alanyl-D-alanine (D-ala-D-ala) terminals of each pentapeptide through hydrogen bonds, obstructing the access of transglycosylases to their substrate, hindering cell wall synthesis [49,5].

1.3.3. Lipopeptides

Daptomycin (DAP) is the first and most well-known member of the recently discovered antibiotic class known as cyclic lipopeptides. DAP displays concentration-dependent bactericidal activity effective against many clinically significant Gram-positive pathogens such as VRE, MRSA and vancomycin-resistant *S. aureus*, being considered a last-resort antibiotic [52].

Mode of action: Daptomycin is a negatively charged antibiotic, depending on the presence of calcium ions to enter the cell membrane. The calcium-DAP complex has a higher affinity for membrane phospholipids, which have a negative electric charge. In model membrane studies, daptomycin antimicrobial activity was generally attributed to the formation of pores in the cell membrane that would presumably depolarize the cell membrane by allowing the influx of sodium ions [52]. However, recent *in vivo* and *in vitro* studies brought to light more knowledge on how this antimicrobial agent acts to kill bacteria, leading to the proposal of a novel DAP working model [53,54,55]. The new model (Figure 2) established that DAP molecules display an affinity for regions of increased fluidity (RIFs) in the cell membrane.
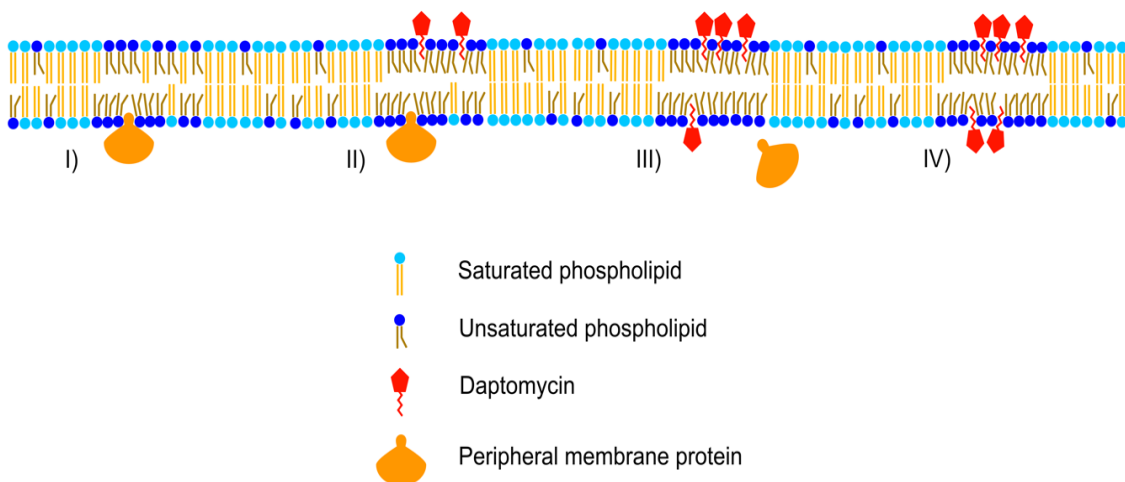
Figure 2. Illustration of the newly hypothesized mode of action of daptomycin. I) RIFs in the cell membrane are rich in unsaturated fatty acids. II) DAP displays affinity for RIFs and after insertion in the external leaflet and a calcium-stimulated oligomerization, it forces the membrane lipids apart, creating membrane distortions. III) This is counterbalanced by the attraction of more fluid lipids, which causes a redistribution of lipids in both the external and internal membrane leaflets, facilitating the progression and flipping of daptomycin oligomers to the inner membrane leaflet. IV) As a result, peripheral membrane proteins are dislodged from RIFs in the inner leaflet, and restricted from binding again. The presence of DAP oligomers in RIFs clusters the surrounding fluid lipids into inflexible domains, causing the withdrawal of some of these lipids from the regions and increasing overall membrane rigidity. Adapted from Müller A et al., 2016 [55]

After insertion, DAP causes an increase of overall membrane rigidity and the displacement of peripheral membrane proteins from RIFs. The bactericidal activity of this antibiotic could be explained by the delocalization and subsequent function impairment of proteins that participate in cell wall biosynthesis and lipid synthesis, and by the changes in the membrane bilayer organization [55].

1.3.4. Quinolones and fluoroquinolones

Quinolones were first introduced into the clinical practice in the late 1960s, and originally had a limited spectrum of activity (restricted to Gram-negative pathogens) and low systemic availability [50,56]. Fluorinated quinolones (*e.g.,* enrofloxacin and ciprofloxacin) were developed in the next few decades, with improved pharmacokinetics and a broader spectrum of action, that includes

Gram-negative and Gram-positive bacteria [50,56]. Quinolones and fluoroquinolones can be bacteriostatic or bactericidal, depending on concentration.

In veterinary medicine, quinolones are often prescribed to treat uncomplicated urinary tract infections in small animals, and fluoroquinolones are frequently used for infections caused by Gram-negative bacteria and intracellular pathogens such as *Mycoplasma* spp. and some *Mycobacterium* spp. (in companion animals, particularly in cats) [49,57].

Mode of action: During deoxyribonucleic acid (DNA) replication and transcription, helicases separate the double helix DNA into two single-stranded structures that act as templates for new complementary strands synthesized by DNA polymerase enzymes. As this process continues, positive supercoiling of the DNA accumulates ahead of the progressing replication fork. A topoisomerase II type enzyme named DNA gyrase can unwind the DNA by introducing negative supercoils and relieving the existing topological stress through the cleavage and re-ligation of DNA strands, in adenosine triphosphate (ATP)-dependent process [50,56].

Another topoisomerase known as topoisomerase IV acts at the end of bacterial replication, partaking in the relaxation of the supercoiled circular DNAs of the two interlinked DNA daughter molecules, allowing them to separate and eventually segregate to form two new bacterial cells [50,56].

Although the mechanisms of action of quinolones and fluoroquinolones are not entirely understood, it is known that they act mainly by binding at the topoisomerase-DNA interface in the cleavage-ligation active sites, promoting permanent breaks in the bacterial genome [58]. These breaks trigger DNA repair pathways (such as the SOS response), which are ultimately overwhelmed, resulting in an inability to efficiently repair DNA breakage [58,59].

1.3.5. Macrolides

Introduced in the market in the early 1960s, macrolides — in particular spiramycin and tylosin — were frequently used as growth promoters in food producing animals within the European Union until their ban in 1998 (EC2821/98 of 17 December 1998). They are currently still abundantly used in veterinary medicine to treat many common infections in small and large animals [60]. These antibiotic substances are active against many Gram-positive and Gram-negative bacteria, along with spirochetes and *Mycoplasma* spp. Macrolides possess mostly bacteriostatic activity and are often used in combination with other antibiotic classes, such as aminoglycosides and polymyxins [50,60]. Erythromycin, azithromycin, and tylosin are well-known members of this class.

Mode of action: The second step of DNA translation is elongation, and it is during this step that amino-acids carried via aa-tRNA (aminoacyl-transfer ribonucleic acid) are continuously bound to the growing peptide chain, converting codon information into proteins. Through translocation, the mRNA-tRNA (messenger RNA-transfer RNA) complex advances on the ribosome, allowing the next codon in the A site to be decoded. As the nascent polypeptide grows in length, it passes through a tunnel — the nascent peptide exit tunnel — which begins at the peptidyl transferase center in the 50S subunit, and leaves the ribosome [50,61].

Macrolides bind preferentially and reversibly to the 23S ribosomal RNA (rRNA) of the 50S subunit in the NPET, blocking translocation. This leads to the loss of incomplete peptides and dissociation of the ribosomal subunits [50].

Resistance to macrolides, lincosamides and streptogramins is often linked, because binding sites overlap in these antibiotics. This group of antibiotics is frequently referred to as MLS_B.

1.3.6. Streptogramins

There are two types of streptogramins: streptogramin A (a polyunsaturated macrolactone) and streptogramin B (a cyclic hexadepsipeptide). Separately, they exhibit bacteriostatic activity, but when combined in a specific ratio, they display potent bactericidal activity, working synergistically. They are often prescribed for the treatment of severe Gram-positive infections [62]. A typical combination of streptogramin B and streptogramin A is quinupristin-dalfopristin, prepared in a ratio of 30:70.

Mode of action: Dalfopristin (streptogramin A) and quinupristin (streptogramin B) can block protein synthesis by binding sequentially and adjacently to the 50S ribosomal subunit.

Dalfopristin binds first to the peptidyl transferase center (PTC) changing the conformation of the ribosome, improving its binding affinity to quinupristin. Dalfopristin also acts by inhibiting the binding of aa-tRNA to the ribosome A site, preventing the peptidyl transferase reaction. Quinupristin can then bind to the nascent peptide exiting tunnel (NPET) and block the nascent polypeptide chains elongation in a similar manner to macrolides [50].

1.3.7. Amphenicols

Amphenicols are a class of broad-spectrum antibiotics with mainly bacteriostatic activity, effective against Gram-positive, Gram-negative and anaerobic bacteria.

The use of chloramphenicol (a well-known member of this class) was associated with the development of irreversible aplastic anemia and dose-dependent bone marrow suppression in humans, leading to the ban of its use in food producing animals within the EU [63]. In turn, florfenicol — another amphenicol that lacks the mentioned toxic side effects — has become a successor to chloramphenicol, being widely used in farm animals, particularly calves, poultry, and pigs [64]

In human medicine, chloramphenicol is still used to treat MDR bacterial infections when other antibiotics are ineffective [49].

Mode of action: Amphenicols disrupt protein synthesis by reversibly binding to the site A region of the ribosomal 50S subunit. This affects the binding of aa-tRNA to the A site, making it inaccessible to peptidyl transferase, the enzyme that would allow the formation of peptidic bonds between the nascent polypeptide and the tRNA bound amino-acids [50].

1.3.8. Oxazolidinones

Linezolid is a recently developed bacteriostatic antibiotic that, along with tedizolid, belongs to a class of synthetic antibiotics known as oxazolidinones. Linezolid and tedizolid possess great oral bioavailability and are mainly active against Gram-positive bacteria. Linezolid is considered a last-resort antibiotic for the treatment of severe infections caused by Gram-positive multi-resistant bacteria, such as VRE, MRSA, and MDR pneumococci [65].

Mode of action: Linezolid has a unique method of action that differs from that of other antibiotics that inhibit protein synthesis: it prevents translation by binding to the 23S rRNA on the 50S subunit in the PTC, seemingly to inhibit the formation of a functional 70S initiation complex (consisting of the 50S subunit, the 30S subunit, mRNA, N-formylmethionine (fMet)-tRNA, and initiation factors 2 and 3) [65].

1.3.9. Aminoglycosides

Aminoglycosides are a bactericidal, concentration-dependent antibiotic class extensively used in veterinary medicine for a variety of infections. In the clinical setting, they are mostly used to treat infections caused by Gram-negative bacteria and staphylococci but are ineffective against anaerobic bacteria. Because some microorganisms display decreased permeability to these antimicrobials, they are often prescribed together with cell-wall inhibitors such as β-lactams, broadening their spectrum of activity [66]. Gentamicin, streptomycin, kanamycin, spectinomycin, and neomycin are notable members of this class.

In the European Union, neomycin and hygromycin-B were added to poultry feed as growth promoters before their use for this purpose was prohibited in 1976 [66].

Mode of action: This class of antibiotics binds irreversibly and with high affinity to the A site on the 16S rRNA of the 30S ribosomal subunit, allowing non-cognate tRNA to attach to the mRNA on the ribosome during translation, promoting translational misreading. This leads to an error-prone protein synthesis that produces non-functional proteins causing damage to the cell [67].

1.3.10. Tetracyclines and Glycylcyclines

Tetracyclines are a class of bacteriostatic antibiotics abundantly used in veterinary medicine. Their broad spectrum of activity and lack of toxicity have supported their therapeutic and prophylactic use for various infections in food producing animals [68]. Tetracycline, doxycycline, and oxytetracycline are well-known members of this class.

Tigecycline is a glycylcycline, a class of bacteriostatic antibiotics consisting of semisynthetic derivatives of tetracyclines. Tigecycline was developed to overcome the main existing resistance mechanisms to tetracyclines and is known to be less susceptible to efflux pumps and ribosome protection mechanisms [67]. It can be used as a last-resort drug when other antibiotic classes fail to treat a bacterial infection.

Mode of action: Tetracyclines and tigecycline have a similar action mechanism: they are actively transported into bacterial cells and bind reversibly to the 30S ribosomal subunit, halting protein synthesis by preventing the attachment of aa-tRNA to the ribosomal site A [50,67].

1.4. Antibiotic resistance in *Enterococcus* spp.

Antibiotic resistance is the ability of a microorganism to grow in the presence of an antimicrobial agent through various mechanisms which may result in the decrease of antibiotic influx, increased antibiotic efflux, bypass or modification of the antibiotic target and antibiotic inactivation [69]. Antibiotic resistance may be described as phenotypic and/or genotypic, and can be quantified by the minimum inhibitory concentration (MIC) of the drug necessary to prevent visible bacterial growth [69] or by detecting de presence of genetic determinants.

Resistance can be intrinsic — when associated with innate properties universally observed within a species or genus — or acquired — through the occurrence of mutations in the genome or by the acquisition of new genetic material through horizontal gene transfer [70].

Moreover, it is important to distinguish between cross-resistance and co-resistance. Co-resistance involves the acquisition of several resistance genes by a bacterial isolate and/or the occurrence of mutations in different *loci* conferring resistance to different antimicrobials [71]. The acquisition of genetic material or mutations that result in resistance to various antimicrobial agents of the same class is known as cross-resistance [71]. Cross-resistance can also occur in relation to antibiotic agents from different classes (usually with targets that overlap).

Bacteria can sometimes exhibit tolerance to an antibiotic (instead of resistance), when the bacterial population is able to transiently survive high concentrations of the drug without growing [68], requiring longer treatment durations. It can be quantified by the minimum duration for killing 99% of the population (MDK$_{99}$) [69].

E. faecalis and *E. faecium* display intrinsic resistance to antibiotics from several different classes, including β-lactams, aminoglycosides, streptogramins (in the case of *E. faecalis*), sulphonamides (and combination trimethoprim/sulfamethoxazole) and fusidic acid [72]. In addition, various enterococci strains have also acquired resistance to many last-resort antibiotics, such as vancomycin, daptomycin, linezolid and tigecycline [73].

The main mechanisms of acquired antibiotic resistance in *E. faecalis* and *E. faecium* are included in Table 2 [73-75].

