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Identification of major vancomycin resistant *Enterococcus faecium* reservoirs in livestock and the environment

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Identification of major vancomycin resistant *Enterococcus faecium* reservoirs in livestock and the environment

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Abstract

Enterococci are commensals of the gastrointestinal tract of humans and animals, that under certain conditions, become opportunistic pathogens. Vancomycin-resistant *Enterococcus faecium* (VRE) is one of the leading causes of nosocomial infections worldwide. VRE are considered endemic in the United States and reached a prevalence of 16.8% in European hospitals in 2020. The present study aimed to identify VRE reservoirs among livestock and in the environment, and to determine the epidemiological relationship with VRE responsible for infections in Portuguese hospitals.

A total of 294 samples from livestock (pigs, n=80 and cows, n=55) and from the environment (buses, n=89; hands of passengers, n=18; urban environment close to hospitals, n=52) were screened for VRE. Speciation, detection of vancomycin resistance and virulence genes were performed by PCR. Antimicrobial resistance was characterized by disk diffusion, Etest® and agar dilution. Genomic analysis of 23 selected *E. faecium* was performed by cgMLST (chewBBACA v2.5) and detection of antimicrobial resistance, virulence and plasmids by ResFinder_v3.1, VirulenceFinder_v2.0 and PlasmidFinder_v2.0.

Overall, 470 *Enterococcus* spp. isolates were recovered from 90% (266/294) of the screened samples, and six species were identified: *E. faecalis* (30%), *E. casseliflavus* (20%), *E. gallinarum* (14%), *E. faecium* (13%), *E. hirae* (5%) and *E. durans* (< 1%). *E. faecium* was most prevalent among environmental samples [urban environment (27%), hands of bus passengers (23%) and buses (19%)] compared to livestock [pigs (7%) and cows (5%)]. Despite the fact that all isolates were susceptible to glycopeptides and no *vanA* or *vanB* genes were found, 23% of *Enterococcus* harbored the *vanC2/3* and 17% carried the *vanC1*, including 12 (40%) *E. faecalis*. These *E. faecalis*, showed a resistance pattern mainly associated to intrinsic resistance, while *E. faecium* showed high resistance to aminoglycosides and macrolides (100%; *aac(6')-Ii* and *msrC* genes), to ampicillin (83%; associated with mutations in *pbp5*), tetracyclines (39%; *tetM* and *L* genes) and high-level resistance to streptomycin (35%; *ant(6)-Ia*) and gentamicin (4%; *aac(6')-aph(2'')*). Different virulence determinants were identified among both *E. faecalis* (*asa1*, *gelE* and *ace*) and *E. faecium* (*ace*, *acm*, *scm*, *sgrA*, *efaAfm* and IS16).

E. faecium population showed a high genetic variability, with a different sequence type (ST) per isolate, and including isolates from clonal complex (CC) 17 (ST32) and CC94 (ST296, ST1205, ST800 and ST2206), which are highly associated to hospital infections and human carriage. Although no epidemiological link could be drawn by cgMLST, between *E. faecium* isolates from this study and *E. faecium* isolated in Portuguese hospitals, the same clonal backgrounds defined by MLST were identified in both collections.

Although none of the five settings screened in this study appear to be a VRE reservoir in Portugal, a high prevalence of antibiotic resistance and virulence determinants was identified among *E. faecium* and *E. faecalis* commensal isolates. Our results suggest that an active surveillance of *Enterococcus* reservoirs, beyond VRE, to prevent antimicrobial resistance spread is strongly recommended.

Keywords: *Enterococcus*, Livestock, Environment, VRE, Antimicrobial resistance

Resumo

Os enterecocos são bactérias comensais do trato gastrointestinal de humanos e animais, que em condições específicas se tornam agentes patogénicos oportunistas. *Enterococcus faecium* resistentes à vancomicina (VRE) são uma das principais causas de infeções nosocomiais mundialmente. Os VRE são endémicos nos Estados Unidos e atingiram uma prevalência de 16,8% nos hospitais Europeus em 2020. Este estudo teve como objetivo identificar reservatórios de VRE em amostras de animais de produção e do meio ambiente, e identificar a sua relação epidemiológica com os VRE responsáveis por infeções nos hospitais portugueses.

Foram rastreadas 294 amostras de animais de produção (porcos, n=80 e vacas, n=55) e do ambiente (autocarros, n=89; mãos de passageiros, n=18; ambiente urbano próximo de hospitais, n=52) para pesquisa de VRE. Especiação, deteção de genes de resistência à vancomicina e de virulência foram realizados por PCR. A resistência aos antibióticos foi estudada por difusão em disco, Etest® e diluição em agar. A análise genómica de 23 *E. faecium* foi realizada por cgMLST (chewBBACA v2.5) e a deteção de resistência aos antimicrobianos, virulência e plasmídeos por ResFinder_v3.1, VirulenceFinder_v2.0 e PlasmidFinder_v2.0.

Um total de 470 isolados de *Enterococcus* spp. foram identificados em 90% (266/294) das amostras, incluindo seis espécies diferentes: *E. faecalis* (30%), *E. casseliflavus* (20%), *E. gallinarum* (14%), *E. faecium* (13%), *E. hirae* (5%) e *E. durans* (< 1%). *E. faecium* foi mais prevalente nas amostras do ambiente [ambiente urbano (27%), mãos dos passageiros (23%) e autocarros (19%)] comparativamente aos animais [porcos (7%) e vacas (5%)]. Apesar de todos os isolados serem suscetíveis aos glicopéptidos e de não terem sido detetados os genes *vanA* e *vanB*, 23% dos *Enterococcus* continham o *vanC2/3* e 17% o *vanC1*, incluindo 12 (40%) *E. faecalis*. Estes *E. faecalis*, apresentaram um padrão de resistência associado principalmente a resistência intrínseca, enquanto os *E. faecium* apresentaram elevada resistência aos aminoglicosídeos e macrólidos (100%; genes *aac(6')-Ii* e *msrC*), à ampicilina (83%; mutações na *pbp5*), tetraciclinas (39%; genes *tetM* and *L*) e elevada resistência à estreptomicina (35%; *ant(6)-Ia*) e gentamicina (4%; *aac(6')-aph(2'')*). Foram identificados diferentes determinantes de virulência em *E. faecalis* (*asa1*, *gelE* e *ace*) e *E. faecium* (*ace*, *acm*, *scm*, *sgrA*, *efaAfm* e IS16).

A população de *E. faecium* mostrou uma elevada variabilidade genética, tendo sido identificado um "sequence type" (ST) diferente por isolado, incluindo STs pertencentes aos complexos clonais (CC) 17 (ST32) e CC94 (ST296, ST1205, ST800 e ST2206), frequentemente associados a infeções hospitalares e a colonização. Embora não tenha sido possível traçar uma ligação epidemiológica por cgMLST, entre os *E. faecium* deste estudo e *E. faecium* isolados nos hospitais portugueses, foram identificados os mesmos patrimónios clonais definidos por MLST em ambas as coleções.

Apesar de nenhuma das cinco coleções rastreadas neste estudo constituir um reservatório de VRE em Portugal, foi detetada uma elevada prevalência de resistência aos antibióticos e determinantes de virulência em isolados comensais de *E. faecium* e *E. faecalis*. Estes resultados sugerem que uma vigilância ativa de reservatórios de

Enterococcus, para além dos VRE, é fortemente recomendada na prevenção da disseminação global da resistência aos antimicrobianos.

Palavras-chave: *Enterococcus*, Animais de produção, Meio ambiente, VRE, Resistência aos antimicrobianos