Table 2. *Acquired Mechanisms of Antibiotic Resistance of Enterococcus spp.*

Antibiotic	Mode of Action	Resistance Mechanism	Associated gene/target	Common Species	Additional notes
β-lactams	Inhibition of cell wall synthesis	Decreased affinity to the antibiotic	*pbp4*	*E. faecalis*	Confers high level resistance to β-lactams
β-lactams	Inhibition of cell wall synthesis	Decreased affinity to the antibiotic	*pbp5*	*E. faecium*	Confers high level resistance to β-lactams
β-lactams	Inhibition of cell wall synthesis	Antibiotic inactivation	*blaZ*	*E. faecalis* *E. faecium*	Confers low-level constitutive resistance, that is frequently missed on normal screenings
Glycopeptides	Inhibition of cell wall synthesis	Target modification – alternative cell wall precursor	*van* operons	*E. faecalis* *E. faecium*	The *vanA* phenotype is characterized by vancomycin and teicoplanin resistance while the *vanB* phenotype is teicoplanin susceptible. Exceptions have been reported.
Daptomycin	Disruption of cell membrane; impairment of cell wall and lipid synthesis	Diversion of antibiotic from its preferred target	*liaFSR*	*E. faecalis*	Confers initial tolerance (MIC ≈ 3-4 µg/ml)
Daptomycin	Disruption of cell membrane; impairment of cell wall and lipid synthesis	Diversion of antibiotic from its preferred target	*cls*	*E. faecalis*	Can give rise to high-level resistance if associated with *liaFSR* mutations
Daptomycin	Disruption of cell membrane; impairment of cell wall and lipid synthesis	Diversion of antibiotic from its preferred target	*gdpD*	*E. faecalis*	Can give rise to high-level resistance if associated with *liaFSR* mutations
Daptomycin	Disruption of cell membrane; impairment of cell wall and lipid synthesis	Repulsion of antibiotic from cell envelope	*liaFSR*	*E. faecium*	Most common mutation in resistant isolates
Daptomycin	Disruption of cell membrane; impairment of cell wall and lipid synthesis	Repulsion of antibiotic from cell envelope	*yycFG*	*E. faecium*	
Daptomycin	Disruption of cell membrane; impairment of cell wall and lipid synthesis	Repulsion of antibiotic from cell envelope	*cls*	*E. faecium*	Only confers resistance if associated with mutations of *liaFSR*
Aminoglycosides	Inhibition of protein synthesis (initiation, elongation, termination, recycling)	Antibiotic inactivation	*aac(6')-aph(2'')*	*E. faecalis* *E. faecium*	Confers resistance to all aminoglycosides except streptomycin
Aminoglycosides	Inhibition of protein synthesis (initiation, elongation, termination, recycling)	Antibiotic inactivation	*aph(3')-Iiia*	*E. faecalis* *E. faecium*	Confers low-level kanamycin resistance
Aminoglycosides	Inhibition of protein synthesis (initiation, elongation, termination, recycling)	Antibiotic inactivation	*ant(4'')-Ia*	*E. faecalis* *E. faecium*	Confers low-level kanamycin, tobramycin, amikacin and neomycin resistance
Aminoglycosides	Inhibition of protein synthesis (initiation, elongation, termination, recycling)	Antibiotic inactivation	*aph(2'')-Ib*	*E. faecium*	Confers high-level gentamicin resistance
Aminoglycosides	Inhibition of protein synthesis (initiation, elongation, termination, recycling)	Antibiotic inactivation	*aph(2'')-Ic*	*E. faecalis* *E. faecium*	Confers high-level gentamicin resistance
Aminoglycosides	Inhibition of protein synthesis (initiation, elongation, termination, recycling)	Antibiotic inactivation	*aph(2'')-Id*	*E. faecium*	Confers high-level gentamicin resistance
Aminoglycosides	Inhibition of protein synthesis (initiation, elongation, termination, recycling)	Antibiotic inactivation	*ant(3'')-Ie*	*E. faecium*	Confers high-level streptomycin resistance
Aminoglycosides	Inhibition of protein synthesis (initiation, elongation, termination, recycling)	Antibiotic inactivation	*ant(6')-Ia*	*E. faecalis* *E. faecium*	Confers high-level streptomycin resistance

Table 2. *Acquired Mechanisms of Antibiotic Resistance of Enterococcus spp.*

Oxazolidinones and Amphenicols	Inhibition of protein synthesis (initiation)	Target modification	*cfr/ cfr*(B)	*E. faecalis* *E. faecium*	Confers the PhLOPS$_A$ phenotype; gene can be silent
		Target modification	23S rRNA	*E. faecalis* *E. faecium*	MICs depend on the number of mutated alleles
		Target protection	*poxtA*	*E. faecalis* *E. faecium*	Contributes to resistance to amphenicols, oxazolidinones and tetracyclines
		Target protection	*optrA*	*E. faecalis* *E. faecium*	LZD MICs depend on protein variant and genetic context; Can confer resistance to oxazolidinones and amphenicols
		Antibiotic inactivation	*catA/ catB*	*E. faecalis* *E. faecium*	Contributes to chloramphenicol resistance
		Efflux pumps	*fexA/ fexB*	*E. faecalis* *E. faecium*	Contributes to chloramphenicol resistance
		Target modification	*rplC/ rplD*	*E. faecalis* *E. faecium*	Mutations in ribosomal proteins L3/L4, respectively
MLS	Inhibition of protein synthesis (initiation)	Target modification	*erm(A)/ erm(B)*	*E. faecalis* *E. faecium*	MLS$_B$ Phenotype
		Antibiotic inactivation	*vatD/ vatE*	*E. faecium*	Confers streptogramin A resistance
		Antibiotic inactivation	*vgbA/ vgbB*	*E. faecium*	Confers streptogramin B resistance
		Target protection	*eat(A)*	*E. faecium*	LS$_A$P phenotype
Tetracyclines and Tigecycline	Inhibition of protein synthesis (elongation)	Efflux pumps	*tet(K)/ tet(L)*	*E. faecalis* *E. faecium*	Confer resistance to tetracyclines but not minocycline; Overexpression of *tet(M)* and *tet(L)* can confer TGC resistance
		Target protection	*tet(M)/ tet(S)*	*E. faecalis* *E. faecium*	Confer resistance to tetracycline and minocycline; Overexpression of *tet(M)* and *tet(L)* can confer TGC resistance
		Target modification	*rpsJ*	*E. faecalis* *E. faecium*	Mutations in this gene can confer resistance to TGC
Fluoroquinolones	Inhibition of DNA replication	Target modification	*gyrA, parC*	*E. faecalis* *E. faecium*	Mutations in the QRDRs can confer resistance

Adapted from [73], [74] & [75].
MIC: minimal inhibitory concentration; PhLOPS$_A$: phenicols/ lincosamides/ pleuromutilins/ streptogramin A;
TGC: Tigecycline; LZD: linezolid; MLS: macrolides/ lincosamides/ streptogramins; MLS$_B$: macrolides/lincosamides/streptogramin B;
LS$_A$P: lincosamides/ streptogramin A/ pleuromutilins; TGC: tigecycline; QRDR: Quinolone resistance-determining regions

1.4.1. Molecular mechanisms of resistance

β-lactams resistance

Intrinsic low-level β-lactam resistance can often be attributed to the expression of the low affinity, species-specific class B penicillin-binding protein known as PBP5 (sometimes referred to as PBP4 in *E. faecalis*).

The overproduction of PBP5 in *E. faecium* appears to be responsible for moderate ampicillin resistance but alone cannot confer high-level resistance to this antibiotic [76]. In *E. faecium,* the two main clades found in humans — subclade A1, associated with the hospital environment and clade B, the community-associated clade — exhibit different levels of resistance to ampicillin. The different susceptibility has been attributed to the existence of two allelic forms of the *pbp5* gene, one displaying higher ampicillin MICs, commonly ≥ 16 µg/ml (*pbp-R*), and the other with lower MICs to ampicillin, frequently ≤ 2 µg/ml (*pbp-S*). Subclade A1 strains are usually resistant to ampicillin, carry the consensus *pbp5-R* allele and express increased *pbp5* mRNA and PBP5 levels compared to those detected in clade B strains, which are usually susceptible to ampicillin and harbor the consensus *pbp5-S* allele. Subclade A2 strains, which are typically related to animals, exhibit MICs from 0.5 to 128 µg/ml and carry an intermediate allele between *pbp5-S* and *pbp5-R.* Strains can present ampicillin-resistant or susceptible phenotypes depending on the *pbp5* amino acid sequence, the levels of *pbp5* mRNA and PBP5 [77,78].

High-level resistance to β-lactams in *E. faecium* has been primarily attributed to amino acid sequence modifications in the C-terminal transpeptidase domain of PBP5, especially when these amino acid substitutions are located close to the active site of the enzyme and when some mutations are present in combination [79,80].

In addition, an L,D-transpeptidase named Ldtfm that is constitutively expressed in *E. faecium* has also been reported to provide intrinsic β-lactam resistance *in vitro*, by utilizing an alternative substrate (a tetrapeptide) to incorporate into the cell wall, bypassing DD-transpeptidation [81].

In *E. faecalis*, the expression of *pbp4* confers low intrinsic tolerance to ampicillin, and the overproduction of the associated PBP produces higher resistance to the same antibiotic. However, the latter mechanism does not result in a substantial increase of MICs values as those observed in *E. faecium* [74,82]. Resistance to penicillin, ampicillin, and imipenem is rare in *E. faecalis* and it has been associated with point mutations of PBP4 [83,84] which decrease the affinity of these proteins to the antibiotics, and mutations upstream of the putative *pbp4* promoter region that would increase the transcription of the gene [84].

Another possible but rare mechanism of β-lactam resistance in *E. faecalis* and *E. faecium* is the presence of the *blaZ* gene cluster (initially described in staphylococci), which encodes the synthesis of a β-lactamase capable of hydrolyzing the β-lactam ring, rendering the antibiotics in this class ineffective unless a β-lactamase inhibitor is present [85,86]. Enterococci express this gene constitutively at low levels, meaning that *in vitro* susceptibility testing may assess isolates that harbor this gene to be susceptible, when they could express resistance at high inoculum, such as during infection [87].

Cephalosporins

The molecular basis for the intrinsic resistance of enterococci to cephalosporins is not entirely understood. In addition to the low affinity exhibited by PBP5 to cephalosporins (particularly significant in *E. faecium*), other determinants of resistance to these antibiotics have been described. A eukaryote-like Ser/Thr kinase and phosphatase pathway named Irek/P [88], the two-component signal transduction system (TCS) CrosR/S [89] and the UDP-N-acetylglucosamine 1-carboxyvinyltransferase MurAA [90] appear to be required for intrinsic cephalosporin resistance in *E. faecalis*, suggesting they may influence one another or share a common pathway [90,91].

Glycopeptide resistance

Glycopeptide resistance is usually mediated by the *van* operons. To date, nine *van* operons (*vanA*, *-B, -C, -D, -E, -G, -L, -M* and *N*) expressing distinguishable levels of glycopeptide resistance, transferability and inducibility have been identified in *Enterococcus* [93].

The *van* operon consists of a cluster of genes encoding a response regulator system sensitive to glycopeptides, and a group of enzymes that work together to assemble an alternative pentapeptide cell wall precursor, eliminating simultaneously the normal pentapeptide precursors. The alternative cell wall precursor carries either D-alanyl-D-lactate (D-ala-D-lac) or D-alanyl-D-serine (D-ala-D-ser) amino-acid terminals instead of the typical vancomycin susceptible D-alanyl-D-alanine (D-ala-D-ala) ending precursors (Figure 3). Compared to the normal pentapeptide precursors, there is a 1000-fold decrease in binding affinity to vancomycin for D-ala-D-lac and a 7-fold decrease for D-ala-D-ser [93-95]. The *vanA* and *vanB* operons present a similar genetic organization. However, the *vanA* gene cluster contains an additional gene — *vanZ* — which provides resistance to teicoplanin through an unknown mechanism [95].

The *vanA* phenotype is defined by inducible, high-level vancomycin resistance and teicoplanin resistance [93]. The typical *vanB* phenotype is characterized by moderate to high-level inducible resistance to vancomycin alone [92], but *vanB*-carrying *Enterococcus* can also express low-level inducible vancomycin resistance [96,100] or, very rarely, the *vanA* phenotype [98]. Although less frequently, *vanB* phenotype-*vanA* genotype enterococci have been observed in several countries [99-101]. Vancomycin-susceptible enterococci carrying the *vanA* gene have also been reported [102, 103] and, alarmingly, some of these strains (known as vancomycin-variable enterococci) can convert into resistant phenotypes during antimicrobial therapy [104]. The *vanC* gene cluster is typically expressed constitutively and provides low-level resistance to vancomycin [105]. Enterococci such as *E. gallinarum*, *E. casseliflavus,* and *E. flavescens* intrinsically possess, respectively, the *vanC-1*, *vanC-2*, and *vanC-3*, subtypes of the *vanC* operon.
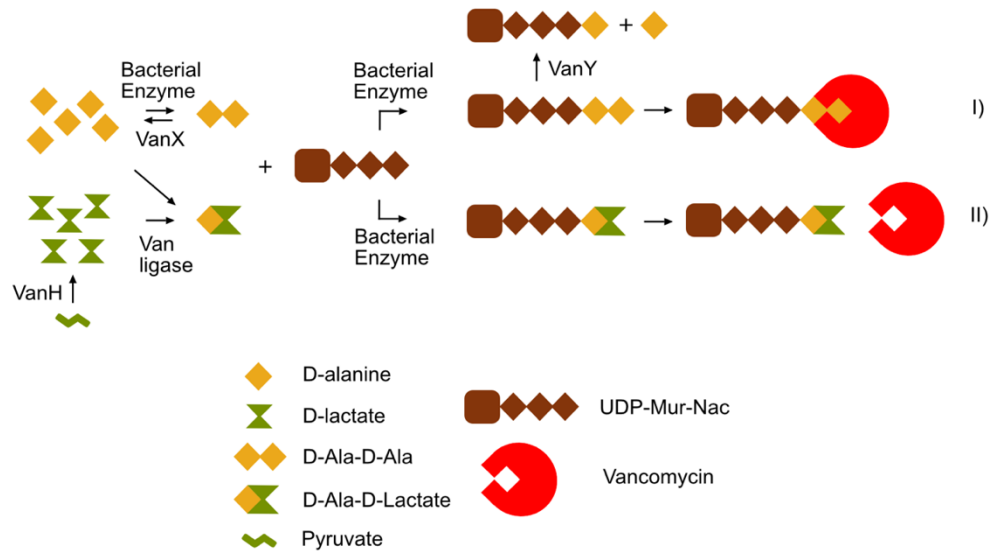
Figure 3. Vancomycin's mode of action and resistance mechanism.
I) Vancomycin acts by binding to the D-alanyl-D-alanine (D-ala-D-ala) terminals of the pentapeptide precursors, hindering normal cell wall biosynthesis. II) The *vanA* operon contains a two-component response regulator system that responds to the presence of glycopeptides and to the cell wall disruption caused by these antimicrobials by activating the downstream *vanA* operon genes: *vanH, vanA* and *vanX*. VanH reduces pyruvate to D-lactate and VanX breaks down the D-ala-D-ala repeats, providing substrate for the VanA ligase, which then binds D-alanine to D-lactate. These will attach to the tripeptide precursor (UDP-Mur-Nac) and the resulting pentapeptide will display low binding affinity to vancomycin. VanY cleaves the peptide bond between the D-ala-D-ala terminals of the regular pentapeptide precursors, further reducing the pools of pentapeptides with high binding affinity to vancomycin. Adapted from Faron et al., 2016 [94].

Intrinsic resistance to vancomycin can also be observed in strains of *Enterococcus* that harbor the same L, D-transpeptidase that confers resistance to β-lactams — Ldtfm — using an alternative substrate (that is not targeted by vancomycin) to incorporate into the cell wall [106].

Lipopeptide resistance

The prevalence of daptomycin non-susceptible enterococci (DNSE) in humans is low. Although non-susceptible strains have been isolated in patients without prior exposure to daptomycin, the development of resistance during antibiotic therapy has been observed [107,108].

The underlying mechanisms for DAP resistance in enterococci are not entirely understood. Over 30 genes have been connected to daptomycin resistance in this genus, most of them involved with the cell envelope stress response or with the metabolism of important cell membrane phospholipids [73,109]. Studies performed in *E. faecalis* strains suggested that daptomycin accumulates at the division septum when this antibiotic increases to or above the MIC (Figure 4, I), and mutations in LiaFSR, a three-component system that regulates the cell envelope stress response, were crucial to DAP resistance [110-112]. One study demonstrated that in *E. faecalis* DAP tolerant strains, a deletion in *liaF* altered the distribution of cardiolipin (CL) enriched domains (usually localized at the division septum and cell poles), diverting them from the division septa (Figure 4, II) [73]. Mutations in *cls* and *gdpd* — two genes involved in phospholipid metabolism — which alter the cell membrane phospholipid composition, by reducing its content in phosphatidylglycerol (a phospholipid that appears to be necessary for daptomycin to bind to

the membrane), together with the activation of the LiaFSR system, can deflect DAP molecules from binding to the septum and decrease oligomerization, leading to the occurrence of resistant phenotypes [109-111]. Modifications in the LiaFSR pathway usually confer an initial tolerance to DAP (MIC 3-4 μg/ml) and higher MICs can be achieved with the additional mutation of *cls* or *gdpD* [110-112].

In *E. faecium*, the mutation of LiaFSR is the most common pathway to DAP resistance in clinical isolates, followed by modifications in the essential two-component system YycFG, involved in the control of peptidoglycan biosynthesis [73,113,114]. Deletions of *liaR* in strains that harbored either one of the mutations mentioned above resulted in hypersusceptibility independent of the genetic background [115]. In contrast with *E. faecalis*, there is no evidence of phospholipid rearrangement being a mechanism for resistance in *E. faecium*. Instead, mutations that confer DAP resistance in *E. faecium* predominantly result in a more positively charged envelope surface and repulsion of the calcium-bound antibiotic (Figure 4, III) [73,116].
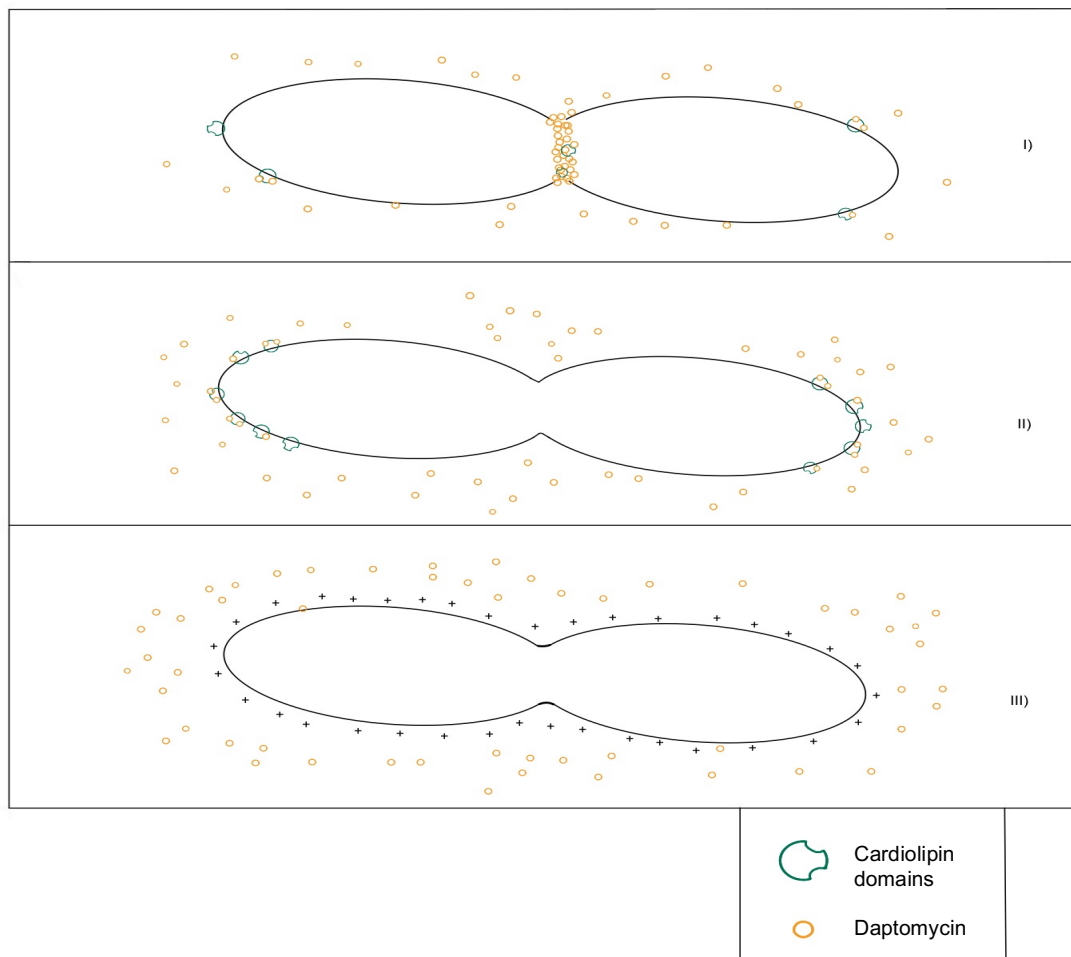
Figure 4. Proposed mechanisms of action and resistance to daptomycin in *Enterococcus* spp. I) The daptomycin-calcium complex binds preferentially to the division septum and accumulates there in DAP-susceptible enterococci. II) DAP- resistant *E. faecalis* diverts the antibiotic away from the septum in a process that involves the redistribution of the cardiolipin micro-domains, which results in inefficient binding of the drug to the cell membrane. III) DAP-resistant *E. faecium* strains can repeal the calcium-bound daptomycin through changes in cell envelope composition that increase the overall net charge of the cell surface. Adapted from Tran et al., 2015 [115].

A recent study found that the environment influences the development of DAP resistance in *E. faecium* [117]. Strains that grew in flask environments favored resistance by repulsion of the antibiotic from the cell surface, while bacteria that could produce biofilm in planktonic environments selected for both repulsion and diversion of daptomycin from the division septa. Regardless of the adaptive response that conferred DAP resistance, all evolutionary trajectories culminated in mutations in genes affecting membrane homeostasis, most often in *cls* [117]. While mutations in these genes alone may not directly grant DAP resistance, they appear to play an important role in it [73,117].

Fluoroquinolone resistance

Enterococci intrinsically display low-level resistance to fluoroquinolones.

Two multidrug resistance efflux pumps denominated EmeA (homologous to NorA, found in *Staphylococcus aureus*) that belongs to the major facilitator superfamily (MFF) and EfrAB, an ATP-binding cassette (ABC) transporter, were found to contribute to intrinsic resistance to some fluoroquinolones in *E. faecalis* and *E. faecium* [118,119].

Overexpression of *qnr*-like genes has also been linked to intrinsic fluoroquinolone resistance in both *E. faecalis* and *E. faecium*. These genes encode putative pentapeptide repeat proteins homologous to other Qnrs proteins, which block quinolone inhibition of both topoisomerases [120].

DNA gyrase and topoisomerase IV, the target enzymes of quinolones and fluoroquinolones, are homologous tetramers comprised of two pairs of two subunits: GyrA and GyrB (for DNA gyrase) or ParC and ParE (in the case of topoisomerase IV). Mutations of the quinolone resistance-determining regions (QRDR) of the corresponding encoding genes *gyrA* and *parC*, have been described in fluoroquinolone-resistant *E. faecalis* and *E. faecium* strains and are thought to lower the binding affinity of these antibiotics to the respective enzymes [74,121-123].

Macrolide, lincosamide and streptogramin resistance

Concerning streptogramins, while the quinupristin-dalfopristin preparation can be effective against *E. faecium* strains, *E. faecalis* harbors the chromosomal gene known as *lsa*, which causes this antibiotic combination to become inefficient [74]. The *lsa* gene is responsible for intrinsic resistance to lincosamides and streptogramin A [75]. It encodes a putative protein that is structurally similar to ABC-efflux pumps (although the exact function of this protein remains unknown) [75].

In *E. faecium*, low-level intrinsic streptogramin B resistance can be attributed to the presence of the *msrC* gene, which encodes a predicted ABC-efflux pump [124,125]. If an additional streptogramin A resistance determinant is acquired, *msrC*-carrying *E. faecium* strains express high-level resistance to quinupristin-dalfopristin [124]. However, *msrC* is not distributed evenly through all *E. faecium* isolates [125].

Cross-resistance to macrolides, lincosamides, and streptogramin B (MLS$_B$ phenotype) is frequently observed in enterococci and is usually involved with the acquisition of *erm* genes (especially *erm(B)*), which encode ribosomal methyltransferases that modify the 23S rRNA target site shared by this group of antibiotics [116,126].

The *msrA* gene encoding antibiotic resistance (ARE) ABC-F protein that mediates macrolide and streptogramin B resistance through ribosomal protection, has also been discovered in *Enterococcus* strains [124,127].

Acquired resistance to streptogramins can also be found in *Enterococcus* strains capable of producing enzymes that alter these antibiotics: VatA and VatB — two acetyltransferases that target streptogramin A — and VgbA and VgbB — lactonase enzymes that cleavage the ring structure of streptogramin B [128].

Another possible mechanism leading to quinupristin-dalfopristin resistance is through mutation on the *eat*(A) (standing for *Enterococcus* ABC-transporter) gene. For example, previously susceptible *E. faecium* strains showed the LS$_A$P phenotype exhibiting resistance to lincosamides, streptogramin A and pleuromutilins after mutation [128,129].

Oxazolidinone and amphenicol resistance

The plasmid-borne *cfr* gene and the *cfr*-like gene *cfr*(B) have been detected in strains of *Enterococcus* [73]. These genes encode S-adenosyl-l-methionine (SAM) enzymes that catalyze the methylation of a specific nucleotide in the 23S rRNA, located in the PTC [73,130]. The *cfr* gene was initially found in *Staphylococcus sciuri* but has spread across bacterial species and genera [73].

The presence of *cfr* can confer resistance to several antibiotic classes, including phenicols, lincosamides, oxazolidinones (only linezolid), pleuromutilin and streptogramin A (PhLOPS$_A$ phenotype) [130]. However, the gene can also remain silent and fail to express the PhLOPS$_A$ phenotype altogether [73,131,132].

Resistance to oxazolidinones (including tedizolid) and amphenicols can also be mediated by the *optrA* gene [73], which encodes an ARE ABC-F protein that presumably protects the ribosome from antibiotic-mediated inhibition [124]. This gene can be chromosomally encoded or included in a variety of plasmids or transposon structures [73].

Multiple variants of the OptrA protein have been described, with distinct amino-acid sequences. The OptrA variant and the genetic context surrounding the *optrA* gene have been shown to play a role in the expression of resistance to linezolid, influencing the MIC values [73,133].

Another recently discovered ARE ABC-F protein, PoxtA, has been reported in *Enterococcus* isolates. It is associated with a putative mobile element, and confers decreased susceptibility to phenicols, oxazolidinones, and tetracyclines [134].

The chloramphenicol-florfenicol resistance genes *catA* and *catB* — chloramphenicol acetyltransferases — and *fex*A and *fexB* — encoding florfenicol exporters — have also been found in enterococci strains [135].

Aminoglycoside resistance

In enterococci, low-level intrinsic resistance to aminoglycosides can generally be attributed to the limited drug uptake into the cell (which can be overcome by the additional use of cell wall inhibitors) and the expression of an aminoglycoside modifying enzyme (AME) or a ribosome modifying enzyme.

E. faecium strains carry a chromosomally encoded 6' acetyltransferase (AAC(6')-Ii), an AME that confers intrinsic tolerance to tobramycin, kanamycin, netilmicin, and sisomicin. The AME alters the mentioned aminoglycosides in a way that reduces their affinity to bind to the ribosome, rendering them ineffective [136].

The *efmM* gene encodes the *E. faecium* methyltransferase, an enzyme that modifies a 16S rRNA nucleotide on the 30S subunit's A site, decreasing the affinity of a subset of aminoglycosides (namely tobramycin and kanamycin) to bind to their ribosomal target [137].

Acquired resistance to aminoglycosides in enterococci is usually mediated by genes encoding aminoglycoside modifying enzymes (AMEs). These enzymes alter the conformation of the aminoglycoside, compromising the binding of the antibiotic to its target in the 30S subunit.

AMEs belong to one or two of three subclasses: aminoglycoside phosphotransferases (APHs), aminoglycoside nucleotidyltransferases (ANTs) or aminoglycoside acetyltransferases (AACs).

High-level resistance to gentamicin is usually conferred by the expression of a bi-functional enzyme — AAC(6')-Ie/APH(2')-Ia — that is capable of both phosphorylation at the 2'hydroxy position of gentamicin and acetylation at the 6' hydroxy position of other aminoglycosides, except streptomycin [75]. The enzymes APH(2'')-Ib, APH(2'')-Ic and APH(2'')-Id can also contribute to gentamicin resistance, to a smaller extent [138-140, referenced in 136].

High-level resistance to streptomycin can arise due to a single-step ribosomal mutation or to the acquisition of genes coding for either ANT(6')-Ia or ANT(3'')-Ie [136].

Other AMEs can cause aminoglycoside resistance (Table 2). Nevertheless, as gentamicin and streptomycin are the most efficient members of this class against enterococcal infections, high-level resistance to these two antibiotics is the most concerning.

Tetracycline and glycylcycline resistance

In enterococci, resistance to tetracyclines usually occurs through either target protection or by antibiotic efflux.

Ribosome protection can be mediated by the *tet(M)* and *tet(O)* genes, which encode ribosomal protection proteins that, much like ABC-F proteins, bind to the ribosome and promote the dissociation of the antibiotics from their binding sites. These genes confer resistance to tetracycline, doxycycline, and minocycline [74,141].

Tetracycline-specific extrusion is exhibited by strains containing efflux pumps encoded by the *tet(L)* and *tet(K)* genes, which may confer resistance to tetracycline but not minocycline [74].

A recent study has demonstrated that tigecycline resistance can be connected to the expression of plasmid-encoded *tet(M)* and *tet(L)* (which were co-transcribed) and a high number of plasmid copies carrying both genes [73,141].

Mutations of the *rpsJ* gene, encoding for the S10 ribosomal protein of the 30S subunit, have been associated with tigecycline resistance in multiple bacterial genera [143], particularly those found in the regions of the S10 protein near the 16S rRNA tigecycline target [73,143]. These findings support that the mutations observed in resistant phenotypes may give rise to conformational changes in the 16S rRNA, resulting in decreased tigecycline binding affinity and/or reduced translational inhibition [73, 143].

1.4.2. Resistance to critically important, last-resort antibiotics in Europe in humans and animals

Last-resort antibiotics are the last line of antimicrobial agents to be used in the treatment of severe bacterial infections, when common first line drugs have failed to effectively treat the infection. These are Critical Important Antibiotics (CIA) that should be prescribed very sparingly, only in highly specific settings, and they should be included in national and international stewardship programs, as resistance to these antibiotics is of great global concern [144].

Resistance to the last-resort antibiotics linezolid, daptomycin, and tigecycline has been reported in enterococci strains from European countries including Portugal. Because the use of these antibiotics in food producing animals is prohibited in the EU, reports of resistant strains are more frequent in humans.

Linezolid (LZD): The overall prevalence of linezolid resistance among enterococci from humans and animals in European countries is below 1%. However, linezolid resistant enterococci (LRE) strains have been identified in humans from hospitals all over Europe (including in Denmark, Poland, Spain, Ireland, France, and Portugal) [73] and, regarding food producing animals, linezolid resistant *E. faecium* and *E. faecalis* isolates from pigs of Spanish origin have also been reported [149].

In Portugal, LRE have been isolated from hospitalized patients, hospital wastewaters and community-associated wastewaters [73,145].

Linezolid exposure is a risk factor for LRE infection and, more recently, the use of florfenicol in food producing animals has also been related to linezolid resistance emergence [145]. Resistance rates to linezolid may depend on the species of *Enterococcus* as showed by Tian et al., 2014, in reporting a greater occurrence of LZD resistance in *E. faecalis* compared to *E. faecium* [146].

Daptomycin: Enterococci resistance to daptomycin is rare and with no increasing trend in a five-year study between 2009 and 2013 in European countries [148]. Only a few DAP resistant enterococci from humans have been reported in Germany, Ireland, Spain, and Denmark [73]. Although spontaneous resistance occurs at low frequencies *in vitro*, DAP non-susceptibility is

usually associated with exposure to this antibiotic [73]. To our knowledge, daptomycin non-susceptible enterococci have not been isolated in Portugal in humans or animals.