Table of contents

List of abbreviations

AMP- Ampicillin

AMR- Antimicrobial resistance

AREfm- Ampicillin resistant *Enterococcus faecium*

ATCC- American Type Culture Collection

BHI- Brain heart infusion media

C- Chloramphenicol

CA- Community-associated

CARD- Comprehensive Antibiotic Resistance Database

CAT- Chloramphenicol acetyltransferase

CC – Clonal complex

CDC- Center for Disease Control and Prevention

CIP- Ciprofloxacin

CGE - Center for Genomic Epidemiology

CLSI- Clinical & Laboratory Standards Institute

cgMLST- Core genome multilocus sequence typing

CN- Gentamicin

CRISPR- Clustered regularly interspaced short palindromic repeats

CT- Clonal type

DLV- doble-locus variant

DNA – Deoxyribonucleic acid

dNTP – Deoxyribonucleotide triphosphate

E- Erythromycin

EARS-net- European Antimicrobial Resistance Surveillance System Network

EMA- European Medicines Agency

EUCAST- European Committee on Antimicrobial Susceptibility Testing

F- Nitrofurantoin

FDA- United States Food and Drug Administration

FIB- Fecal indicator bacteria

GIT- Gastrointestinal tract

HA- Hospital-associated

HAI- Healthcare associated infections

HGT- Horizontal gene transfer

HLAR- High-Level Aminoglycoside Resistance

HLGR- High-Level Gentamicin Resistance

HLSR- High-Level Streptomycin Resistance

HS- High-sensitivity

ID- Identity

IS- Insertion sequence

ITQB- Instituto de Tecnologia Química e Biológica António Xavier

LEV- Levofloxacin

LNZ- Linezolid

LRE-Finder - Linezolid Resistant Enterococci- Finder

MDR – Multidrug resistance

MIC – Minimal inhibitory concentration

MLST – Multilocus sequence typing

MRSA- Methicillin-resistant *Staphylococcus aureus*

MSCRAMM- Microbial Surface Components Recognizing Adhesive Matrix Molecules

NaCl- Sodium chloride

NCBI- National Center of Biotechnology Information

PAI- Pathogenicity islands

PBP - Penicillin-binding protein

PCR – Polymerase chain reaction

PYR- Lpyrrolidonyl- B-naphthylamide

QD- Quinupristin- dalfopristin

RNA- Ribonucleic acid

S- Streptomycin

SLV- Single-locus variant

SNP- Single nucleotide polymorphism

ST- Sequence type

SXT- Trimethoprim-sulfamethoxazole

TC- Teicoplanin

TE- Tetracycline

TGC- Tigecycline

TLV- Triple-locus variant

TSA- Tryptic Soy Agar

TSB- Tryptic Soy Broth

UNL - Universidade Nova de Lisboa

US or USA- United States of America

VAN- Vancomycin

VFDB- Virulence Factor Database

VRE- Vancomycin- resistant *Enterococcus*

VRSA- Vancomycin-resistant *Staphylococcus aureus*

W- Trimethoprim

WGS- Whole genome sequencing

1. Introduction

1.1 *Enterococcus*: historical perspective

In 1899, the word "Entérocoque*"* (French) was originally used by Thiercelin to designate the intestinal origin of a Gram-positive diplococcus recovered from human feces (1). The English translation, *Enterococcus*, was adopted to generally characterize the genus of Gram-positive cocci belonging to the family *Enterococcaceae*, order *Lactobacillales* and class *Bacilli* of the phylum *Firmicutes* (2).

Prior to the establishment of the *Enterococcus* genus, enterococci were categorized as members of the *Streptococcus* genus. In 1906, Andrewes and Horder named *Streptococcus faecalis* an organism isolated from a patient with endocarditis that was very similar to cocci isolated from human intestine (3,4). In the following years other *Streptococcus* species differing in the fermentation patterns of *S. faecalis* were described. In the 1930s, Sherman proposed a classification scheme which divided streptococcus into four groups including the non-enterococcal streptococci (pyrogenic, viridians and lactic) and the enterococci, based on the physiologic features as temperature, pH and saline growth conditions (3,4). This classification correlated to the Lancefield serological typing scheme proposed in 1933, that classified enterococci as group D *Streptococcus* (5). In 1984, Schleifer and Kilpper-Bälz distinguished *S. faecalis* and *Streptococcus faecium* from other members of the streptococci family based on DNA:DNA and DNA:rRNA hybridization and 16S rRNA sequencing studies. Since then, the group D *Streptococcus* (except *Streptococcus bovis*) were considered genetically and biochemically different from the *Streptococcus* and therefore considered a separated genus, the *Enterococcus* (6). *S. faecalis* and *S. faecium* were then renamed *Enterococcus faecalis* and *Enterococcus faecium*, respectively. Nowadays, the genus *Enterococcus* is composed by eighty species and three subspecies (https://lpsn.dsmz.de/search?word=enterococcus; accessed on 18 September 2022).

1.2 The *Enterococcus* genus

Enterococci are Gram-positive bacteria, with ovoid shape (coccus) that grows in single cells, pairs, or short chains. They are non-mobile, except for the species *Enterococcus gallinarum* and *Enterococcus casseliflavus* (3), non-sporulating facultative anaerobic and catalase negative organisms with the ability to hydrolyze esculin in the presence of 40% bile salts and Lpyrrolidonyl- B-naphthylamide (PYR) (7,8). These microorganisms are remarkably resistant to numerous environmental stresses since they are able to grow in a wide range of temperatures (10-45 ºC), although their optimal temperature is 35-37 ºC; are tolerant to saline up to 6.5% of NaCl and to wide pH gradients (pH 4.5–10.0), allowing them to survive and grow in harsh environments (2,9). These microorganisms are ubiquitously distributed in nature with a remarkable array of habitats, including the gastrointestinal tract (GIT) of humans and vertebrate and invertebrate animals, as well as soil and sediments, plants, fresh and marine waters and different types of foods (including dairy products, fermented vegetables, meat, fish and sea foods) (3,4,9,10). *E. faecalis* and *E. faecium* have the widest range of habitats and are the most common species associated to human gut (4,9,10).

The enterococci are commonly used as fecal indicator bacteria (FIB). FIB are broadly commensals of the gastrointestinal tract of animals that are shed in their excretions at high densities and therefore regularly detected in contaminated waters (10). Usually, increased enterococci levels detected in the environment have been linked to an increased risk of gastroenteritis in humans (11).

Enterococcus have a genome with a low-GC content (34 to 45%), ranging in size from 2.3 to 5.4 Mb with 2,154 to 5,107 predicted genes, and a genus core genome comprising about 605 to 1,037 genes (12,13). The enterococci large pangenome is promoted by their highly flexible genomes and niche adaptation capacity (14). Enterococci have the ability to acquire new genes by horizontal gene transfer (HGT) involving mobile genetic elements, such as plasmids and transposons (9). The absence of CRISPR (clustered regularly interspaced short palindromic repeats) system, a mechanism that gives bacteria a defense system against incoming DNA, contribute to enterococci genome flexibility (15). This is particularly true for *E. faecium*, given its high

recombination rates, namely for genes associated to antimicrobial resistance (AMR) that have an important role in the pathogen implementation and dissemination in the hospital environment (9,16).

1.3 *Enterococcus* spp. colonization and infection

Enterococci have been considered commensal microorganisms with a widespread pattern of colonization in the gastrointestinal tract (GIT) and feces of mammals (71.3%), reptiles (85.7%), birds (31.8%) as well as insects (53%), which likely place them among the earliest members of GIT microbiota of their hosts (3) (Figure 1).

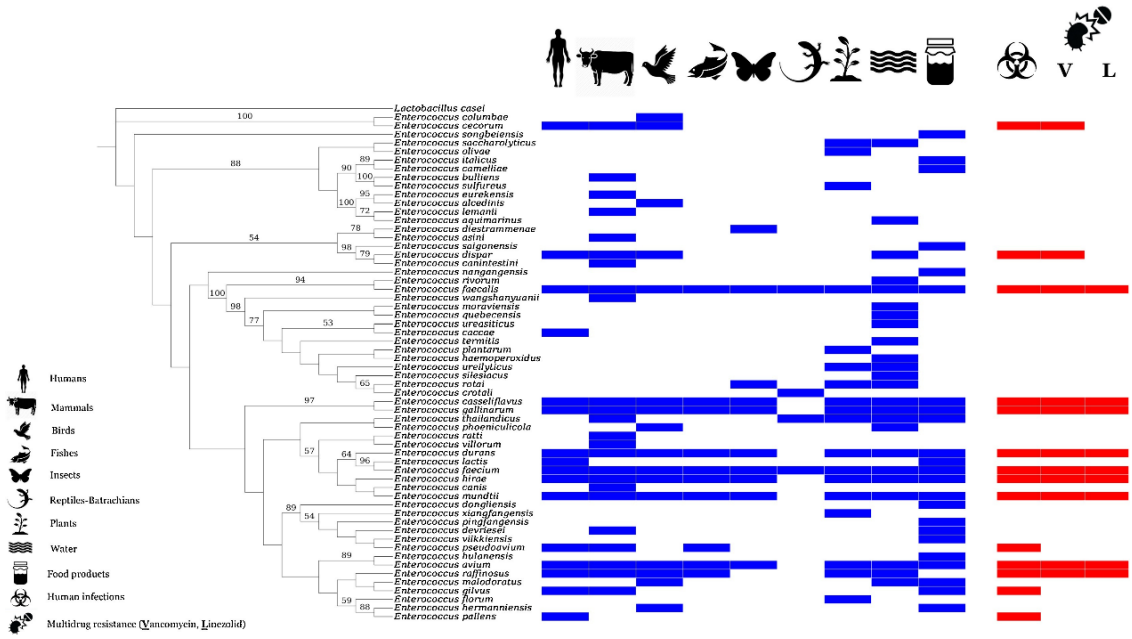


Figure 1. Phylogenetic tree and distribution of *Enterococcus* species in different hosts. Adapted from (9).

Although the natural habitat of enterococci in humans is the lower gastrointestinal tract, they can also colonize the upper gastrointestinal tract, the lower and upper genital tracts, and the oral cavity, including saliva, tongue, vestibular mucosa and dental plaque (17). *Enterococcus* spp. represent less than 1% of the intestinal microbiota of an adult,

but are able to increase their density and even transit to infection, when other members of the microbiota are depleted by antibiotics during host treatment courses (15,18). *E. faecalis* and *E. faecium* are the enterococcal species most frequently found in human gut, but the human GIT can also be occasionally colonized by other enterococcal species including *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus hirae*, *Enterococcus mundtii* and *Enterococcus raffinosus* (14,19).

Beside humans, also animals' GIT is a common reservoir for enterococci. The proportions of *Enterococcus* species among animals are highly variable and influenced by some factors like diet, age or heath condition of the host (17). *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* are the enterococcal species most commonly found in the gut of mammals (9,20). Additionally, bovine and swine microbiota occasionally contain other species, such as *E. casseliflavus*, *E. gallinarum*, *E. avium*, and *Enterococcus cecorum* but in a lower prevalence (20). In insects, *E. faecalis* and *E. faecium* are the most predominant species, while other species are reported less frequently (3).

Although many *Enterococcus* spp. have been sharing a commensal lifestyle with their hosts for hundreds of millions of years, certain species evolved as human opportunistic pathogens (15). *E. faecalis* and *E. faecium* are, by far the most frequent species responsible for nosocomial infections in humans (14), as urinary tract and intra-abdominal infections, to most serious and life-threatening conditions including bacteremia and endocarditis (9). Historically, the overwhelming majority of human enterococcal infections (80–90%) have been attributed to *E. faecalis*, but *E. faecium* has been increasingly reported in hospitals usually associated to high antibiotic resistance, namely to vancomycin, and virulence (13). In fact, a major hallmark of enterococci pathogenicity is its capacity of acquiring antimicrobial resistance, virulence determinants, and adaptation to hostile environments (14).

Enterococcus asymptomatic colonization can last for long periods, and is a risk factor for infection, once the immunocompromised host barriers are disrupted (21–23). This is of particular significance in critical ill and immunocompromised patients, receiving long-term antimicrobial treatments, being hospitalized in long-term facilities,

surgical or intensive-care units and having indwelling medical devices, such as catheters (15,24).

Colonized individuals or animals are potential reservoirs for the spread of multidrug resistant *Enterococcus* spp., specially vancomycin-resistant *Enterococcus faecium* (VRE) to other persons and to the environment, which is of major concern in hospital settings. These reservoirs represent a significant source of pathogen transmission, supporting the inclusion of carriers' surveillance and environmental screening in infection control strategies (26).

Transmission can occur through direct contact with colonized or infected patients or, through indirect contact via the hands of health-care workers, either contaminated patient-care equipment or environmental surfaces (22). Outside the hospitals, multidrug resistant *Enterococcus* spp. can be transmitted to humans through direct contact with colonized farm workers or professionals working in slaughterhouses, contaminated water (including surface water used for recreation, drinking water, irrigation of crops, and domestic, hospital and industrial wastewater) and food, as vegetables or foods from animal origin (9,25). It is recognized that the food chain constitutes a major route for the acquisition of antibiotic-resistant bacteria by humans, which could be transferred via direct animal contact, or by consumption of food of animal origin (26). Also, livestock, pets and wild animals may also be exposed to VRE by drinking or foraging in contaminated water and soils (25) (Figure 2).

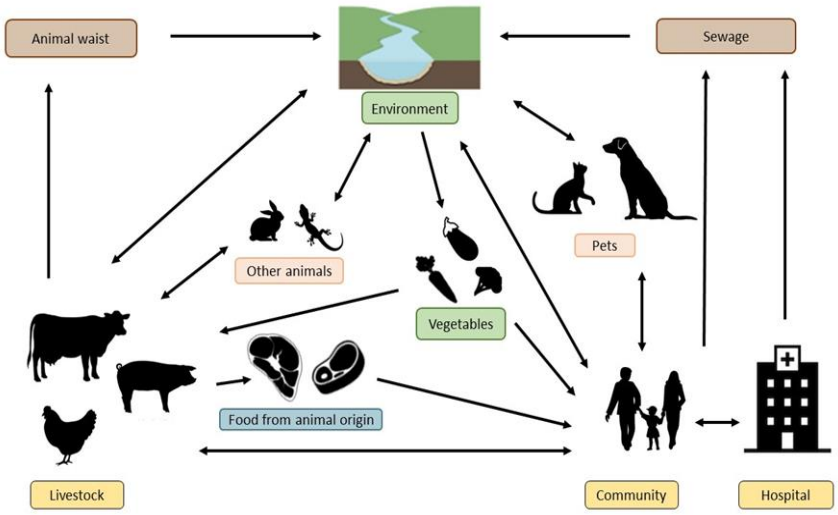


Figure 2. Routes of transmission of multidrug resistant *Enterococcus* spp. and/or VRE among different reservoirs.

VRE can persist and be maintained as a commensal in the community, supported by different reservoirs, until achieve optimal conditions for infection and become a major pathogen (21,27–29).

1.4 Antibiotic resistance in enterococci

A major challenge in the treatment of enterococcal infections is the limited therapeutic options, given the enterococci wide antimicrobial resistance with intrinsic and acquired resistance profiles against different antibiotic classes. Intrinsic resistance, is inherent to the species and therefore encoded within the core genome of all members of the species, while acquired antibiotic resistance is a property of some members of the species (30,31) resulting from mutations or acquisition of foreign DNA, mainly by horizontal gene transfer via mobile genetic elements (17). Although resistance is a genus-wide trait, the clinically relevant species *E. faecium* and to a less extent *E. faecalis* have the highest antibiotic resistant profiles (32).

1.4.1 Intrinsic resistance

One of the most widely recognized characteristics of enterococci is their intrinsic resistance to several antibiotics, namely β-lactams and cephalosporins, aminoglycosides, lincosamides and combination of trimethoprim-sulfamethoxazole.

β-lactams and cephalosporins interfere with cell wall synthesis by binding to the penicillin-binding proteins (PBPs), which are transpeptidases involved in peptidoglycan cross-linking (17). The production of low affinity penicillin-binding proteins, known as PBP4 in *E. faecalis* and PBP5 in *E. faecium* (14) avoid the attachment and inactivation of these transpeptidases by the antibiotic, preventing the cell wall synthesis disruption resulting in low-level resistance to penicillin and moderate to high-level resistance to cephalosporin (7,33).

Likewise, *Enterococcus* spp. are also intrinsically resistant to clinically achievable concentrations of aminoglycosides, a class of antibiotics that interfere with protein

synthesis by binding to 16S rRNA of the 30S subunit of the ribosome (17). Resistance is thought to be caused by the inability of the antibiotic to enter the cytoplasm since enterococcal cell wall is naturally impermeable to these antibiotics (34). This is commonly overcome in clinical practice, to treat enterococcal infections such as endocarditis, by the addition of a cell wall active agent, like β-lactams or glycopeptides to the aminoglycoside for a synergistic killing effect (7). Some species, such as *E. faecium* (*aac(6')-Ii*), *E. hirae* (*aac(6')-Iih*) and *E. durans* (*aac(6')-Iid*), intrinsically express chromosomal encoded acetyltransferases that confer resistance to tobramycin, kanamycin, and amikacin (35). Additionally, an rRNA methyltransferase (*efmM*), have been associated with low-level resistance to dibekacin, tobramycin and kanamycin in *E. faecium* (36).

Lincosamides and streptogramins inhibit protein synthesis by binding onto the subunit of 50S ribosome (37). *E. faecalis* is intrinsically resistant to clindamycin (a lincosamide), quinupristin (streptogramin B class) and dalfopristin (streptogramin A class) through the expression of the efflux-pump related *lsa* gene (7). *msrC* gene encodes a different putative ABC-efflux pump in *E. faecium* providing low-level resistance to class B streptogramins and macrolides. Resistance to quinupristin-dalfopristin (QD), (which is of major clinical significance given the its usage in the treatment of Gram-positive infections), requires that enterococci must be resistant to both streptogramin A and streptogramin B, which explains the ineffectiveness of QD against *E. faecalis* (7,32).

Trimethoprim-sulfamethoxazole (SXT) antibiotic combination inhibits the folate synthesis pathway and kills a broad spectrum of bacterial species that are not able to take in folate from the environment to produce nucleic acids (7). *Enterococcus* have this unusual capacity to absorb folic acid from the environment, and besides its apparent susceptibility when tested in folic free media *in vitro*, trimethoprim-sulfamethoxazole is not effective in treating serious enterococcal infections (38).

1.4.2 Acquired resistance

High-level resistance to β-lactams in enterococci is associated to the acquisition of either a plasmid or transposon-encoded β-lactamase enzyme, or by mutations in

PBP4/5 (7,17). β-lactamases are enzymes, codified by the *bla* genes that disrupt the β-lactam ring in the antibiotic structure and strip it of their antimicrobial properties (32). Although enterococcal β-lactamase production has been infrequent and mostly linked to *E. faecalis* (7), a small number of *E. faecium* strains positive for β-lactamase have been reported (39). Accumulation of point mutations in the *pbp5* genes have been associated to long courses of β-lactam antibiotics usage. These mutations lead to an extremely low affinity of PBP5 active site region to β-lactams leading to high-level penicillin/ ampicillin resistance MICs (32). Acquired ampicillin resistance due to *pbp5* mutations is considered an hallmark of hospital-associated strains of *E. faecium* (40).