Tigecycline: Resistance to tigecycline in enterococci isolates remains low globally. In Europe, tigecycline non-susceptible enterococci of human origin have been reported in Spain, Germany, Italy, Ireland, and Portugal [73]. A few tigecycline clinically resistant *E. faecium* isolates were also found in pigs from France [149]. In our country, a few non-susceptible strains have been isolated over the last 20 years from various sources, including hospitalized patients, healthy humans, hospital wastewaters, chicken meat and swine [73, 145, 150].

SECTION 2: OBJECTIVES

This thesis will focus mainly on the resistance mechanisms to the antibiotic classes more often used in the treatment of human enterococcal infections caused by *Enterococcus faecium* and *Enterococcus faecalis*.

This study aims to:

- Investigate the frequency of colonization of pigs and cattle intestines by *Enterococcus* spp.
- Identify the species of *Enterococcus* isolated from pig and cattle gut flora through molecular and biochemical profile-based methods.
- Characterize the susceptibility patterns of *Enterococcus faecalis* and *Enterococcus faecium* strains to a panel of antibiotics by minimum inhibitory concentration (MIC) determination, using the agar diffusion technique and the microdilution technique.
- Detect and characterize antimicrobial resistance determinants to critically important antibiotics by polymerase chain reaction (PCR) and whole genome sequencing (WGS).

SECTION 3: MATERIALS AND METHODS

3.1. Biological samples and Enterococci isolation

Cecal samples obtained from randomly selected clinically healthy bovine (n= 205) and swine (n= 254) were collected from various Portuguese slaughterhouses throughout 2017, under the scope of the national surveillance program of antimicrobial resistance in zoonotic and commensal bacteria, according to Commission Decision 2013/652/EU. Samples were collected after evisceration at the slaughtering line, kept in plastic containers at a temperature of 4 – 8ºC, and sent to the laboratory for bacteriological analysis within two days.

On the day of arrival at the laboratory, 1 g of each sample was inoculated in a separate tube containing 9 ml of Heart Infusion Broth with 6.5 % NaCl and incubated at 37 ºC for 18 h. The broth cultures were then streaked on the selective medium BBL™ Enterococcosel™ Agar (Becton, Dickinson Company) and incubated at 37 ºC for another 18 h.

The BBL™ Enterococcosel™ Agar has esculin in its composition, a glycoside that is hydrolyzed by enterococci to dextrose and esculetin, the latter reacting with an iron salt (ferric ammonium citrate) also present in the medium to form dark brown to black colored zones in the agar under translucent colonies. This selective medium also incorporates sodium azide and oxgall, which inhibit the growth of gram-negative and other gram-positive bacteria, respectively.

A single isolated presumptive enterococci colony was transferred onto Colombia Blood Agar and incubated for 18 to 22 h at 37 ºC. A few streaks of bacterial culture were frozen in Tryptone Soy broth with 15 % glycerol and stored at -80 ºC until further analysis.

3.2. DNA extraction and quantification

DNA extraction of bacterial isolates followed the boiling lysis procedure. Briefly, a few colonies were dissolved into 100 µl of Tris-EDTA (TE) buffer, boiled for 10 min and centrifuged at 20.000 *xg* for 5 min at 4 ºC. The supernatant was then transferred to a new micro-centrifuge tube and stored at -20 ºC.

DNA quantification was performed using the NanoDrop™ 2000 (Thermo Scientific™, Thermo Fisher Scientific) Spectrophotometer to assess the DNA concentration and purity of the samples by measuring absorbance at 260 nm and the 260/280 and 260/230 ratios, respectively.

3.3. Identification of *Enterococcus* spp.

3.3.1. Molecular identification

Confirmation of presumptive enterococci isolates was performed by PCR amplification targeting the 16S rRNA gene according to Deasy et al., 2000 [151]. To identify enterococci at the species

level, four PCR assays were carried out, each containing different primer sets. The primers used for the identification of five enterococci species, namely *E. faecalis*, *E. faecium*, *E. durans*, *E. hirae* and *E. casseliflavus* were designed by Jackson et al., 2004 [152] (Table 3).

All PCR reaction mixtures were performed in a total volume of 25 µL and contained 1× gel load reaction buffer NZYTech, 1.5 mM of NZYTech MgCl2, 400 µM of deoxynucleotide triphosphate, 1 U of Taq polymerase (NZYTech), a variable concentration of specific-group primers (Supplementary Table 1) and 1-2µl DNA template. Amplifications were performed on Tone Biometra (Analytik Jena) thermocycler using optimized thermal cycling conditions. The primer sets, annealing temperatures, and positive control strains used for the PCR assays are described in Table 3.

PCR products were analyzed by 2 % to 2.5 % agarose gel electrophoresis stained with RedSafe^{TM} (iNtRON Biotechnology) and visualized under UV lighting using the BioDoc – It^{TM} Imaging System (UVP).

Sanger sequencing of the 16S rRNA gene amplification with primers E1 and E2 (Table 3) was performed for bacterial isolates (n=18) that displayed the genus band but failed to be identified by the PCR assays for species identification.

PCR products were purified using ExoSAP-IT™ (Applied Biosystems^{TM}, Thermo Fisher Scientific), a commercially available combination of two hydrolytic enzymes, according to the manufacturer's recommendations. The quality of the purified DNA fragments was assessed by agarose gel electrophoresis and sequenced by Eurofins Genomics Europe Sequencing GmbH, Germany.

DNA sequences were read with ChromasProTM v2.1.8.0 (Technelysium Pty Ltd) and analyzed with the Basic Local Alignment Search Tool (BLAST). Consensus DNA sequences were generated using BioEdit v7.2.5.0 (Tom Hall Ibis Therapeutics) sequence alignment editor and fasta files were further analyzed with BLAST.

3.3.2. Biochemical identification

Biochemical identification was performed using the commercially available API$^{\circledR}$ 20 Strep (bioMérieux, France) on bacterial isolates for which molecular confirmation was not conclusive (n= 35).

For this purpose, isolates were grown for 24 h in Columbia blood agar at 37 °C and suspended in ampoules of API Suspension Medium adjusted to obtain turbidity greater than 4 McFarland opacity standards. The microcupules of the API 20S strips were prepared and inoculated as described by the manufacturer and the strips were incubated at 37 °C in aerobic conditions for 4 h to attain the first reading, and for 24 h to obtain a second reading if required.

3.4. Antimicrobial Susceptibility Testing

3.4.1. Agar dilution

The agar dilution method was performed according to the guidelines provided by the Clinical & Laboratory Standards Institute [153] (CLSI), following procedure from the National Reference Laboratory for Antimicrobial Resistance (NRL-AMR) on *E. faecalis* (n= 85) and *E. faecium* (n= 49) strains.

The agar dilution plates were prepared for a total of 11 antibiotics, in eight different concentrations (except for chloramphenicol, which was prepared in six concentrations): vancomycin (1-128 µg/ml), teicoplanin (0.5-64 µg/ml), daptomycin (0.5-32 µg/ml), linezolid (0.5-64 µg/ml), tetracycline (1-128 µg/ml), tigecycline (0.03-4 µg/ml), ampicillin (0.5-64 µg/ml), ciprofloxacin (0.12-16 µg/ml), erythromycin (1-128 µg/ml), gentamicin (8-1024 µg/ml) and chloramphenicol (4-128 µg/ml).

Briefly, the procedure was as follows:

- Bacteria were cultured on Columbia blood agar and incubated at 37 ºC in aerobic conditions for 18-24 h.
- A few colonies were suspended in 0.85 % NaCl ampoules to obtain turbidity of 0.5 McFarland standard, adjusted using the Densimat (BioMérieux, France) densitometer.
- Suspensions were further diluted in saline solution to reach a final inoculum concentration of approximately 10^5 colony forming units (CFU)/ml.
- The antibiotic-supplemented agar dilution plates were inoculated by means of a multichannel pipette, which dispensed 2 µl of bacterial suspension from four different strains in a row. This process was repeated six times for each agar plate, to obtain plates containing 24 different bacterial strains (Supplementary Figure 1). For quality control, the reference strain *E. faecalis* ATCC 29212 was always inoculated last.
- Three Tryptose Soya Agar (TSA) plates were also inoculated in a similar manner in the beginning, middle and end of the assay to assess the viability of the strains.
- To ascertain the purity of the cultures, all the bacterial suspensions were streaked onto TSA agar plates.
- The agar plates were incubated for 24 h at 37 ºC.

After incubation, plates were read manually over a dark surface and the MIC value was determined as the lowest antibiotic concentration necessary to inhibit the growth of bacteria (growth of 1-2 colonies was neglected). For bacteriostatic antibiotics, the MIC was read as the concentration that reduced bacterial growth by at least 80 %, compared to the positive control. The MIC values of the quality control strain had to be within acceptable ranges according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [154], for the assay results to be considered valid.

Epidemiological cut-off values (ECOFF) established by EUCAST were used to categorize the isolates as susceptible or resistant. MIC_{50} and MIC_{90} values [155], the lowest antibiotic

concentration values that inhibit the growth of 50% and 90% of the isolates, respectively, were determined analyzing the MIC distribution for each antibiotic.

Although enterococci isolates were tested for daptomycin and tigecycline susceptibility using this method, the results were not validated and thus, this method was not employed to assess phenotypic resistance to tigecycline and daptomycin.

3.4.2. Broth microdilution – Commercial microplates

The antimicrobial susceptibility patterns of 19 strains of *E. faecium* (n= 6) and *E. faecalis* (n=13) that exhibited MICs above or one twofold below the ECOFF of daptomycin and tigecycline in the agar dilution technique were further determined using commercially available broth microdilution Sensititre™ EUVENC plates (Thermo Scientific™, Thermo Fisher Scientific). The 96-well EUVENC microplate contains a total of 12 antibiotics in eight different concentrations, which include vancomycin, teicoplanin, daptomycin, linezolid, tetracycline, tigecycline, chloramphenicol, quinupristin-dalfopristin, ampicillin, ciprofloxacin, erythromycin, and gentamicin (Supplementary Figure 2). All procedures were performed according to manufacturer instructions.

Briefly, the inoculum was prepared as above described in 3.4.1, using Sensititre™ cation-adjusted Mueller-Hinton broth (Thermo Scientific™, Thermo Fisher Scientific) instead of saline solution. Microplates were inoculated by using an automated delivery system Sensititre™ AIM™ (Thermo Scientific™, Thermo Fisher Scientific), that added 50 µL of the broth to each well. The plates were then sealed and incubated at 37 °C for 24 h. Likewise, bacterial cultures were checked for purity control and reference strain *E. faecalis* ATCC 29212 was used for assay quality control.

The antibiotic panels were read using a semi-automatic Digital MIC Viewing System (Sensititre VizionTM, ThermoFisher Scientific) and the Scientific™ Sensititre™ SWIN™ Software System (Thermo Scientific™). MIC values were established as the lowest antibiotic concentration inhibiting visible growth, and MIC reading and interpretation followed the recommendations by EUCAST. ECOFF established by EUCAST were used to categorize the isolates as susceptible or resistant.

3.4.3. Broth microdilution – *in house* procedure

Microdilution technique was performed, according to CSLI recommendations [153], on selected isolates carrying the o*ptrA* gene (n= 9) to further confirm linezolid MIC values.

The procedure was performed as follows:

- A fresh stock solution of linezolid (Glentham Life Sciences, UK) was prepared and diluted in Sensititre™ cation-adjusted Mueller-Hinton broth (MHB-Thermo Scientific™, Thermo Fisher Scientific) as described in Table 4 to obtain eight working solutions.

- Sterile 96-well microplates were prepared according to the layout of Figure 6; the wells were filled with 50 µl of the working solutions to obtain two times the desired antibiotic concentrations, achieved after the addition of the bacterial inoculum to each well (Supplementary Table 2; Supplementary Figure 3). The negative and positive control wells were filled with 100 µl and 50 µl of MHB, respectively.

- The sealed antibiotic microdilution plates were stored at ≤ -20 ºC (to be used in a period inferior to 3 months) and unfrozen the day of the assay.

- Bacteria were cultured on Columbia blood agar and incubated at 37 ºC in ambient air for 18–24 h. Three to five colonies were suspended in 0.85 % NaCl ampoules to obtain turbidity of 0.5 McFarland standard. Bacterial suspensions were further diluted in MHB with TES (Thermo Scientific™, Thermo Fisher Scientific) to reach a final bacterial concentration of about $5x10^5$ CFU/ml in each well.

- The wells were then inoculated with 50 µl of bacterial suspension using a multichannel pipette (Supplementary Figure 3), except for the negative control wells.

- The microplates were sealed and incubated at 37 ºC for 24 h.

The plates were read manually using a mirror viewing device. The MIC values were established using ECOFFs and following EUCAST recommendations for linezolid, disregarding pinpoint growths [154].

3.5. Molecular characterization of resistance

Molecular screening of antibiotic resistance determinants to vancomycin and linezolid was performed by PCR.

DNAs extracted from all *E. faecalis* (n= 85) and *E. faecium* (n= 49) isolates were screened for the *vanA* gene. The *vanB* gene was assessed in isolates that exhibited vancomycin MIC values of 2 and 4 µg/ml (n= 45) either in agar dilution or Sensititre™ EUVENC microdilution plates.

The presence of genes conferring resistance to linezolid and chloramphenicol was analyzed by two PCR assays targeting, respectively, *optrA* and *cfr* genes. Chloramphenicol-resistant isolates (MIC> 32 µg/ml) were screened by PCR assay for the detection of *cfr* gene (n= 20). A PCR assay targeting the *optrA* gene was performed on isolates with linezolid MICs ≥2 µg/ml (n= 83) and isolates exhibited resistance to chloramphenicol and linezolid MICs of 1 µg/ml (n= 7) in agar dilution or Sensititre™ EUVENC microdilution plates.

The primers, annealing temperatures, and positive control strains used for PCR assays are described in Supplementary Table 3 and performed as previously described [156-159].

3.6. Whole Genome Sequencing

The complete genome sequence of one *E. faecium* and two *E. faecalis* isolates from swine were studied, using the short-read sequencing method of the Illumina platform. The DNA was extracted using PureLink® Genomic DNA mini kit (Invitrogen™, Thermo Fisher Scientific), according to manufacturer instructions. The concentration and quality of the extracted DNA were assessed using the NanoDrop™ 2000 (Thermo Scientific™, Thermo Fisher Scientific) spectrophotometer and the Quantus™ Fluorometer (Promega).

The library preparation and pair-end DNA sequencing were performed using Illumina® HiSeq sequencing technology (Novogene Europe, UK). All pre-processed reads were assembled with SPAdes 3.12.0. The assembly stats of all sequenced isolates were calculated using QUAST-5.0.2. Contigs with sizes lower than 500 bp were removed and bioinformatic analysis using tools available at the Center for Genomic Epidemiology (CGE) website [160] were performed. Multi-locus sequence type (MLST), acquired antimicrobial resistance genes and virulence genes were identified using MLST version 2.0, ResFinder version 4.1, and VirulenceFinder version 2.0, respectively. Raw sequence data from these isolates were submitted to the European Nucleotide Archive (ENA) under study accession numbers: ERS6142029, ERS6142031, ERS6142033

3.7. Statistical analysis

For the statistical analysis of data, the Fisher's exact test on Microsoft Excel was used, since the Chi-square test (χ^2) may not be reliable on samples that are too small (< 1000) and exact tests should be used in such cases. To test the independence of two nominable variables, Fisher's exact test was used with a 95% confidence level (a p-value of ≤ 0.05 being considered significant).