Gentamicin and streptomycin have been significant antibiotics in the treatment of serious enterococcal infections given its synergistic killing effect on enterococci when combined to β-lactams, and evasiveness from the activity of intrinsically produced enterococcal enzymes (41). However, acquired high-level resistance to aminoglycosides, namely MIC > 500 μg/mL for gentamicin and MIC > 2000 μg/mL for streptomycin, have been described in both animal and human *E. faecium* and *E. faecalis* (7,20). High-level resistance to gentamicin is mainly due to a bifunctional enzyme encoded by *aph(2'')Ia-aac(6')-Ie* that inactivates gentamicin and structurally identical aminoglycosides, except for streptomycin (7,42). Moreover, expression of other genes could be also involved in high-level resistance as: *aph(2")-Ic*, *aph(2")-Id*, *aph(2")-Ie*, and *aph (2")-Ib* genes (7). *aph(2"")-Ic* seems to be more frequent in enterococci of animal origin, and some farm animals could be a reservoir of this gene (43). Globally, up to 55% of clinical enterococcal isolates reported high-level resistance to gentamicin (17). On the other hand, high-level resistance to streptomycin occurs typically by single point mutations in the ribosome, but enzymatic inactivation of the antibiotic due to Ant(6')-Ia and Ant(3")-Ia adenylyltransferases also occurs (7,42). Other aminoglycoside-modifying enzymes have been identified in enterococci, including aminoglycoside phosphotransferase, Aph(3')-IIIa, that confers resistance to kanamycin and Ant(4")-Ia, a nucleotidyltransferase that confers resistance to tobramycin, amikacin and kanamycin (42).

Quinupristin-dalfopristin (QD), a streptogramin B/A combination is commonly used to treat infections caused by vancomycin-resistant *E. faecium*. Extensive resistance among enterococci isolated from farm animals and agricultural sewage was associated to

the widespread use of virginiamycin, a veterinary streptogramin A compound (7). Hence, QD resistance is more common in environmental samples, but nosocomial infections due to resistant enterococci are becoming more prevalent (7). Three mechanisms of acquired resistance to lincosamides and streptogramins in enterococci were described: (i) the acquisition of genes encoding a new ion pump, (ii) inactivation of the antibiotic by enzymatic acetylation, or (iii) methylation of 23S rRNA leading to disruption of the antibiotic binding site on the 50S ribosome subunit (32). A total of 12 different genes, including those encoding drug efflux pumps (*lsa*, *vgaD* and *mefA*), drug-acetylating enzymes (*vatD*, *E*, and *H*), and rRNA-methylating enzymes (*ermA* and *B*) have been associated with this resistance (17). Furthermore, most of these genes are thought to be acquired through horizontal transfer of transposons, especially Tn*917*, a member of the Tn*3* family, which is responsible for enterococcal resistance to streptogramin B, macrolides, and lincosamides (MLSb phenotype) (44).

Nowadays, resistance to other antibiotics such as macrolides, tetracyclines, chloramphenicol, fosfomycin, rifampin and quinolones is so widespread that these antibiotics are rarely used in the treatment of human enterococci infections (7). However, due to their significance, it is important to highlight some of the resistance mechanisms to these antibiotics.

The macrolide antibiotics tylosin, spiramycin, and virginiamycin were often used as a growth promoter in animals, until being banned in Europe, in 2006. After their restriction in animal feed regimens, *Enterococcus* strains from animals showed a considerable reduction in erythromycin resistance (45). Acquired resistance to macrolides, with erythromycin being the most representative, can be attributed to various genetic determinants. *erm(B)* gene is the most prevalent resistance determinant, which is often carried by transposon Tn*917*, and is commonly found in human and animal isolates. Other gene conferring resistance to macrolides, but less prevalent, is the efflux gene *mef(A)* (20).

The tetracycline family is one of the most used antibiotics in veterinary medicine, which could be responsible for the widespread resistance among animal isolates. Resistance is mediated by multiple genes, although the most frequent ones in enterococci are those implicated in ribosomal protection, (*tet(M)*, *tet(O)*, *tet(S)*) and in efflux or enzymatic inactivation (*tet(K)*, *tet(L)*) (20). The ribosomal protection protein mechanism

encoded by the *tet(M)* gene is the most common, and this gene is often carried by conjugative transposons related to Tn*916* (46).

Enterococci can also acquire high-level resistance to fluoroquinolones, namely by the accumulation of point mutations in *gyrA* and *parC* genes (14) and through the acquisition of *qnr* genes. Less frequently, resistance to fluoroquinolones could be attributed to efflux pumps such as *NorA*-like for *E. faecium* and *EmeA* for *E. faecalis* (47).

Chloramphenicol, a broad-spectrum antibiotic was banned in Europe in 2006 from additive usage in animals due to side effects, and is now severely restricted in human and veterinary medicine (48). A number of genes have been found in enterococci from animals, foods, and humans that code for resistance to phenicols (*cat*, *fexA*, *fexB*), and both phenicols and oxazolidinones (*cfr*, *optrA*). However, the main mechanism of chloramphenicol resistance appears to be the production of chloramphenicol acetyltransferase (CAT) enzymes (20). The most common *cat* variants are *catA7*, associated with the Inc18 plasmid family, widely disseminated in food, farm animals and healthy humans, and *catA8*, mostly in clinical and swine isolates. Although less frequent, the *catA9* gene was already identified in *E. faecalis* from swine (20,49).

Since most multidrug resistant *Enterococcus*, namely VRE, are resistant to first line antibiotics, such as ampicillin and aminoglycosides, only a few last-resort antimicrobials, including oxazolidinones (linezolid, tedizolid), novel tetracyclines (tigecycline) and lipopeptides (daptomycin) remain valid options to treat these infections (40).

Linezolid is one of the last resort antibiotics for the treatment of VRE and methicillin-resistant staphylococci infections in humans authorized by US Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) (15,50). Linezolid is a bacteriostatic antibiotic that inhibits protein synthesis by interfering with the placement of the aminoacyl tRNA at the A site of the bacterial ribosome (51). Linezolid resistance can be caused by point mutations in the 23S rRNA gene, specially G2576T and G2505A, or through acquisition at least one of the five mobile oxazolidinone resistance genes, namely *cfr*, *cfr(B)*, *cfr(D)*, *optrA* and *poxtA* (52,53). The *cfr* gene codes for a 23S rRNA methyltransferase that confers resistance to oxazolidinones, phenicols, lincosamides, pleuromutilins, and streptogramin A (PhLOPSA phenotype) (20). *cfr* gene variants *cfr(B)* and *cfr(D)* were reported in clinical *E. faecium* (53,54), whereas *cfr* has

been also reported in *E. faecalis* from both animal and human origin (55,56). The *optrA* gene encodes an ABC transporter that confers resistance to oxazolidinones (linezolid and telizolid) and phenicols (chloramphenicol and florfenicol), and has been more prevalent in *E. faecalis* than in *E. faecium*, and from food-producing animals compared to human isolates (20,57). The *poxtA* gene codes for an ARE ABC-F protein that confers reduced susceptibility to oxazolidinones, phenicols and tetracyclines (52). This gene was initially identified in a clinical methicillin-resistant *Staphylococcus aureus* (MRSA) and then identified in both *E. faecium* and *E. faecalis* isolated from swine animals (53).

Daptomycin is an antimicrobial lipopeptide used in the treatment of Gram-positive bacterial infections including ampicillin- and vancomycin resistant *Enterococcus* (15,17). Daptomycin resistance has been documented in both *E. faecalis* and *E. faecium*, but in general it is more prevalent in the latter (7). In the presence of physiologic calcium concentrations, this antibiotic incorporates into the cell membrane of Gram-positive organisms and promotes leakage of intracellular potassium into the extracellular space, resulting in cell death (7). Although daptomycin resistance seems to be multifactorial, acquired resistance appears to be associated with mutations in intrinsic genes involved in cell wall synthesis and homeostasis (58).

Tigecycline is a synthetic derivative of the broad spectrum tetracycline antibiotic minocycline, which acts on the 16S rRNA of the ribosome 30S subunit by inhibiting its association with aminoacyl-tRNAs (59). This antibiotic has bacteriostatic activity against a broad range of bacterial pathogens, including Gram-positive and Gram-negative bacteria, anaerobes and atypical organisms (40). Initially, the emergence of resistance was observed in *E. faecalis* (60), but as already been described in *E. faecium*, including clinical VRE isolates (61,62). In enterococci, resistance to tigecycline can be mediated through up regulation of tetracycline resistance determinants such as *tet(L)* (encoding an efflux pump) and *tet(M)* (offering ribosomal protection), and mutations in the ribosomal protein *rpsJ* (61,63). The description of enterococcal isolates with reduced susceptibility to tigecycline in animals intended for food consumption seems to be a consequence of widespread use of tetracyclines in veterinary settings (64).

1.4.2.1 Glycopeptides resistance

Glycopeptides are glycosylated cyclic or polycyclic nonribosomal peptides used for the treatment of severe human infections caused by multidrug-resistant Gram-positive pathogens, such as *Staphylococcus aureus*, *Enterococcus* spp., and *Clostridioides difficile* (19). These agents are bactericidal and act by inhibiting cell wall synthesis, binding to the D-Alanine-D-Alanine terminus of the pentapeptide precursor of the peptidoglycan, preventing the cross-linking of the peptidoglycan chain and their incorporation into the cell wall (20). The leading mechanism of glycopeptide resistance in enterococci implicates the alteration of the peptidoglycan terminus D-Ala-D-Ala to either D-Ala-D-Lactate, resulting in an almost 1,000-fold decrease in affinity of glycopeptides for peptidoglycan precursors, or to D-Ala-D-Serine which decreases the binding affinity in 6-fold, leading to variable expressions of resistance (65). While high-level resistance (vancomycin MIC >64 μg/ml) is attributed to D-Ala-D-Lac precursors, D-Ala-D-Ser confer low-level resistance (vancomycin MIC, 4 to 32 μg/ml) (20).