SECTION 4: RESULTS AND DISCUSSION

4.1. *Enterococcus* recovery, diversity and distribution

A total of 323 presumptive *Enterococcus* spp. isolates were recovered from cecal samples of 459 animals (205 bovine and 254 swine). Genus-specific PCR assay confirmed 301 isolates as belonging to the *Enterococcus* genus, 142 recovered from cattle and 159 from pigs. Sixteen isolates with similar colony morphology to enterococci were determined to be *Aerococcus viridans*, through biochemical identification (n= 9) and Sanger sequencing (n= 7). Therefore, recovery rates averaged around 69% for bovine and 63% for swine samples, which is comparable to what has been described in other reports [19].

Species identification was accomplished in 84.7% (n= 255) of isolates by PCR assays targeting five species of *Enterococcus*: *E. hirae* (n= 107*), E. faecalis* (n= 88), *E. faecium* (n= 49)*, E. casseliflavus* (n= 7) and *E. durans* (n= 4). However, PCR assays failed to recognize 18 *E. hirae* strains. Hairpinning (which may happen when a primer loops on itself) or primer-dimerization (when the two primers anneal with one another) were the most likely causes. These isolates were identified either by API®20 Strep (n= 12) or 16S rDNA amplification and Sanger sequencing (n= 6, ≥ 99.87% identity).

Apart from the above-mentioned *E. hirae* isolates, the API® 20 Strep system also identified *E. casseliflavus* (n= 4)*, E. durans* (n= 2)*, E. faecium* (n= 5) and *E. faecalis* (n= 3). However, three of the strains initially identified as *E. casseliflavus* by biochemical testing were later recognized as *E. hirae* (n= 2) by PCR assay, and *E. mundtii* (n= 1) by Sanger sequencing (≥ 99.74% of sequence identity).

Globally, Sanger sequencing of the 16S rRNA gene allowed identification of 14 *Enterococcus* isolates: *E. faecalis* (n= 1), *E. hirae* (n= 6), *E. mundtii* (n= 3), *Enterococcus asini* (n= 3) and *Enterococcus thailandicus* (n= 1). Three of these species had not yet been identified in our study: *Enterococcus thailandicus, E. mundtii* and *E. asini*, with sequence identities of 99.5%, ≥99.61%, and ≥ 99.47%, respectively. The identification of four *Enterococcus* strains through Sanger sequencing was not possible because the 16S rRNA sequences were identical or almost identical to two different species: *E. thailandicus*/*E. durans* (n= 1), *E. faecium*/*E. hirae* (n= 2) and *E. hirae*/*E. azikeevi* (n= 1).

Identification at the species level of enterococci can be challenging. The biochemical characteristics of enterococci have been shown not to be reliable for the identification of *Enterococcus* species [161]. Due to intraspecies variability (*i.e.*, strains from the same species do not share identical 16S rRNA gene sequences), there is no consensus definition of bacterial species by comparing 16S rDNA sequences, with different authors suggesting from ≥ 99% to ≥ 99.8% homology between sequences to define a species [162]. Thus, some strains could be misidentified since the 16S rRNA gene sequencing has low discriminatory power when

differentiating closely related enterococcal species [163]. Sequencing other genes such as the *sodA* or *tuf* genes could be of help in confirming the identity at the species level of isolates that displayed relatively low sequence homology with a species, or that could not be identified by Sanger sequencing of the 16S rRNA gene.

Alternatively, or in complement with the previously mentioned methods, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) could be used in the identification of *Enterococcus* species, as it is also a reliable and rapid tool for identifying *Enterococcus* species with greater discriminatory power than biochemical methods [164].

The isolates without identification to the species level (n= 16) remained classified solely as *Enterococcus* spp. The distribution and diversity of *Enterococcus* species in the cecal samples of cattle and pigs are illustrated in Figure 5.
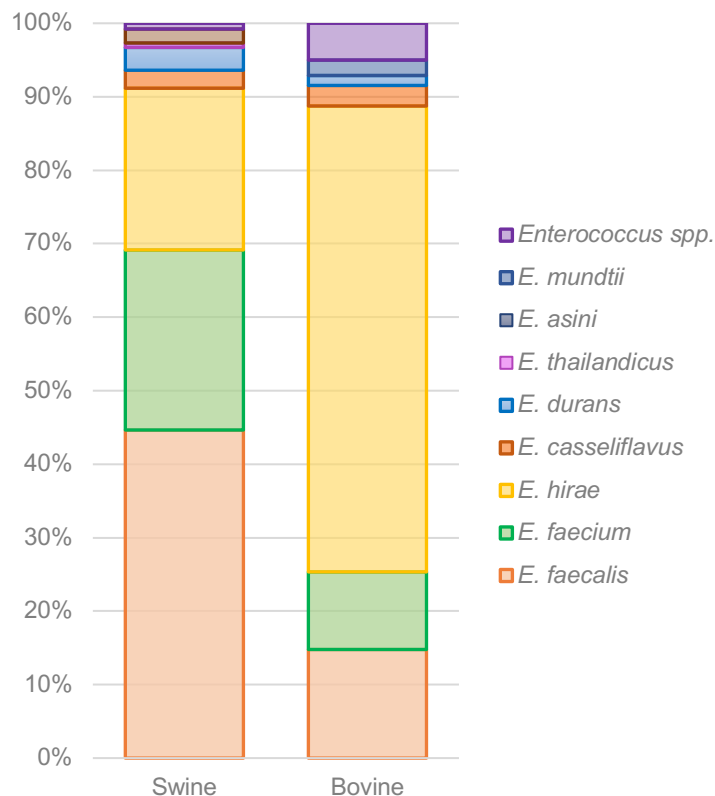
Figure 5. Distribution of *Enterococcus* species in cattle and pigs

In pigs, *E. faecalis* (44.6%, n= 71), *E. faecium* (24.5%, n= 39) and *E. hirae* (22%, n= 35) were the most prevalent species of *Enterococcus*, comprising over 90% of the 159 isolates (Fig. 5). Other species of *Enterococcus*, namely *E. durans* (n= 5) and *E. casseliflavus* (n= 4) were also found. These are all commonly found enterococci in pig cecal samples [6,15]. Other two species were recovered from pigs: *E. thailandicus*, a species firstly discovered in fermented sausage in

Thailand [26], but also isolated in bovine and swine [165,166] and *E. asini,* which is almost exclusively reported in donkeys [6,167], but has also been found in fecal samples from broilers [168] and pigs [165], and on raw cow milk [169]. *Enterococcus thailandicus* and *E. asini* have been reported in the gastrointestinal tracts of pigs in a study that also detected the *cfr* antibiotic resistance gene in strains of *E. thailandicus* collected from a pig farm [165].

With the exception of *E. thailandicus* and *E. asini*, the other species of *Enterococcus* identified in the current study had also been reported in pig fecal samples from Portugal [15,170]. Other species of enterococci have been reported at low prevalence in pigs, such as *E. cecorum, E. avium* and *E. gallinarum* [6,171,172]

E. hirae was the predominant species recovered from the bovine cecal samples, representing 63.4% (n= 90) of the 142 isolates (Fig. 5). Other species recovered from cattle included *E. faecalis* (n= 21), *E. faecium* (n= 15), *E. casseliflavus* (n= 4), *E. durans* (n= 2) and *E. mundtii* (n=3). *Enterococcus casseliflavus* and *E. mundti* are often associated with vegetation and forage crops, but are also found at low frequencies in the intestine of cattle and other animals [20,173,174]. The high prevalence of *E. hirae* in bovine feces along with the presence of the other species reported in the current study, have also been previously described by other authors, including Kühn (2003) [19] and Zaheer et al., 2020 [20]. Other species of *Enterococcus* identified in the intestine of cattle at low frequencies include *E. raffinosus, E. villorum,* and *E. gallinarum* [6,19]. Although these species were not found in this study, they could be among the isolates classified as *Enterococcus* spp.

In summary, we found similar *Enterococcus* species diversity in swine (six species) and cattle (seven species). The identification of enterococci to the species level in this study was difficult, although both biochemical and molecular methods were used. Therefore, some isolates remained classified simply as *Enterococcus* spp. Nevertheless, species not initially targeted in this study were identified namely, *E. thailandicus* and *E. asini* in pigs and *E. mundtii* in cattle.

High numbers of *E. faecium* and *E. faecalis* were found in pigs. These findings suggest that pigs may represent a larger reservoir of *E. faecalis* and *E. faecium* than bovine in Portugal.

4.2. Phenotypic Antimicrobial Resistance in *E. faecium* and *E. faecalis*

4.2.1. Prevalence and distribution of antibiotic resistance phenotypes in cattle and pigs

The antibiotic susceptibility profiles of 85 *E. faecalis* and 49 *E. faecium* isolates from cattle (n= 31) and pigs (n= 103) were obtained by the agar dilution technique. The frequency of resistance, MIC_{50} and MIC_{90} of these strains are described in Tables 3 and 4.

The MIC values of the quality control strain *E. faecalis* ATCC 29212 to daptomycin and tigecycline were consistently outside of the expected range provided by EUCAST in the agar diffusion method and thus, the results were not validated for these two antibiotics.

Table 3. *Frequency of resistance, MIC_{50} (µg/ml) and MIC_{90} (µg/ml) of E. faecalis isolates from cattle and pigs*

Antibiotic	ECOFF (µg/ml)	*E. faecalis* (N= 85)					
		Cattle (N= 19)			Pigs (N= 66)		
		MIC_{50}	MIC_{90}	Resistance (%)	MIC_{50}	MIC_{90}	Resistance (%)
VAN	4	≤ 1	4	0	≤ 1	2	0
TEI	2	≤ 0,5	≤ 0,5	0	≤ 0,5	≤ 0,5	0
TET	4	≤ 1	64	47*	128	128	98*
CIP	4	1	2	9	1	2	9
ERY	4	≤ 1	128	16*	> 128	> 128	86*
LZD	4	2	2	0	2	2	1.5
GEN	64	≤8	16	0	≤8	32	11
AMP	4	≤ 0,5	2	0	1	2	0
CLO	32	≤ 4	8	0*	8	64	29*

VAN: Vancomycin; TEI: Teicoplanin; TET: Tetracycline; CIP: Ciprofloxacin; TET: Tetracycline;
ERY: Erythromycin; LZD: Linezolid; GEN: Gentamicin; AMP: Ampicillin; CLO: Chloramphenicol;
ECOFF: Epidemiological cut-off values; * p-value ≤ 0.05

Table 4. *Frequency of resistance, MIC_{50} (µg/ml) and MIC_{90} (µg/ml) of E. faecium isolates from cattle and pigs*

Antibiotic	ECOFF (µg/ml)	*E. faecium* (N= 49)					
		Cattle (N= 12)			Pigs (N= 37)		
		MIC_{50}	MIC_{90}	Resistance (%)	MIC_{50}	MIC_{90}	Resistance (%)
VAN	4	≤ 1	≤ 1	0	≤ 1	2	0
TEI	2	≤ 0,5	1	0	≤ 0,5	1	0
TET	8	32	128	41*	128	> 128	78*
CIP	8	2	4	0	1	2	0
ERY	4	4	> 128	0*	128	> 128	60*
LZD	4	2	2	0	1	2	0
GEN	32	≤ 8	≤ 8	0	≤ 8	≤ 8	0
AMP	4	1	1	0*	1	8	30*
CLO	32	≤ 4	≤ 4	8	≤ 4	16	3

VAN: Vancomycin; TEI: Teicoplanin; TET: Tetracycline; CIP: Ciprofloxacin; TET: Tetracycline;
ERY: Erythromycin; LZD: Linezolid; GEN: Gentamicin; AMP: Ampicillin; CLO: Chloramphenicol;
ECOFF: Epidemiological cut-off values; * p-value ≤ 0.05

Overall, the level of antibiotic resistance was higher in *E. faecalis* than in *E. faecium* strains and also in swine compared to bovine. Resistance to tetracycline was the most prevalent in *E. faecium* and *E. faecalis* strains regardless of animal origin. No isolates were resistant to vancomycin and teicoplanin.

In *E. faecalis*, the frequencies of tetracycline and erythromycin resistance were very high and more prevalent in pigs. Resistance to ciprofloxacin, gentamicin, chloramphenicol and linezolid was only observed in swine isolates. Ciprofloxacin resistance (n= 6) and linezolid resistance (n= 1) were rare, while ampicillin resistant strains were absent (Table 3).

Regarding *E. faecium*, tetracycline resistance was high and present in both animal species, while erythromycin resistance was also high, but found only in isolates from pigs. Ampicillin resistance was moderate and also shown by isolates of porcine origin. Chloramphenicol resistance was observed very infrequently (n= 2) in isolates from both animal species, and no isolates displayed phenotypic resistance to ciprofloxacin or linezolid (Table 4).

Tetracycline resistance was widespread in *E. faecium* and *E. faecalis* and more frequent in the isolates collected from pigs. Erythromycin resistance was also significantly more prevalent in *E. faecium* and *E. faecalis* of porcine origin than in bovine enterococci; in fact, no erythromycin-resistant *E. faecium* isolates were recovered from cattle (Tables 3 and 4).

The frequency of resistance to chloramphenicol was generally low, except in *E. faecalis* strains from pigs (29%), which displayed a significantly higher prevalence of antibiotic resistance than *E. faecalis* from bovine (p-value ≤ 0.05) (Table 3).

High levels of tetracycline and macrolide resistance were anticipated since the antimicrobial consumption in our country reported on the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) [175] indicates that the most purchased antibiotics in Portugal between 2010 and 2018 have been tetracyclines, followed by penicillins, macrolides and polymyxins. Other frequently used antibiotics according to ESVAC include aminoglycosides (with a rise in consumption in 2018), pleuromutilins, fluoroquinolones, sulfonamides, licosamides and amphenicols.

Ciprofloxacin resistance was absent in *E. faecium* and in *E. faecalis* isolates from bovine, only being noticed in 9% of *E. faecalis* isolates from pigs. Low frequencies of ciprofloxacin resistance can be related to the use of fluoroquinolones in animals, which has overall decreased since 2014 in Portugal, being consumed more moderately than tetracyclines, penicillins, macrolides, polymyxins and pleuromutilins [175].

Ampicillin resistance was only displayed by *E. faecium* isolates from swine. It was expected that ampicillin resistance would only be observed in *E. faecium* isolates since resistance to this antibiotic is very rare in *E. faecalis* strains [86, 153] (Table 4).

The prevalence of gentamicin resistance was low and only found in *E. faecalis* isolated from pigs. Nevertheless, high-level gentamicin resistance (MIC > 128 µg/ml) was detected in four isolates. High-level resistance to gentamicin in enterococci is often associated with the presence of the bifunctional aminoglycoside modifying enzyme AAC(6')-Ie/APH(2')-Ia that inactivates other aminoglycosides (except streptomycin) thus, isolates that display this level of resistance are usually considered resistant to most aminoglycosides [75,176].

In the present study, resistance to both ampicillin and gentamicin was not observed in any animal species. These antibiotics are frequently used in combination for the treatment of enterococcal infections and resistance to both is infrequent in enterococci [148,179].

Resistance to linezolid was found in *E. faecalis* of swine origin (n= 1, MIC = 8 µg/ml). Of notice, this strain was also resistant to chloramphenicol (Table 3).

The *in-house* microdilution method confirmed linezolid MIC values, corroborating the results obtained by the agar diffusion technique. Although linezolid resistance in food producing animals in Europe is still rare, some linezolid resistant strains have been isolated from food producing animals, including *E. faecium* and *E. faecalis* isolates from pigs reported in Spain in 2013-14 [149] and *Enterococcus* spp. from cattle, collected from Lithuania in 2009 [177].

Phenotypic resistance to glycopeptides (vancomycin and teicoplanin) was not observed in any isolate. Other European countries have also reported low to absent levels of VRE between 2004 and 2014 [149,179]. The absence of VRE in our study is likely a direct result of the ban on the use of glycopeptides on food producing animals in 1997.

In general, the results herewith presented are in accordance with a previous study by Jong et al., 2018 which assessed the antibiotic susceptibility of enterococci from healthy food producing animals collected by the European Antimicrobial Susceptibility Surveillance in Animals (EASSA) program from 2004 to 2014 in various European countries (including Spain, France, Italy, Denmark, and the United Kingdom, among others) [179]. Jong at al. observed widespread, high-level resistance to tetracycline and erythromycin and a low prevalence of linezolid, tigecycline and vancomycin resistance. Ampicillin resistance was also reported more frequently associated with *E. faecium* from swine and chicken than from bovine and, ampicillin-resistant *E. faecalis* strains were scarce [179]. Most *E. faecium* strains remain susceptible to gentamicin, while gentamicin resistance was most frequently associated with *E. faecalis* strains from swine [149,179].

In the present work, some antibiotics such as teicoplanin, linezolid, and ciprofloxacin displayed MIC_{50} and MIC_{90} values below the ECOFF for *E. faecium* and *E. faecalis* from cattle and pigs, which indicates the presence of susceptible enterococci populations in both animal species. The discrepancy between MIC_{50} and MIC_{90} values for tetracycline in *E. faecalis* isolates of porcine origin and erythromycin in *E. faecium* isolates from bovine may indicate the presence of at least

two subpopulations of each species, with very distinct susceptibility to the respective antimicrobial agents.

The resistance level in enterococci from cattle was generally lower than that observed in swine corroborating previous studies [149, 179]. The selective pressure caused by antibiotic use is well-known to promote the development and persistence of antibiotic resistance in bacteria [180]. The significantly higher prevalence of antibiotic resistance (p-value ≤ 0.05) observed in pigs for tetracycline, erythromycin, chloramphenicol and ampicillin could be associated with the more frequent use of these antibiotics (or antibiotics of the same class, as is the case of chloramphenicol) in that animal species. These results may mean that pigs have higher antimicrobial intake than cattle, which could be explained by the more intensive livestock production practices for this species in our country when compared to bovines. In the 2012 and 2013 Portuguese annual reports of surveillance on antimicrobial consumption surveillance, swine were the animal species associated with the highest percentage of antibiotic purchases [181,182]. However, the association between animal species and frequency of chloramphenicol and ampicillin resistance was not found among strains of *E. faecium* (for chloramphenicol) and *E. faecalis* (for ampicillin, since no ampicillin resistant *E. faecalis* were isolated) (p-value > 0.05).

4.2.1.2. Multidrug resistance

Isolates were characterized as multidrug resistant when they presented non-susceptibility to at least one agent of three or more antibiotic classes.

The frequency of MDR was 34.1% (29/85) in *E. faecalis* isolates and 20.4% (10/49) in *E. faecium* strains (Figure 6), which was not significantly different (p-value > 0.05).
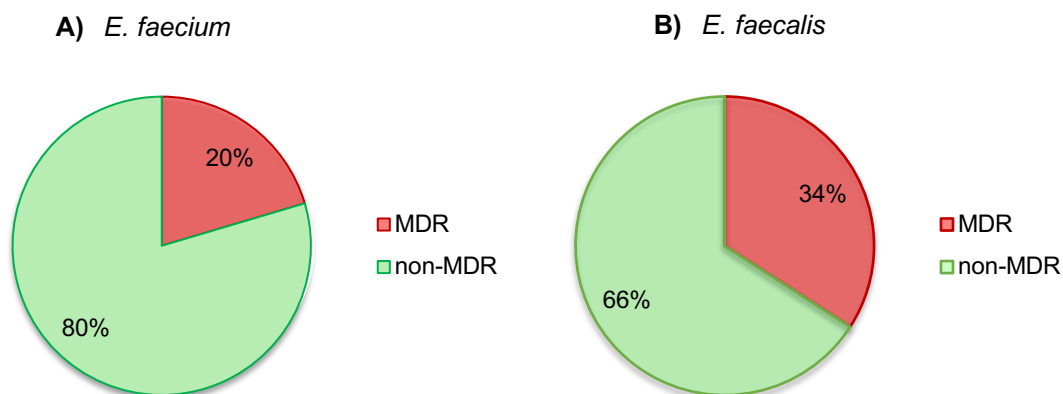

Figure 6. Frequency of multidrug resistance of *E. faecium* **A)** and *E. faecalis* **B)**

Multidrug resistance was absent in cattle. Full susceptibility was observed in 26.5% of *E. faecium* (n= 13) and 11.8% of *E. faecalis* (n= 10), and found more often in bovine (14/31) compared to swine (9/103).

The frequency of MDR *Enterococcus* in the present study is lower than reported in food producing animals from the United States [172], China [183], and Malaysia [184], and similar to that described in Danish [185] and Estonian [186] studies.

Eight multidrug antibiotic resistance profiles were reported in *E. faecalis* strains and two in *E. faecium* (Table 5).

Table 5. *Multidrug resistance profiles of E. faecium and E. faecalis from pigs*

E. faecium (N=49)		
Multiresistance profiles	**Swine (N= 37)**	**%**
TET-ERY-AMP	9	18.4
TET-ERY-CLO	1	2.0
E. faecalis (N=85)		
Multiresistance profiles	**Swine (N= 66)**	**%**
TET-ERY-CIP-GEN-CLO	3	4.7
TET -ERY-CIP-CLO	2	2.3
TET -ERY-CIP-GEN	3	4.7
TET-ERY-LZD-CLO	1	1.2
TET-ERY-GEN-CLO	4	4.7
TET-ERY-GEN	1	1.2
TET-ERY-CLO	14	16.5
TET- ERY-CIP	1	1.2

TET: Tetracycline; ERY: Erythromycin; LZD: Linezolid; AMP: Ampicillin; CLO: Chloramphenicol;
CIP: Ciprofloxacin; GEN: Gentamicin

Resistance to five antibiotics was only displayed by three *E. faecalis* isolates. Tetracycline and erythromycin resistance were present in all multidrug resistance profiles. The profile TET-ERY-AMP was the most prevalent pattern in *E. faecium* (18.4%), and was also unique to this species due to the absence of ampicillin resistance in *E. faecalis* strains. On the other hand, the most frequently noticed pattern in isolates of *E. faecalis* was TET-ERY-CLO (16.5%), also found in *E. faecium*. *Enterococcus faecalis* isolates also exhibited seven profiles exclusive to this species.

The resistance to tetracycline and macrolides appears to be ubiquitous in MDR profiles of both enterococci species, likely reflecting the widespread use of these antibiotics in pig farming, as above mentioned.

4.2.2. Susceptibility to daptomycin and tigecycline

Considering the importance of daptomycin and tigecycline as last-resort antibiotics used in the treatment of multidrug resistant infections, it is important to monitor and investigate resistance to these antibiotics among strains of *E. faecalis* and *E. faecium,* two important agents in nosocomial infections worldwide. Therefore, Sensititre™ EUVENC microdilution plates were used to assess the antimicrobial susceptibility patterns of 20 strains of *E. faecium* (n= 6) and *E. faecalis* (n= 14). These isolates showed the highest MIC values to tigecycline and daptomycin in the agar diffusion susceptibility test (MIC values either above the ECOFF values for these antibiotics or one dilution step below, obtained in non-validated tests). The results of the susceptibility testing using the microplates are shown in Table 6.

Table 6. *Phenotypic profiles of E. faecium and E. faecalis isolates tested using EUVENC microplates*

	Strain	Animal species	Antibiotic resistance profile	DAP MIC (µg/ml)	TIG MIC (µg/ml)
E. faecium (n=6)	181P	Bovine (n=2)	TET	4	0.12
	387P		-	4	0.06
	207P	Swine (n=4)	TET-ERY-Q/D	4	0.12
	292P		-	1	≤0,03
	334P		TET-ERY-CLO	2	0.12
	INIAV004		TET-ERY-LZD-CLO	4	0.25
E. faecalis (n=14)	230P	Bovine (n=2)	TET	2	0.06
	379P		TET-DAP	**8**	0.125
	38P	Swine (n= 12)	TET-ERY	2	0.12
	41P		TET-ERY	2	0.25
	100P		TET-ERY-LZD-CLO	2	0.12
	101P		TET-ERY-CLO	2	0.12
	INIAV006		TET-CIP-ERY-GEN-CLO	1	0.25
	197P		TET-ERY	2	0.25
	223P		TET-ERY-LZD-CLO	1	0.12
	224P		TET-ERY-GEN	2	0.25
	226P		TET-ERY-GEN-CLO	4	≤0.03
	227P		TET-ERY-CLO	2	0.12
	260P		TET-DAP-ERY	**16**	0.12
	INIAV005		TET-DAP-ERY	**8**	0.25

DAP: Daptomycin; TIG: Tigecycline; Q/D: Quinupristin-dalfopristin; TET: Tetracycline;
ERY: Erythromycin; CLO: Chloramphenicol; LZD: Linezolid; GEN: Gentamicin
ECOFFs: DAP$_{E.faecium}$ (MIC = 8 µg/ml); DAP$_{E.faecalis}$: (MIC = 4 µg/ml); TIG (MIC=0.25 µg/ml);
MICs above the ECOFF values (indicating resistance) are in bolt.
E. faecalis are intrinsically resistant to quinupristin-dalfopristin

MIC values obtained with the EUVENC microplates were mostly consistent with the MIC values obtained by the agar diffusion technique (data not shown). However, two isolates (*E. faecium* INIAV004 and *E. faecalis* 223P) susceptible to linezolid in the agar diffusion test (MIC = 4 µg/ml), shifted to the resistant phenotype in the EUVENC microplate assays, presenting MICs one dilution step higher (MIC = 8 µg/ml). Small discrepancies in MIC values are common to any antimicrobial

susceptibility testing method due to inherent variability, even for replicate MIC values measured for a single isolate with reference broth microdilution plates [187]. Nevertheless, it can be problematic for resistance interpretation when MIC values are near the ECOFF, as was the case. Resistance to tigecycline was not observed in any isolate. Tigecycline resistance remains low in food producing animals from European countries, although it has been reported in France, in *E. faecium* isolates from pigs in 2013-2014 [149]. The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) also reported no tigecycline resistance in enterococci isolated from pigs in 2019 [185].

Resistance to quinupristin-dalfopristin (MIC > 4 µg/ml) was found in all *E. faecalis* isolates (n= 16)*,* while only one *E. faecium* strain isolated from pigs was resistant (MIC = 8 µg/ml). Resistance to this antibiotic combination in *E. faecalis* was expected since this species is intrinsically resistant [188]. In a European study, *E. faecium* strains from food producing animals displayed high levels of resistance to quinupristin-dalfopristin, particularly in samples from swine and chicken. Our work only tested six strains of *E. faecium* for this antibiotic and thus, no conclusions can be drawn about the prevalence of quinupristin-dalfopristin in this species.

A total of three *E. faecalis* isolates were non-susceptible to daptomycin (MIC > 4 µg/ml). Two of the daptomycin non-susceptible enterococci (DNSE) were sourced from pigs and one was from cattle. The prevalence of daptomycin non-susceptible isolates in Europe is still low [73], and to our knowledge, no isolates had been reported in Portugal in humans or animals until the present work.

In an EASSA study that evaluated the susceptibility profiles of enterococci isolated from farm animals from European countries, in the years of 2013-14, only one sample of *E. faecium* from a pig in Germany was considered non-susceptible to daptomycin out of a total of approximately 2259 strains of *Enterococcus* isolated from livestock in that period [149,179]. In 2009, DAP non-susceptible *Enterococcus* spp. were noticed in samples of cattle and pigs from Lithuania [177] and in 2019, daptomycin-resistant strains made up 3% of a total of 91 *E. faecalis* isolates from pigs in the DANMAP report from Denmark [185].

Daptomycin non-susceptibility (DNSE) is usually associated with exposure to the antibiotic, although DNSE have been reported in patients without prior exposure (*de novo* DNSE) [189]. The transmission of daptomycin resistance genes between animals and humans has been speculated as a possible cause of *de novo* DNSE, as well as sporadic emergence [190].

Since no daptomycin formulation has been approved for animal use in the EU, the presence of daptomycin non-susceptible enterococci in farm animals is most likely due to spontaneous mutations, although the inappropriate use of this drug cannot be completely discarded as a possible cause.

Of notice, DNSE have also been identified in probiotic products used in cattle and pigs in the United States of America [191]. Problematic strains of enterococci in probiotics could be the

source of dissemination of antibiotic resistance determinants, and the contribution of these strains to resistance to critically important and last-resort antibiotics such as daptomycin should be assessed on a global level. It is essential to assess and monitor the safety of probiotic strains before incorporating them in feeds, foods, and pharmaceutical preparations.

Three multidrug resistance profiles were observed in *E. faecium* (Table 6). In *E. faecalis* strains, six MDR profiles were expressed. These differed from the patterns obtained by agar diffusion due to the additional three antimicrobial agents being tested (quinupristin-dalfopristin, daptomycin and tigecycline) and because of the detection of two additional linezolid-resistant strains.

All isolates resistant to linezolid were also resistant to chloramphenicol, which could suggest a common resistance mechanism for both antibiotic agents in these strains since resistance to both antibiotic classes is often associated among enterococci [73, 130, 133].

4.3. Antibiotic Resistance Determinants

4.3.1. Oxazolidinone resistance genes

The three linezolid resistant isolates, as well as isolates with low-level linezolid resistance (MICs ≥ 2 µg/ml) and isolates that were chloramphenicol resistant but linezolid susceptible (MIC= 1 µg/ml), were subjected to the PCR assays targeting the *optrA* gene, which confers resistance to oxazolidinones and chloramphenicol. Overall, the gene was detected in susceptible (MIC≤4mg/L) strains of *E. faecalis* (n=4) and *E. faecium* (n=2), and in non-susceptible (MIC>4mg/L) strains of *E. faecalis* (n=2) and *E. faecium* (n=1), all strains being all from pigs (Figure 8). Among the isolates carrying the *optrA* gene, three resistant and four susceptible strains to linezolid were also resistant to chloramphenicol, whereas two linezolid susceptible isolates (one *E. faecalis* and one *E. faecium*) were also susceptible to chloramphenicol.

Among the linezolid and chloramphenicol resistant isolates (n= 22), none harbored the *cfr* gene, known to confer resistance to linezolid, phenicols, lincosamides, pleuromutilins and streptogramin A.
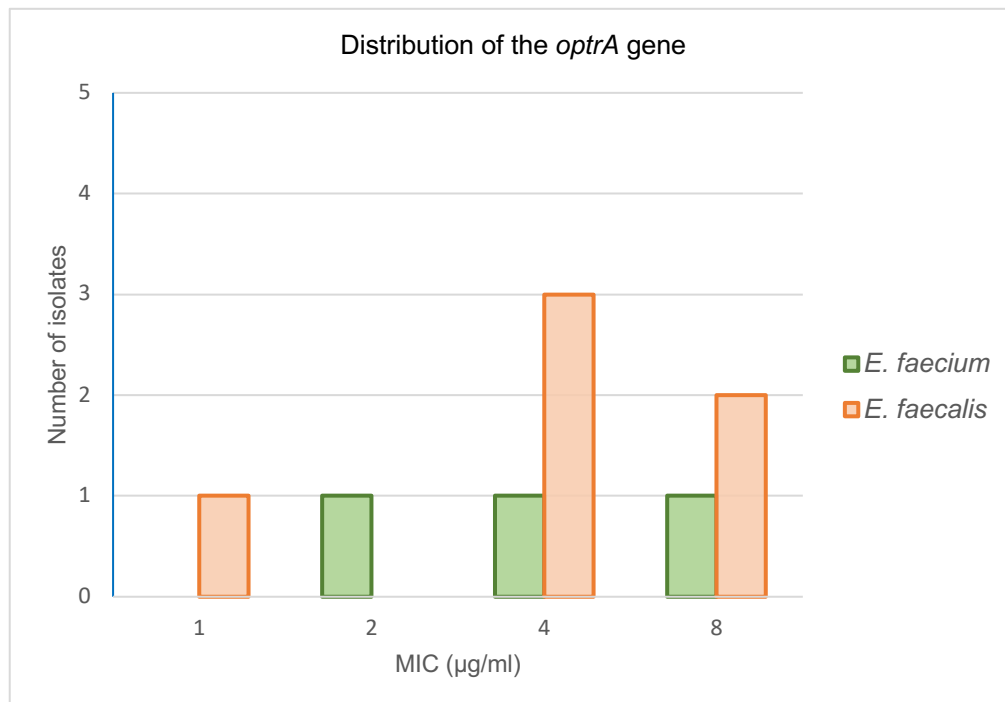
Figure 7. Linezolid MIC distribution of *Enterococci* carrying *optrA*

The *optrA* gene has been detected more frequently in *Enterococcus* from food producing animals including poultry [192], cattle [193] and pigs [193], than from humans [194]. Linezolid susceptible *optrA*-carrying enterococci were reported in China in a study, also revealing that linezolid MICs may depend on the genetic context surrounding the *optrA* gene, and on the amino-acid sequence of the OptrA protein (OptrA variant) [133].