In the 1950s, the first glycopeptide antibiotic was discovered: vancomycin. Given its high toxicity, other antibiotics as β- lactams, were preferred to treat staphylococcal infections until the late 1970s, when MRSA strains and extensive β-lactam resistance became more prevalent and vancomycin started to be widely used (21,66). The introduction and spread of VRE parallels the increased use of vancomycin for the treatment of MRSA infections (21). The majority of vancomycin resistance has been identified among *E. faecium* (77%) but it can also be associated to *E. faecalis* (9%) and other *Enterococcus* species (14%) usually less implicated in serious infections, such as *E. gallinarum*, *E. casseliflavus*, *E. avium*, and *E. raffinosus* (67,68).

Moreover, an additional concern associated to increased prevalence of VRE is the potential transfer of mobile genetic elements containing the vancomycin resistance determinants to other bacterial species, such as vancomycin-resistant *Staphylococcus aureus* (VRSA) (20,69). The first VRSA was isolated in 2002 in the US, from a catheter tip of a 40-year-old diabetic patient undergoing dialysis (67). Although the transference of vancomycin resistance determinants between these species is not frequent, 52 VRSA strains have been identified worldwide until 2020 (70). The unique VRSA identified in

Europe was isolated in Portugal in 2013, and in this case MRSA acquired the vancomycin resistance determinant from a vancomycin resistant *E. faecalis* co-isolated from a diabetic foot ulcer (71).

1.4.2.1.1 Mechanisms of vancomycin resistance

Vancomycin and teicoplanin are the most commonly used glycopeptides in clinical practice, and therefore, herein used as surrogate for all glycopeptides regarding resistance mechanisms.

Vancomycin resistance is mediated by *van* operons, composed by several genes encoding a two-component sensor-transducer system as *vanS*-*vanR*; enzymes involved in the production of new altered pentapeptide precursors as d-lactate dehydrogenase (*vanH* gene) or peptide ligases and enzymes involved on the destruction of normal D-Ala-D-Ala precursors as D-Ala-D-Ala dipeptidase (*vanX* gene) (7). So far, 9 different genes encoding the peptide ligase have been identified (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*) (67). Of these, multiple subtypes have been described for *vanB* (*vanB1*, *vanB2*, and *vanB3*), *vanC* (*vanC1*, *vanC2*, and *vanC3*), *vanD* (*vanD1* to *vanD5*) and *vanG* (*vanG1*, *vanG2*) (20). Moreover, *van* operons can be divided into two groups regarding the altered precursor produced: those where the terminal D-Ala is replaced by a D-Lac (like *vanA*, *vanB*, *vanD*, and *vanM*), all conferring resistance to vancomycin and teicoplanin, although the *vanB* operon is not induced by the presence of teicoplanin, and those where the terminal D-Ala is replaced by a D-Ser (*vanC*, *vanE*, *vanG*, *vanL*, and *vanN*), conferring low levels resistance to vancomycin, but are still susceptible to teicoplanin (14).

Using the *vanA* cassette as a model (Figure 3), the expression of the *van* operon genes is regulated by a two-component sensor-transducer system (*vanR* and *vanS*). The sensor of the system (*vanS*) in the presence of vancomycin (or other glycopeptide) in the environment is activated (by autophosphorylation) and activates the regulator *vanR*, by phosphorylation. Once activated, *vanR* interacts with specific promoter regions (including its own promoter) increasing *vanR*, *vanS* and the other operon genes transcription (7,72). The transcription of *vanH* will produce a dehydrogenase that

converts the cellular pyruvate to d-lactate (D-Lac) which is the first step in the expression of vancomycin resistance, followed by the VanA ligase that binds D-Ala to D-Lac (72). Host enzymes ligate D-Ala-D-Lac to the tripeptide precursor, resulting in a low affinity peptidoglycan pentapeptide precursor. VanX hydrolyzes D-Ala-D-Ala to produce its individual amino acids, allowing D-Ala-D-Lac to serve as the only substrate for the cell wall synthesis (73). Thus, vancomycin fails to bind to the altered pentapeptide which is incorporated into the bacterial cell wall and results in vancomycin resistance. Additionally, VanY hydrolyzes the terminal D-Ala from any normal pentapeptide precursor, making it unusable for normal cell wall synthesis. VanZ provides lower sensitivity to teicoplanin when present, yet it is uncertain how it increases resistance (7,74).

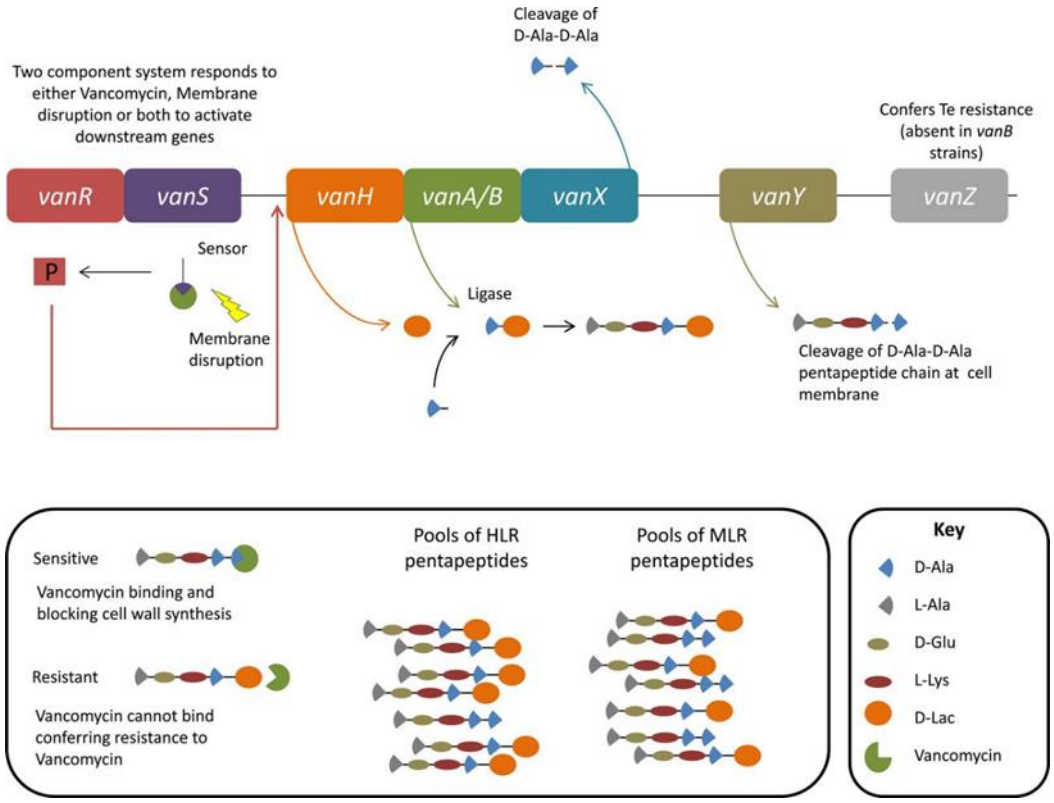


Figure 3. Vancomycin resistance mechanism and structure of typical *vanA* operon. Adapted from (67).

The mechanism for the *vanB* cluster is similar to the *vanA*, but with some significant differences. The $vanS_B$ is not activated in the presence of teicoplanin, so strains with *vanB* remain susceptible to this antibiotic. Though, the continue usage of teicoplanin

for the treatment of infections can promote the development of mutations in the *vanS*$_B$, resulting in the activation of the system that can lead to constitutive expression of the operon and consequently resistance to this antibiotic (72).

vanA and *vanB* are the most prevalent genotypes of VRE (namely in *E. faecalis* and *E. faecium*) with acquired resistance mechanisms in humans and animals, while *vanC*, a chromosomally encoded gene cluster that confers low-level resistance to vancomycin, are intrinsic to *E. gallinarum* (*vanC1*), *E. casseliflavus* (*vanC2*), and *E. flavescens* (*vanC3*) (20,72).

Horizontal transfer of the *van* genes occurs through a variety of mechanisms. The transposon Tn*1546* of the Tn*3* family, carries the *vanA* operon, that can be found on chromosomes or transferrable plasmids (14). The *vanB* operon has been associated to different transposons, such as Tn*1547*, Tn*5382* and the most prevalent among *vanB*-type enterococci Tn*1549*, typically located in the chromosome and less frequently on plasmids (75).

1.5 Virulence factors in enterococci

A virulence factor is an effector molecule that increases microorganism's ability to cause disease (76). Bacterial pathogenesis in most infections, including enterococci, are promoted by a series of events starting by initial colonization, followed by adhesion to the host cells, capacity to invade tissues, and evade non-specific immunologic defensive mechanisms (77). Virulence factors have an important role in *Enterococcus* pathogenicity since strains with a high virulence potential can cause more severe infections.

The most common and well described virulence determinants in enterococci could be divided in two categories: a) surface factors that promote colonization of host cells, and b) virulence factors that damage the host tissues and promote invasion. Moreover, the overwhelming majority of these virulence determinants are known to be transmissible between enterococcal isolates and to other non-enterococcal bacteria (78,79).

a) surface factors that promote colonization of host cells

Enterococci have the ability to adhere and colonize host tissues. Even though, colonization itself is not proof of pathogenicity, but when combined with other virulence factors and with the presence of antibiotic resistance genes, it become a risk factor for the development of infection (77). Adhesins play a major role in the colonization and persistence of *Enterococcus* spp. in the intestinal tract, which serves as a significant natural reservoir for both commensal and infection-associated strains in humans, and facilitate the translocation through damaged intestinal epithelium to promote infection (80,81). Aggregation substance (AS) proteins, collagen- fibrinogen-adhesins (Ace, Scm, Acm and SgrA), enterococcal surface protein (Esp), and cell wall adhesin (EfaA) are some of the virulence determinants involved in and promoting colonization (82).

Aggregation substance includes a range of highly homologous adhesins, as Asa1 or Asp1, which mediate the specific attachment of enterococci to epithelial cells for colonization and induce bacterial cells aggregation during conjugation, facilitating the exchange of plasmids containing virulence and antibiotic resistance genes (76).

Ace (accessory colonization factor), another surface protein with adhesive properties, encoded by the *ace* gene was isolated from *E. faecalis* in both healthy carriers and infections (77,83). This adhesin belongs to the subfamily of bacterial surface adhesins denominated Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) which binds specifically to the protein layer of the extracellular matrix of the host (84). Ace also participate in biding to types I and IV collagen, laminin and dentin. Other collagen-binding MSCRAMMs, homologous to Ace, is Acm (adhesin of collagen from *E. faecium*) protein, encoded by the *acm* gene, is mainly present in *E. faecium* clinical isolates, promoting adherence to collagen type I and to a lesser extent with collagen type IV (76,85). While Scm (second collagen adhesin of *E. faecium*) promotes *E. faecium* binding to collagen type V and fibrinogen (80), SgrA, a LPxTG surface adhesin binds to fibrinogen and nidogen involved in biofilm formation, and contribute to the pathogenesis of hospital-acquired *E. faecium* infections (86).

Enterococcal surface protein (Esp) is a surface protein associated to cell-to-cell adhesion, particularly to eukaryotic cells. It is encoded by *esp* gene, located on pathogenicity islands (PAI) in both *E. faecalis* and *E. faecium*, as is more common among infection isolates than commensals. In *E. faecium*, Esp is highly expressed and was found

to contribute to the bacterial pathogenicity in experimental endocarditis, urinary tract infection and bacteremia. Additionally, Esp can enhance biofilm formation and colonization (76,77,82).

The cell wall adhesin EfaA (endocarditis antigen), encoded by the *efaA*$_{fs}$ gene in *E. faecalis*, and by *efaA*$_{fm}$ in *E. faecium*, was one of the principal virulence factors associated to infective endocarditis (77), but can be found in both clinical and food isolates (87).

b) Virulence factors that affect tissues

Pathogenic *Enterococcus* spp. are able to secrete toxic substances during colonization process, which can cause damage in the host tissue (77). Major virulence factors secreted by enterococci include: cytolysin (Cyl), gelatinase (GelE) and hyaluronidase (Hyl).

Cytolysin (Cyl) is a bacteriocin-type exotoxin, with bactericidal properties against Gram-negative bacteria and toxic effects (β-haemolysis) towards erythrocytes, leukocytes and macrophages (76,77). Cytolysin has been found in both *E. faecium* and *E. faecalis* from infection and from commensal microbiota in humans, as well as among isolates from animals and food (animal and plant origin) (77).

Gelatinase is a zinc-dependent metallo-endopeptidase, with a high potential to hydrolyze gelatin, elastin, collagen, hemoglobin, as well as other bioactive peptides (78). It promotes the host tissue damage by cleaving fibrins, allowing *Enterococcus* bacterial cells migration and proceed to invasive infection (76). Gelatinase also contributes to biofilm formation enhancing enterococci colonization of tissues and persistence in some infection sites by inhibiting phagocytosis and promoting bacterial evasion (76,78). Chromosomal *gelE* gene can be found among *E. faecalis* and punctually in *E. faecium*, from both clinical and food sources (77).

Hyaluronidase is a protein encoded by the *hyl* gene, which has been mostly identified in clinical strains of *E. faecium* and less frequently in *E. faecalis*. This proteolytic protein is implicated in the degradation of mucopolysaccharides that connect tissue and cartilage and, consequently, involved in spreading of bacteria (78).

Globally, enterococci of clinical origin usually contain more virulence determinants than commensal isolates (77). Moreover, *E. faecalis* harbors a higher arsenal of virulence factors compared to *E. faecium*, whose pathogenicity is also strongly supported by antimicrobial resistance determinants (76).

1.6 Emergence and epidemiology of vancomycin resistant *Enterococcus faecium* (VRE)

The rise of enterococci as major human pathogens was mainly devoted to the emergence of vancomycin resistant isolates. The first reports of vancomycin resistance in clinical *Enterococcus* spp. included strains of *E. faecium* isolated from patients in France and England in 1986, that where resistant to vancomycin and teicoplanin (later identified as *vanA* associated resistance) (88,89). Shortly after, in 1987, *E. faecalis* isolates with resistance to vancomycin, but susceptibility to teicoplanin (later identified as *vanB* associated resistance), were recovered from patients in Missouri in the United States (90).

Since then, the global prevalence of VRE has been continuously rising and spreading worldwide. Nonetheless, VRE emergence and epidemiology observed in Europe and the United States (US) followed a different path (8). The first reports of VRE in Europe in the late 1980s, included isolates from healthy people, farm animals, pets, and retail food products (15,91). The extensive and widespread use of avoparcin, a glycopeptide similar to vancomycin, as a growth promoter in animal husbandry, promoted the selection of strains with resistance genes, namely the *vanA* operon. Therefore, food animals become a large reservoir of VRE, which may have then colonized healthy humans via the food chain, supporting the origin of the community reservoir (9,17). In 1997, the use of avoparcin was banned in Europe, leading to a rapid decrease of the prevalence of VRE fecal carriage in food-producing animals and healthy humans (20). In 1986, the first VRE clinical strains were isolated in Europe (29), but the infection incidence rates in hospitals remained significantly lower until 1999, when it started to follow an increasing trend (75).

In the US, since the first VRE identification in the 1990s, colonization of hospitalized patients has expanded quickly and VRE infections were sooner considered

endemic in hospitals (29). It was a result from the large-scale use of antibiotics, particularly vancomycin and cephalosporins in US hospitals (9), while colonization in healthy people seemed to be absent (8). VRE among food-production animals was infrequently detected because vancomycin was never licensed to be used as a growth promoter, neither in the US nor in Europe (29).

In recent years, VRE as the etiological agent of invasive healthcare associated infections (HAI) have been disseminated worldwide, with spatial focus on the American continent and Australia with countries specific prevalence above 50% (ResistanceMap: https://resistancemap.cddep.org/index.php). Since 2017, VRE was listed by the World Health Organization as a high priority pathogen that threaten human health and deserves new antibacterial drugs development (92). Moreover, the Center for Disease Control (CDC) also considered VRE as a serious threat for human health that warranties continuous surveillance. In the last CDC report on the impact of coronavirus disease COVID-19 pandemic on antimicrobial resistance in the US, the global rate of VRE cases increased 16% from 2019 to 2020, out of which, 14% refers to hospital-onset cases only, reversing the decreasing trend followed since 2012 (93).

In Europe, according to the European Antimicrobial Resistance Surveillance System Network (EARS-Net), the global prevalence of vancomycin resistance in *E. faecium* invasive infections increased from 11.6% in 2016 to 16.8% in 2020. Even though, there are still widely variations between European countries, with VRE prevalence ranging from <1% in countries as Finland, France, Iceland, the Netherlands, Norway, Sweden and Ukraine to >50% in Bosnia and Herzegovina, Lithuania, North Macedonia and Serbia (Figure 4) (94). Contrary to what is observed for other multidrug resistant pathogens, there is no distinct geographical pattern for distribution of VRE in Europe, which could be attributed to polyclonal spread of resistant isolates or spread of resistant mobile genetic elements (94).