The ECOFF allows the distinction between microorganisms without (wild type) and with phenotypically detectable acquired resistance mechanisms (non-wild type) [154]. The presence of several linezolid-susceptible isolates carrying the *optrA* gene in the current study, as well as other reports [133], may suggest that the ECOFFs for this antibiotic need to be revised for both enterococci species.

Three *E. faecalis* isolates carrying the *optrA* gene were sourced from pig samples recovered from the same slaughterhouse, on the same day. Additionally, two of them had the same antimicrobial susceptibility profile (TET-ERY-CLO). Therefore, cross-contamination of the samples in the slaughtering line cannot be excluded.

A recent large-scale study in China observed a higher *optrA* prevalence in swine and poultry farms than dairy cows and beef farms, revealing that the extensive florfenicol and tiamulin use may lead to the emergence of *optrA* genes [146]. Although linezolid is unauthorized for veterinary use in the EU, florfenicol is broadly used in farm animals, including pigs and cattle, which could explain the presence of the *optrA* gene in the nine *Enterococcus* isolates from healthy pigs found in our study.

The *cfr* gene has been associated with multiple linezolid resistant staphylococci outbreaks, including MRSA [195,196]. In enterococci, *cfr* was first reported in *E. faecalis* from food-producing animals, but a study noticed that it doesn't always confer resistance in this genus [197]. Along with other linezolid resistance determinants, its prevalence in farm animals was found to be related to florfenicol use in Chinese provinces [146].

4.3.2. Glycopeptide resistance genes

The presence of the glycopeptide resistance genes *vanA* and *vanB* was assessed through PCR assays, and both glycopeptide resistance determinants were absent in all the tested isolates.

The *vanA* operon is usually found in enterococci strains with high-levels of vancomycin and teicoplanin resistance, thus it is usually searched in isolates with resistance to one or both glycopeptides. However, *Enterococcus* isolates carrying the *vanA* gene and appearing to be susceptible to vancomycin have been described [101,102,103]. Some of these strains termed vancomycin variable enterococci (VVE), can change into the resistant phenotype during the antimicrobial therapy [103]. Therefore, to exclude the presence of VVE, the *vanA* gene was screened in all of the *E. faecalis* and *E. faecium* isolates (n= 134).

Since the *vanB* gene has been detected in isolates displaying a low-level vancomycin resistance [95,96], all isolates with MICs of 2 and 4 µg/ml (n= 45) were included in the PCR assay targeting this gene.

A study in Portugal identified 44 vancomycin resistant enterococci carrying the *vanA* operon among 299 strains isolated from food-producing animals from 2005 to 2012 [178]. In contrast, the present study collected only samples from 2017, two decades after the ban of avoparcin in the EU as a growth promoter; neither isolates were found to be resistant to vancomycin or teicoplanin, nor harbor the v*anA* and *vanB* genes.

Vancomycin resistant enterococci possessing the *van* operon (particularly the *vanA* gene) were still frequently recovered from fecal samples of food-producing animals in the late 2000's, many years after the European ban on avoparcin in 1997 [198-201, referenced in 202].

Different theories have emerged to explain why the "van operon" persisted for such an extensive period after the avoparcin ban. In a Danish study, the use of macrolides was suggested to co-select for vancomycin resistance, since the same plasmid encoded resistance genes for both antibiotics [203]. Similarly, co-selection for copper resistance was also suggested as an explanation [204]. In a Norwegian study, plasmid addiction systems (specific systems encoded within plasmids to ensure their survival) located in the same plasmid as *vanA* were proposed to contribute to the persistence of this gene in glycopeptide resistant *E. faecium* strains in non-selective environments [205].

In recent years, resistance to glycopeptides in *Enterococcus* sourced from farm animals has been decreasing in European countries and, for the first time, no VRE were reported by the EASSA in 2013–14 [149,179], reinforcing the premise that antibiotic resistance will subside when the selective pressure associated with the use of a particular antibiotic agent is withdrawn.

4.4. Genomic Characterization of selected *E. faecium* and *E. faecalis* isolates

Whole-genome sequencing (WGS) was conducted on two *E. faecalis* (INIAV005 and INIAV006) and one *E. faecium* (INIAV004) isolates from swine. Genomic data analyses using CGE bioinformatic tools are shown in Table 7. *Enterococcus faecium* INIAV004 and *E. faecalis* INIAV006 were selected for harboring the *optrA* gene, but displaying different susceptibility phenotypes to linezolid, and *E. faecium* INIAV004 was resistant (MIC = 8 µg/ml), while *E. faecalis* INIAV006 was susceptible (MIC = 4 µg/ml). Isolate INIAV006 was also selected for exhibiting ciprofloxacin and gentamicin resistance. *Enterococcus faecalis* INIAV005 was selected for its non-susceptibility to daptomycin (MIC= 8 µg/ml).

The predicted WGS phenotype generally agreed with the susceptibility phenotype. Isolate *E. faecium* INIAV004 harbored several antibiotic resistance genes, including *tet(M), tet(L), erm(A), msr(C), fexB, aac(6')-Ii, optrA* and *poxtA,* which were consistent with resistance to tetracycline, erythromycin, linezolid and chloramphenicol.

Regarding genes associated with tetracycline resistance, *tet(M)* and *tet(L)*, two resistance determinants encoding a ribosome protection protein [140] and a tetracycline-specific efflux pump [64], respectively, were found. Macrolide and streptogramin resistance genes *erm(A)* and *msr(C)* were also present*, erm(A)* conferring the MLS$_B$ phenotype [125,205] and *msr(C)* providing low-level intrinsic macrolide and streptogramin B resistance in *E. faecium* isolates [123,206]). The resistance determinants *fexB* (an amphenicol exporter [134]) and *aac(6')-Ii* (a gene that encodes an aminoglycoside modifying enzyme (AME) that confers intrinsic tolerance to tobramycin, kanamycin, netilmicin, and sisomicin in *E. faecium* strains [135]) were also identified.

In addition to the *optrA* gene, the ABC-F protein-encoding gene *poxtA*, which confers decreased susceptibility to phenicols, oxazolidinones, and tetracyclines [133] was found. Although the *poxtA* gene has only recently been characterized, it has already been identified in *E. faecium* and *E. faecalis* isolates from food-producing animals or their environments in many countries, including China [145,207], Korea [208], Tunisia [209], Italy [210] and Spain [211]. Together with the *optrA* and *cfr* genes, the presence of *poxtA* genes in enterococci from farm animals has been associated with the use of florfenicol [145]. Other studies have also reported *E. faecium* strains co-harbouring *optrA* and *poxtA* genes [207,211]. The transfer of the *poxtA* gene together with other antibiotic resistance determinants such as the *tet(M)*, *tet(L), fexA* and *fexB* genes have also been reported [207,211,212]. The location of these genes in plasmids or transposons could contribute to the co-selection and dissemination of the *poxtA* gene; however, the genetic context surrounding these genes was not investigated in this report.

Table 7. *Whole Genome Sequencing of E. faecium and E. faecalis strains*

Strain	Species	Sample source	MLST	MDR profile	Antibiotic resistance determinants	Plasmid replicons	Virulence genes	Pathogenicity
INIAV004	*Enterococcus faecium*	Swine	ST22	TET-ERY-LZD-CLO	*aac(6')-Ii* *tet(M), tet(L)* *poxtA, optrA, fexB* *erm(A), msr(C)* *pbp5* (p.T172A), *pbp5* (p.L177I), *pbp5* (p.A216S), *pbp5* (p.P667S), *pbp5* (p.D204G), *pbp5* (p.K144Q), *pbp5* (p.R34Q), *pbp5* (p.S27G), *pbp5* (p.E100Q), *pbp5* (p.A499T), *pbp5* (p.G66E), *pbp5* (p.T324A), *pbp5* (p.A68T), *pbp5* (p.V24A), *pbp5* (N496K), *pbp5* (p.E525D), *pbp5* (E85D)	*rep29, rep33, repUS43, rep1, rep2, repUS15*	*acm, efaAfm*	85.5
INIAV005	*Enterococcus faecalis*	Swine	ST93	TET-ERY-DAP	*aac(6')-aph(2'')* *tet(M), tet(L)* *erm(B)*	*rep9a*	*tpx, hylA, elrA, srtA, ace, cCf10, cOB1, cad, camE, ebpA, ebpC, efaAfs*	89.4
INIAV006	*Enterococcus faecalis*	Swine	ST474	TET-ERY-CIP- GEN-CLO	*gyrA (p.E87G), parC (p.S80I)* *aac(6')-aph(2'')* *fexA, cat, optrA* *tet(M), tet(L)* *erm(A), erm(B)*	*repUS43, rep9a*	*cad, camE, ebpA, ebpC, tpx, elrA, srtA, ace, ccf10, cOB1, efaAfs, fsrB, gelE, hylA, hylB*	89.8

MLST: Multilocus Sequence Typing; MDR: Multidrug Resistance; TET: tetracycline; ERY: Erythromycin; LZD: Linezolid; CLO: Chloramphenicol; DAP: Daptomycin; CIP: Ciprofloxacin; GEN: Gentamicin

Although *E. faecium* INIAV004 was susceptible to ampicillin (MIC = 1 µg/ml), the genomic analysis revealed several point mutations of the *pbp5* gene that encodes the low affinity species-specific class B penicillin-binding protein, PBP5. Different levels of ampicillin susceptibility (MICs of 0.5 to 128 µg/ml) in *E. faecium* strains can be related to different amino acid changes of the PBP5 protein that further decrease the affinity of this protein to β-lactam antibiotics [79,213]. Increased ampicillin MICs have been associated with specific amino acid substitutions occurring mostly around the active-site region of PBP5 (in particular when some of these mutations are present in combination), such as M485A and E629V and mutations associated with the addition of a serine at position 466 [77-79,213]. None of these point mutations were detected in *E. faecium* INIAV004.

Enterococcus faecalis INIAV005, which had displayed phenotypic resistance to tetracycline, erythromycin and daptomycin, was shown to be carrying the corresponding resistance genes *tet(M), tet(L)* and *erm(B)*, together with the *aac(6')-aph(2'')* gene. In this isolate, the aminoglycoside resistance determinant *aac(6')-aph(2''),* which is known to provide resistance to all aminoglycosides except streptomycin, was identified with a match < 100% and match length inferior to the reference length, which could explain why this isolate is susceptible to gentamicin. This strain was non-susceptible to daptomycin; however, the molecular basis of this antibiotic resistance was not made clear yet.

The MDR profile of *E. faecalis* INIAV006 (TET-ERY-CIP-GEN-CLO) was consistent with the genotype determined by WGS, which identified tet(M) and tet(L), *erm(A)* and *erm(B), aac(6')-aph(2'')*, *fexA*, *cat* (a chloramphenicol acetyltransferase). Additionally, mutations in the *gyrA* and *parC* genes were also detected in this strain, which was resistant to ciprofloxacin (MIC > 16 µg/ml). These mutations in DNA gyrase subunit A and DNA topoisomerase IV subunit A are thought to lower the binding affinity of fluoroquinolones to the respective enzymes (DNA gyrase and topoisomerase IV) in enterococci [121,122]. Although strain INIAV006 was linezolid-susceptible, the *optrA* gene was also detected by WGS, corroborating our previous results. To explain linezolid susceptibility in this strain, further investigation of the amino acid sequence of the OptrA protein and the genetic context of the *optrA* gene should be carried out.

Enterococcus faecium INIAV004 belonged to ST22, which has been associated with reports in humans [213], pigs [214] and poultry [215]. *E. faecium* ST22 harboring the *optrA* gene has been reported previously [216]. Vancomycin-resistant *E. faecium* ST22 isolates have been reported [213,214]. Lineage ST22 is known to be the primary ancestor to a sizable amount of other significant and drug-resistant *E. faecium* strains including ST17, the secondary founder of the hospital adapted clonal complex-17, related with most of the nosocomial VRE outbreaks [213]. Isolates *E. faecalis* INIAV005 and INIAV006 belonged to ST93 and ST474 respectively, two lineages that have been found in human infections and animals [217-220]. Both sequence types have also been associated with the *optrA* gene [218-220] also detected in *E. faecalis* INIAV006.

Several plasmid replicons were identified in *E. faecium* INIAV004 namely *rep29, rep33, repUS43, rep1, rep2 and repUS15*. With less plasmid diversity, both *E. faecalis* strains carried *rep9a* replicon and INIAV006 also harboured *repUS43* replicon.

The *rep1* and, especially, *rep2* families are highly prevalent in *E. faecium* strains [221]. The *rep2* family has a substantially limited bacterial host diversity (mainly composed of *E. faecium* and *E. faecalis*) when compared to *rep1*, which is commonly found in several gram-positive bacteria [221]. The *rep9* family seems to be specific for *E. faecalis* and is frequently detected in this species. The *rep1, rep2* and *rep9* plasmids have been found in bacterial isolates from humans and animals and are known to contribute to the dissemination of antibiotic resistance [221]. Of notice, in *E. faecium* INIAV004, the *optrA* gene was found in the same contig as the *rep33* plasmid replicon, suggesting that this gene is encoded within the plasmid.

All isolates showed an over 85.5% probability of being human pathogens. Several virulence genes were found in both *E. faecalis* isolates (INIAV005 and INIAV006) (Table 6). The *tpx* gene encodes a thiol peroxidase (Tpx) which provides resistance against oxidative stress and has been implicated in experimental peritonitis caused by *E. faecalis* [225]. The *hylA* and *hylB* genes encode hyaluronidases [226]. The *elrA* gene is frequently found in *E. faecalis* strains, and it encodes the ElrA protein which assists in evading macrophage phagocytosis, providing cell adherence. It has experimentally been proven to play a role in intensifying interleukin-6 cytokine production and in the development of peritonitis [227,228]. The *srtA* gene encodes a sortase enzyme (SrtA) which is fundamental for the pathogenesis of *E. faecalis*, being related to the production of biofilm [229]. The *ace* gene encodes a collagen adhesin involved in attachment to the extracellular matrix of the organism [230]. The *cCf10, cOB1, cAD* and *camE* genes encode sex pheromones taking part in conjugative plasmid transfer [231-234]. The *ebpA* and *ebpC* genes encode the *E. faecalis* endocarditis and biofilm-associated EbpA pilus protein and the endocarditis and biofilm-associated pilus major subunit EbpC protein, respectively [235]. The *fsrB* gene promotes the activation of the *gelE* gene that encodes a gelatinase (GelE) [236].