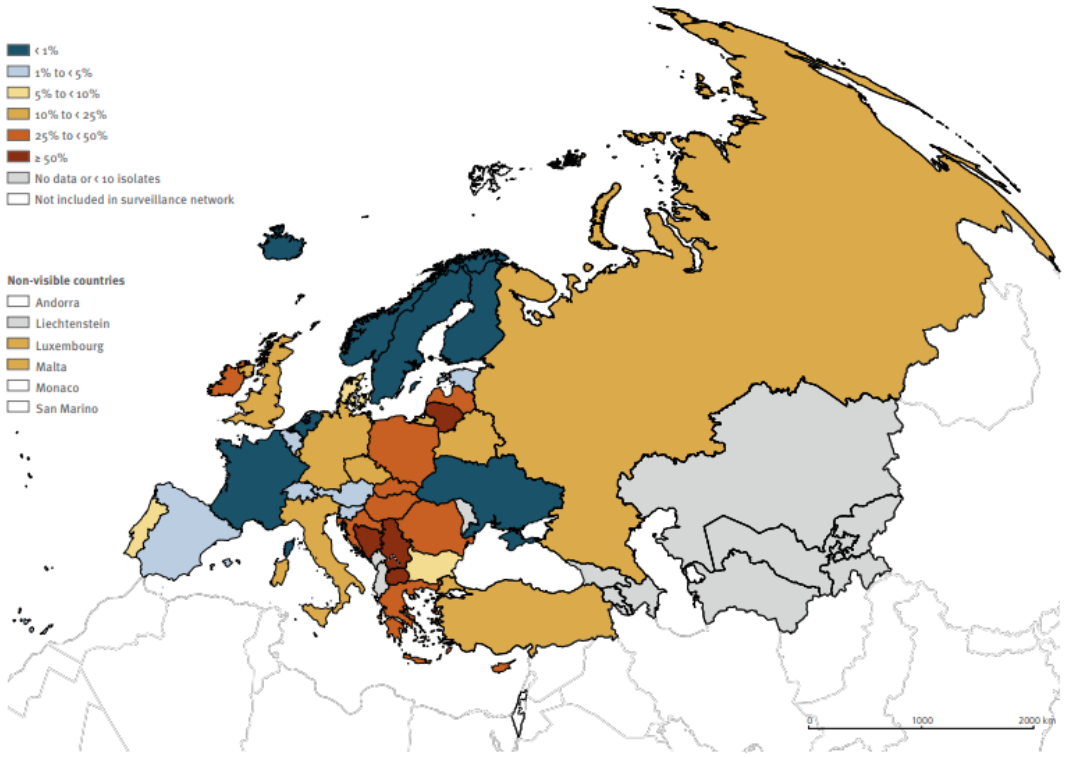


Figure 4. Prevalence of VRE invasive isolates in Europe, 2020. Adapted from (94).

Early *E. faecium* population studies were driven by the increasing number of VRE hospital associated outbreaks. The first recognized *E. faecium* subpopulation mainly associated to hospital outbreaks and human infections, the lineage C1, was described in 2000, and sooner renamed "clonal complex 17" (CC17) based on multilocus sequence typing (95). More recent whole genome sequencing (WGS)-based studies proposed the split of *E. faecium* population in two different clades: the hospital-associated (HA) lineage (clade A) and the community-associated (CA) lineage (clade B) (9,96). Clade A was further divided into two sublineages: clade A1 that tended to replace the former CC17 and includes human clinical strains and clade A2 which represents mostly animal-related strains (97). Nevertheless, CC17 nomenclature was maintained and referred as a globally spread nosocomial lineage characterized by distinct markers such as ampicillin and high-level fluoroquinolone resistance and virulence factors as *esp* and *hyl* (69,98). In addition, when compared to clades A2 and B, clade A1 shows a higher spontaneous mutation rate, as well as larger genomes, that include a high number of mobile genetic elements, pathogenicity island(s), and plasmids or genetic determinants associated to antibiotic

resistance and/or virulence (96,97). Moreover, clade A1 are enriched in a range of determinants that facilitate the adaptation to the hospital environment and therefore being more successful in colonizing and infecting immunocompromised hosts (99). More recent phylogenetic studies support the distinction between clades A and B, although the separation inside clade A was not always verified when using larger collections of isolates (100). Additional evidence from genome data indicate that hospital-adapted lineage evolved rapidly and concurrently with the introduction of antibiotics in clinical practice, from an ancestral population of isolates of animal origin, and not from human commensals (101).

Genomic based studies revealed a polyclonal population structure of *E. faecium* circulating in hospitals, with evidence of inter and intra hospital transmission of particular clones. Clonal lineages, defined by specific clonal complexes determined by multilocus sequence typing, and with particular genetic traits were predominantly associated to specific hosts as humans (CC17, CC22, and CC94) or livestock as swine (CC5), poultry (CC9) or calves (CC1) (102). In general, human community/commensal-associated *E. faecium* isolates belonged to CC94 whereas hospital-associated outbreak infection isolates frequently clustered in CC17 (103). Even though, specific lineages successfully caused outbreaks in hospitals in geographic restricted areas/countries and become endemic, as the case of ST17 widespread in the United States, or ST796 widely disseminated in Australia and New Zealand and further identified as a major lineage in Switzerland (104). Other successful lineages have been reported in different European countries, as the ST80 (with *vanB* gene) in Sweden, Denmark and more recently in Germany (105–107) and ST117 in Germany (107) in Greece, where ST117 *E. faecium* harboring both *vanA* and *vanB* genes was firstly reported (108), and in Spanish hospitals, considered one of the main causes of bloodstream infections (109). Recently, the *E. faecium* ST1421, phenotypically susceptible to vancomycin and known as vancomycin-variable enterococci (VVE), has becoming the predominant *vanA E. faecium* clone in Denmark (110).

Hospital-associated VRE lineages have been also identified in non-clinical backgrounds colonizing healthy humans, livestock and companion animals, wildlife animals, the environment namely sewage waters and foodstuff (111), evidencing the potential for these settings as reservoirs of pathogenic bacteria. On the other hand, strains

from clonal complexes commonly found among animals, namely *E. faecium* CC5 have also been detected in humans (91,112).

Despite the remarkable reduction in the prevalence of VRE carriage among animals observed after the withdrawal of avoparcin in Europe, VRE have frequently been isolated from infection cases in animals, including farm animals and pets (27,113).

1.7 The epidemiology of VRE in Portugal

In Portugal, the first VRE isolates were reported in 1993, with a prevalence in hospitals of about 1.4% (114). In the next years, while the high resistance to ampicillin (70%), high-level resistance to aminoglycosides, gentamicin (43%) and streptomycin (60%) remained stable among *E. faecium*, the percentage of vancomycin-resistant isolates increased from 9% in 1994, to 19.8% in 1996-1997, reaching >25% in some hospitals (115,116) (Figure 5). VRE rates in Portuguese hospitals continued to follow an increasing trend until 2003, when a record of 47% was achieved, mainly attributed to a VRE polyclonal population carrying a *vanA* operon inserted in transposon Tn*1546* (117). Since then, annual surveillance reports from the EARS-Net confirmed a continuous decrease in vancomycin resistance rates among invasive *E. faecium* in Portugal, reaching the lowest prevalence (4.4%) in 2018. Unexpectedly this rate doubled in 2019 and the last surveillance, in 2020, reported 7.8% of VRE, which is still below the European media (16.8%) (Figure 5) (94).

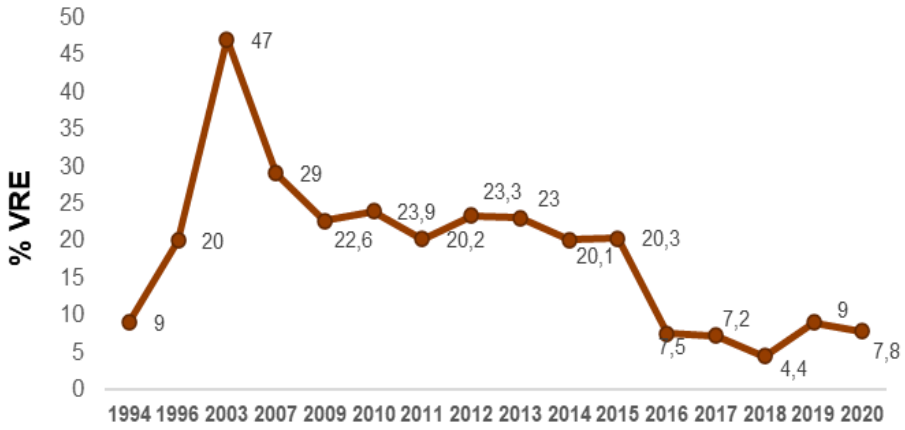


Figure 5. Prevalence of VRE invasive isolates in Portuguese hospitals between 1994 and 2020, based on national and EARS-Net surveillance data (graph kindly provided by Teresa Conceição).

Molecular characterization of VRE isolates responsible for infection in Portuguese hospitals between 2019 and 2020, showed that these isolates belonged to three major clonal lineages associated to CC17: ST17, ST80 and ST494, and to a minor clone, ST117. Moreover, vancomycin resistance was almost exclusively associated to *vanA* gene on Tn*1546*-type transposons. Intra- and interhospital dissemination of VRE, namely ST17 and endemicity of this lineage in some Portuguese hospitals was additionally supported by core genome MLST (cgMLST) data (118,119).

VRE have been also described in asymptomatic colonization in healthy Portuguese individuals without hospital and/or antibiotic exposure (120) as well as in individuals with risk factors such as people living in long-term care facilities or patients undergoing hemodialysis (121,122) In some of these cases, VRE from carriage belong to the same VRE clonal lineages prevalent in hospitals, as CC17(121,122).

In addition, VRE from pets, wild animals, food-producing animals, rivers and wastewater treatment facilities have all been reported in Portugal (102,123–125). Although most of the VRE identified were asymptomatically colonizing animals, cases of urinary tract infections caused by VRE among companion animals, cats and dogs, belonging to the hospital associated CC17, were also identified in Portugal (126), which highlight the versatility of this lineage to colonize and/or infect different hosts.