The virulence genes *acm* and *efaAfm* were identified in *E. faecium* INIAV004. The *acm* gene encodes the Acm protein, a cell wall collagen adhesin that can provide collagen adherence [222]. The *efaAfm* gene also encodes a cell wall adhesin (EfaAfm) and, while the purpose of this gene is not certain, the EfaAfm protein is over half homologous to the cell wall adhesin endocarditis antigen A (EfaA) of *E. faecalis* (encoded by the *efaAfs* gene), and both are thought to participate in cell adherence [223,224].

Both *E. faecalis* strains harbored more virulence genes than *E. faecium* INIAV004, which corroborates that *E. faecalis* strains are more likely to display traits related to virulence [237].

SECTION 5: CONCLUSIONS

The rational use of antibiotics is of upmost importance as the rise of antibiotic resistance threatens the efficiency of current therapies against infections. Although antibiotic resistance mechanisms can emerge in microbial communities in the absence of the selective pressure exerted by antimicrobial agents, the extensive antibiotic use in animal production is a major contributor to the emergence and dissemination of resistant bacteria. The One Health initiative highlights the need to approach health as a holistic, multisectoral, transdisciplinary concept incorporating the relationship networks between humans, animals, and ecosystems.

Enterococci are ubiquitous opportunistic bacteria that constitute part of the intestinal microbiome of humans and animals, the species *E. faecalis* and *E. faecium* being the two most prevalent species found in human infections. These are important nosocomial pathogens known to easily exchange resistance determinants, and evidences suggest that enterococci of animal origin may colonize the intestine of humans, and be able to transfer antibiotic resistance determinants to human-adapted enterococci.

From this study, we highlight the following:

- The most commonly recovered species of *Enterococcus* in pigs were *E. faecalis*, *E. hirae* and *E. faecium*. At lower frequencies, *E. casseliflavus* and *E. durans* were also identified as part of the intestinal flora of swine;

- In cattle, *E. hirae* characterized the majority of the isolates, while *E. faecalis, E. faecium*, *E. casseliflavus* and *E. durans* were also isolated;

- Although some isolates were identified as *E. asini* and *E. thailandicus* (in swine) and *E. mundtii* (in bovine), further confirmation should be ensued for species identification;

- In general, the level of antimicrobial resistance in *E. faecium* and *E. faecalis* from pigs was higher than that of cattle. Multiple MDR profiles were observed in isolates from swine, while no multidrug resistant strains were found in bovine;

- Resistance to tetracycline was widespread. However, with the exception of erythromycin, the prevalence of resistance to critically important antibiotics in human medicine was either low or absent;

- Phenotypic and genotypic resistance to glycopeptides was not detected among *E. faecium* and *E. faecalis* strains;

- Linezolid-resistant enterococci carrying the *optrA* gene were found in pigs. This gene is circulating in intestinal populations of *Enterococcus* from pigs in Portugal, being found also in linezolid susceptible isolates. The frequency of occurrence of the *optrA* gene might be underestimated since this gene is usually only searched in linezolid resistant isolates. A thorough investigation of the genetic context surrounding the *optrA* gene and its nucleotide sequence as well as the presence of other linezolid resistance determinants

among the linezolid-resistant isolates could explain the different linezolid susceptibility profiles expressed by *optrA*-carrying strains;

- The existing evidence suggesting that florfenicol use may play a role in the emergence and persistence of linezolid resistance determinants in *Enterococcus* strains from farm animals could explain the presence of *optrA* and *poxtA* genes in *E. faecalis* and *E. faecium* isolates reported in the current study;

- Genome analysis of three isolates allowed the identification of several antimicrobial resistance determinants (including *poxtA*) and virulence genes, plasmid replicons and sequence type, as well as pathogenicity prediction to humans. Concerningly, all strains were MDR and belong to sequence types previously found in human isolates and were predicted to be potentially pathogenic to humans;

- Although phenotypic resistance to daptomycin was detected in *E. faecalis* strains, the associated molecular basis was not clarified. Further investigation should be ensued to assess the prevalence of resistance to this antibiotic agent, its origin and molecular basis in *enterococci* from food-producing animals;

- To our knowledge, this is the first report of daptomycin resistant *E. faecalis* isolates of animal origin in Portugal. This is also the first description of *E. faecalis* and *E. faecium* carrying *optrA* and *E. faecium* strains co-harboring the *optrA* and *poxtA* genes in food producing animals from Portugal.

The present report underlines the importance of the One Health approach on antibiotic use and surveillance of antibiotic resistance, particularly to critically important antibiotics.

Our findings highlight the impact that antimicrobial consumption has on the emergence and persistence of resistant bacterial strains from the commensal gastrointestinal flora of healthy cattle and pigs. These strains may not only express resistance to antibiotics belonging to the same class to the one that is being administered but also exhibit cross-resistance and co-resistance to other antibiotic classes. The data obtained from these monitoring programmes can be useful when establishing which antibiotics remain useful as well as which should be restricted in veterinary medicine in order to ensure that these agents preserve their efficacy in the treatment of animal and human infections. It is also important to study the mechanisms of resistance to these antibiotics and whether the respective resistance determinants are transmissible to other strains of the same genus, to other bacterial genera and between hosts.

This work provides evidence of *Enterococcus* strains from the gut microbiome of food producing animals exhibiting phenotypic resistance and harboring resistance genes to last-resort antibiotics such as linezolid and daptomycin. It reinforces the need to monitor antibiotic resistance mechanisms and prevalence in the commensal flora of farm animals, as these microorganisms can acquire antibiotic resistance determinants and transfer them to humans and the environment.

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7.1. Abstract submitted to the 2nd International Conference of the European College of Veterinary Microbiology (ICECVM), September 8 – 9, 2020

Antibiotic susceptibility of *Enterococcus faecium* and *Enterococcus faecalis* strains from bovine and swine gut flora

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The intestinal tract of animals is an important reservoir of enterococci, being *Enterococcus faecium* and *Enterococcus faecalis* the most important species associated with human clinical infections. The aim of this study was to evaluate the antibiotic susceptibility profile of both species, isolated from bovine (n=148) and swine (n=160) caecal samples.

Bacterial isolates were obtained after overnight incubation in BHI broth with 6.5% NaCl, followed by inoculation in Enterococcosel Agar. Species identification was performed by multiplex PCR. Minimal inhibitory concentration was determined using the agar dilution technique for 11 antibiotics, and results interpreted according to the ECOFF values of EUCAST. Selected genes encoding resistance were screened using PCR.

Overall, widespread resistance to tetracycline and macrolide was found among isolates from both species. Full susceptibility was observed in 8.2% of *E. faecium* and 7.1% of *E. faecalis*. All isolates were susceptible to vancomycin and teicoplanin. Multidrug resistance was noticed in 26.5% of *E. faecium* and 32.9% of *E. faecalis* isolates. Resistance to linezolid was observed in one *E. faecalis* isolate, harbouring the *optr*A gene. Furthermore, this gene was also found in susceptible isolates of *E. faecium* (n=3) and *E. faecalis* (n=5) of swine origin.

Different levels of antimicrobial resistance were found among isolates from bovine and swine. The frequency of occurrence of *optr*A gene might be underestimated in *E. faecium* and *E. faecalis*, as usually this gene is only searched in isolates resistant to linezolid. This study highlighted the importance of monitoring the antimicrobial resistance mechanisms in bacteria from food-producing animals, representing a potential route for the transmission of resistance to humans and environment.

7.2. Abstract submitted to the 31st ECCMID, July 9-12, 2021

Presence of *poxtA* and *optrA* genes in linezolid resistant *Enterococcus faecium* and *Enterococcus faecalis* from pigs in Portugal

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Background: *Enterococcus* is one of the most important pathogens associated with nosocomial infections worldwide, being *Enterococcus faecium* and *Enterococcus faecalis* the most common species associated with human infections and antibiotic resistance. Although last-resort antibiotics such as linezolid are unauthorized for veterinary use, the emergence of linezolid-resistant enterococci in livestock should be monitored to identify resistance mechanisms and resistant-clones. This study aimed to characterize antibiotic susceptibility profiles and investigate linezolid resistance mechanisms of enterococci from food producing animals.

Methods: Antimicrobial testing was performed on *E. faecium* (n=49) and *E. faecalis* (n=85) isolates from cattle and pigs. Susceptibility to 12 antibiotics was determined by agar dilution and microdilution microplates (EUVENC, Sensititre). MICs were interpreted according to EUCAST ECOFFs. Acquired linezolid resistance genes (*cfr* and *optrA*) were screened by PCR. WGS of linezolid resistant E. faecium and E. faecalis isolates was performed using MiSeq (Illumina) and analysed by bioinformatic tools.

Results: Full susceptibility profiles were observed in 27% (13/49) of *E. faecium* and 12% (10/85) of *E. faecalis*. All isolates were susceptible to vancomycin, teicoplanin and tigecycline. Multidrug resistance was observed in 20% (10/49) of *E. faecium* and 34% (29/85) of *E. faecalis* isolates. Daptomycin resistance was found in *E. faecalis* (n=3), and linezolid resistance was detected in both species (n=3). All linezolid-resistant isolates harboured *optrA* gene but *cfr* gene was not detected. Furthermore, *optrA* gene was found in linezolid-susceptible (MIC≥ 2/ml) isolates of *E. faecium* (n=2) and *E. faecalis* (n=4) from pigs. WGS analysis confirmed the presence of *optrA* gene in both species and revealed that *E. faecium* ST22 co-harboured *poxtA* gene.

Conclusions: To our knowledge, this is the first report of the presence of *optrA* and *poxtA* in enterococci isolates from livestock in Portugal. Moreover, the frequency of occurrence of *optrA* gene might be underestimated, as usually this gene is only searched in linezolid resistant isolates. Of notice, daptomycin-resistant enterococci were found, and the identification of the resistant mechanisms is underway. This study highlighted the importance of monitoring antimicrobial

resistance mechanisms in bacteria from food-producing animals, representing a potential route for the transmission of resistance to humans and environment.

7.3. Supplementary figures

Supplementary Figure 1. Schematic illustration of the distribution of 24 strains within each agar plate in the agar dilution method.
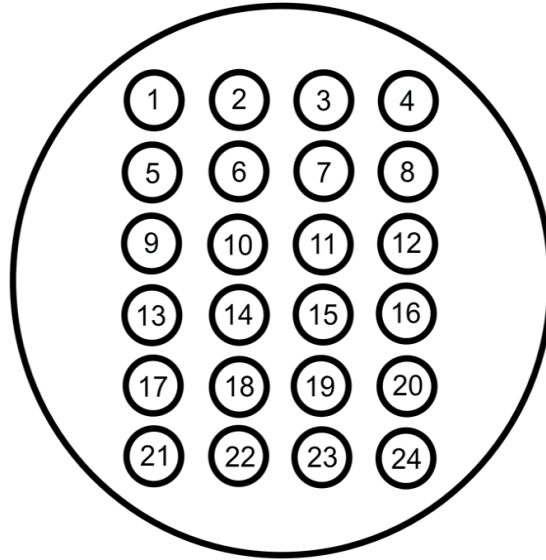
Supplementary Figure 2. Antibiotic panel layout of Sensititre™ EUVENC plate. Retrieved from [238]

Supplementary Figure 3. Illustration of the microdilution plate layout used for the broth microdilution susceptibility assay to linezolid

A representative scheme of the microdilution plates prepared *in-house*, displaying the final concentrations (µg/ml) of linezolid in each well. The positive (CTRL+) and negative (CTRL-) controls occupied the last two filled columns of the plate. The peripheral wells were not used in order to prevent evaporation and contamination of the wells. The last row (G) is inoculated with the reference strain *E.*

7.4. Supplementary tables

Supplementary Table 1. *Categorization of important antibiotics for the treatment of enterococcal infections*

Antibiotic	Category in the CIA list	Category in the VCIA list
Aminopenicillins	CIA	VCIA
Glicopeptides	HP CIA	-
Lipopeptides	CIA	-
Fluoroquinolones	HP CIA	VCIA
Macrolides	HP CIA	VCIA
Streptogramins	HI	VIA (virginamycin)
Amphenicols	HI	VCIA
Oxazolidinones	CIA	-
Aminoglycosides	CIA	VCIA
Tetracyclines	HI	VCIA
Glycylcycline	CI	-

Classification of important classes of antibiotics for the treatment of enterococcal infections according to the World Health Organization's list of Critically Important Antibiotics (CIA) in human Medicine and to the World Health Organization for Animal Health's list of Veterinary Critically Important Antibiotics (VCIA).
CIA/VCIA: Critically Important Antibiotic; HP CIA: Highest Priority Critically Important Antibiotic; HIA/VHIA: Highly Important Antibiotic; VIA: Important Antibiotic

Supplementary Table 2. *Description of primer sets, annealing temperature and DNA from control strains used for the molecular species identification of Enterococcus spp.*

Target taxon	Target genes	Primers	Sequence (5'-3')	Amplicon size	PCR no.	Annealing temperature	PCR-Positive control strain	Ref
Enterococcus	16S rRNA	E1	TCAACCGGGGAGGGT	733 bp	1	55ºC	ATCC 29212	151
		E2	ATTACTAGCGATTCCGG					
E. faecalis	*sodA*	FL1	ACTTATGTGACTAACTTAACC	360 bp	1	55ºC	ATCC 29212	152
		FL2	TAATGGTGAATCTTGGTTTGG					
E. faecium	*sodA*	FM1	GAAAAACAATAGAAGAATTAT	215 bp	1	55ºC	INIAV 127P	152
		FM2	TGCTTTTTTGAATTCTTCTTTA					
E. hirae	*sodA*	HI1	CTTTCTGATATGGATGCTGTC	187 bp	2	48ºC	INIAV 27P	152
		HI2	TAAATTCTTCCTTAAATGTTG					
E. casseliflavus	*sodA*	CA1	TCCTGAATTAGGTGAAAAAAC	288 bp	3	50ºC	AUT 14A	152
		CA2	GCTAGTTTACCGTCTTTAACG					
E. durans	*sodA*	DU1	CCTACTGATATTAAGACAGCG	295 bp	4	50ºC	AUT 49B	152
		DU2	TAATCCTAAGATAGGTGTTTG					

E. casseliflavus AUT 14A and *E. durans* AUT 49B were provided by the University of Trás-os-Montes e Alto Douro.

Supplementary Table 3. *Working dilutions used in the broth microdilution susceptibility assay to linezolid*

Concentration of antibiotic in the stock solution (µl/ml)	Volume of stock solution (ml)	Volume of MHB (ml)*	Concentration of antibiotic (µl/ml)	Well column number
5120	1	9	512	
512	1	3	128	2
512	1	7	64	3
64	1	1	32	4
64	1	3	16	5
64	1	7	8	6
8	1	1	4	7
8	1	3	2	8
8	1	7	1	9

The last line on the table represents the number of the column in the microdilution plate that the working solution is going to be dispensed on, with two times the final concentration of the antibiotic.

Supplementary Table 4. *Description of the primer sets, annealing temperatures and DNA from control strains used for the molecular detection of vanA, vanB, optrA, and cfr*

Target genes	Primers	Sequence (5'-3')	Amplicon size	Annealing temperature	PCR-positive control strains	Ref.
vanA	VanA1	AAAGTGCGAAAAACCTTGC	535 bp	54˚C	*E. faecium* BM4	156
	VanA2	AACAACTTACGCGGCACT				
vanB	EB3	ACGGAATGGGAAGCCGA	647 bp	54˚C	*E. faecalis* V583	157
	EB4	TGCACCCGATTTCGTTC				
optrA	optrA_F	AGGTGGTCAGCGAACTAA	1395 bp	54˚C	*E. faecalis* INIAV 100P	158
	optrA_R	ATCAACTGTTCCCATTCA				
cfr	cfr_F	TGAAGTATAAAGCAGGTTG GGGT	746 bp	48˚C	*Staphylococcus aureus* INIAV001	159
	cfr_R	ACCATATAATTGACCACAG C GC				