1.8 Aim of study

Considering the significant burden of VRE worldwide, and the increasing trend in VRE invasive infections recently reported in United States and European hospitals, the development of global and effective infection control guidelines is warranted. Knowledge on possible VRE reservoirs that represent a source of transmission and spread of this pathogen, will be a first step on VRE management to avoid their dissemination.

In order to contribute to this effort, and to fill the gap on the knowledge of major VRE reservoirs in Portugal, we will follow an "One Health" approach. Different settings will be screened, mainly livestock, human and environmental samples that were previously identified as major reservoirs of other multidrug resistant pathogens, such as methicillin resistant *Staphylococcus aureus*.

In detail, the main objectives were:

i. To identify major VRE in non-clinical settings, such as livestock (pigs and cattle) and environmental samples including public buses, passengers' hands and public environment surrounding hospitals;
ii. To characterize the population structure, resistome and virulome of VRE isolated from non-clinical settings;
iii. To characterize the phylogenetic relationships between *E. faecium* isolates recovered from animals and the environment in this study and identify possible epidemiological links to *E. faecium* infection isolates recovered in Portuguese hospitals between 2019-2020.

The aim is to obtain a picture of the major VRE reservoirs among livestock and environment in Portugal, assessing its significance on the maintenance of hospital associated VRE clonal lineages and/or antibiotic resistance and virulence determinants in nature. This study will provide a valuable information on the guidance of future effective protocols for the management of multidrug resistant pathogens.

2. Material and Methods

2.1 Bacterial collection

For the elaboration of this master thesis, a total of 294 samples from livestock and environmental sources, recovered in previous surveillance studies performed at the laboratory of Molecular Genetics - Microbiology of Human Pathogens Unit at Instituto de Tecnologia Química e Biológica António Xavier (ITQB)/ Universidade Nova de Lisboa (UNL), and previously identified as reservoirs of other multidrug-resistant pathogen (127–130), were selected. All original screening samples were stored on Tryptic Soy Broth (TSB) with 15% glycerol at -80 ºC, at the Laboratory of Molecular Genetics culture collection, and available for the study.

Samples selection criteria included the highest variability and representativeness in terms of dates of collection, source and geographic location and previous identification of methicillin- resistant *Staphylococcus aureus* (MRSA). In cases of a low number of samples in a specific source, the entire collection was screened.

Livestock samples included:

- 80 samples from healthy swine living in farms
- 55 samples from a beef production farm

Environmental samples included:

- 89 samples from public buses
- 18 samples from hands of passengers that have been traveling by public bus
- 52 samples from outdoor urban environment around major hospitals in Lisbon

2.1.1 Livestock samples

2.1.1.1 Healthy pigs from swine farms

A total of 80 out of 215 samples previously recovered from healthy pigs as part of a livestock MRSA surveillance project (127,128), were selected for VRE screening.

Samples were collected with a sterile cotton swab from the nasal cavity of healthy piglets aged 10-11 weeks from two independent Portuguese pig farms located in Alentejo region, in two collection periods: 2016 and 2018. Pigs were born in these farms and live in groups of 32 to 33 animals in 8-12 m^2 stockyards to be further delivered to slaughterhouses for human consumption.

These farms used amoxicillin (0.5%), colistin (0.5%), and zinc oxide (0.15%) in the feed regimen of all animals until 2016, but since then, one farm banned colistin usage, maintaining amoxicillin (0.5%) and zinc oxide (0.15%), while the other farm completely eliminated antibiotics, keeping zinc oxide (0.15%) only to prevent gastrointestinal diseases.

At time of sampling, the main purpose was the identification of MRSA carriage, and therefore all samples were collected from the pigs' anterior nares (128). Although VRE are not common inhabitants of the nasal microbiota, the close living conditions of the animals in the stockyards, made the samples to be considered potential reservoirs for VRE, and therefore selected for this study.

2.1.1.2 Healthy bovines from farm

A collection of 55 samples (36 nasal samples and 19 from the inguinal region), previously recovered from healthy bovines for *Staphylococcus aureus* carriage assessment (130), were screened for VRE.

Samples were collected in January 2016, in a beef production herd in the south of Portugal by swabbing both nares of each animal with a sterile cotton swab and stored in Stuart transport medium, or the udder skin of each animal with a sterile cotton gauze humidified with sterile water and introduced in tubes with Tryptic Soy Broth (TSB) until being processed at the laboratory.

At the time of sampling, none of the animals presented apparent clinical symptoms of infections, or was reported as infected by the farm veterinary, and only two cows have been treated with antibiotics.

The inclusion of samples in this study was based in the fact that the inguinal region, given its location in the animal became easily contaminated with fecal material and therefore potentially contaminated with VRE. Since part of the nasal swabs were collected from the same animals as the inguinal samples, all nasal samples were also included for screening.

2.1.2 Environmental samples

2.1.2.1 Public buses

Eighty-nine samples selected from a collection of 199 samples previously recovered from public buses in Lisbon for MRSA surveillance (129), were included for VRE screening.

Sampling was performed between May 2011 and May 2012, at the end of the day and before any cleaning procedure in the vehicles, using sterile cotton gauzes humidified with TSB. After sampling, the gauzes were placed into 100 ml TSB bottles and transported to the laboratory for incubation. Surfaces with high levels of hand contact (handrails, seat rails, handgrips, stop buttons and the surface in both sides of a bus) were selected for sampling. Buses were randomly sampled, including buses with different routes, some of them serving hospitals.

Although the purpose of the screening was the identification of MRSA contamination in public buses, given the ubiquitous nature of the *Enterococcus*, and the high probability of health care workers, patients and hospital visitors leave the health care institutions and travel by public bus, these surfaces may also constitute a potential reservoir for VRE and were considered for this study.

2.1.2.2 Hands of public buses' passengers

As part of the MRSA contamination surveillance in public buses referred in section 2.1.2.1, individuals who travelled by bus in Lisbon were screened for MRSA hand's contamination, and eighteen samples previously identified as positive for MRSA contamination were selected for VRE screening.

Samples were collected in two periods: in May 2012 for 2 days and in January 2013 for 3 consecutive days. Both hands of each individual were sampled after the bus ride, and before washing their hands or touch other surfaces. Sampling was performed with a sterile cotton gauze moistened with sterile water and placed into TSB tubes and transported to the laboratory.

Hands of passengers travelling by bus could become contaminated with microbial pathogens, as MRSA, by touching surfaces during the bus trip. Therefore, these hands' samples were also considered for this study as potentially contaminated by VRE.

2.1.2.3 Outdoor urban environment

All of 52 environmental samples recovered in June 2013 to assess the probability of the outdoor urban environment around major hospitals in Lisbon, be contaminated with MRSA, were also included for VRE screening.

These samples were collected, by swabbing the surfaces of parking meters, stairwells, automated teller machines, bus stops, public phones and treadmills stops, with sterile cotton gauzes humidified with sterile water and further placed into TSB tubes to be transported to the laboratory.

Since the samples were recovered near hospitals in surfaces that can be touched by health care workers, outpatients and hospital visitors, and some of them were found to be contaminated with MRSA, these surfaces could also be considered as possible VRE reservoirs and therefore included in this study.

2.2 Samples screening and storage of isolates

An aliquot of 100 µl of each sample (stored at -80 ºC) was added to brain heart infusion (BHI) tubes supplemented with 6 µg/mL of vancomycin (to select for resistant microorganisms) and incubated at 37 ºC with agitation at 180 rpm, overnight (Incubator shaker Innova^{TM} 4300). Clinical *E. faecium* isolates available in the lab, with confirmed vancomycin MIC of >256 µg/mL (VRE33) and < 0.75 µg/mL (Efm25) were used as positive and negative controls, respectively. In the following day, test tubes were inspected for bacterial growth, indicated by the opaque appearance of the medium. Aliquots of 100 µl of bacterial growth were then plated in parallel in non-selective agar plates (Tryptic Soy Agar, TSA) and in chromogenic selective media for *Enterococcus* spp. (Compass *Enterococcus* agar; BIOKAR Diagnosis, Beauvais, France) and for vancomycin resistant enterococci (CHROMagar VRE; ChromAgar, Paris, France) (Annex 1, Table 1), and incubated at 35 ± 2 ºC for 24 hours.

Plates were visually inspected and colonies with distinct morphologies (different sizes, shapes or colors) were streaked on BHI agar until a pure culture was obtained. Pure bacterial cultures were conserved in tubes with 1 mL of Tryptic Soy Broth (TSB) with 15% glycerol, quickly froze in liquid nitrogen (LN_2) and stored at -80 ºC.

2.3 Molecular characterization of isolates

2.3.1 Total DNA extraction

Total DNA was extracted by heat lysis method, from bacterial cultures previously plated on BHI agar and incubated overnight at 35 ± 2 ºC. Three up to four colonies of each isolate were resuspended with a white loop (1 µL) in 20 µL of TE buffer 1X (Annex 2, Table 2) and 2 µL of lysozyme [20 mg/mL] (Sigma-Aldrich Co., St. Louis, USA) (Annex 3, Table 3). These suspensions were incubated at 37ºC for 60 minutes followed by 15 minutes at 95ºC, for cell lysis and enzyme denaturation, respectively. After incubation, it was added 180 µL MilliQ sterile water to each suspension and centrifuged

at 13.000 rpm for 5 minutes (Biofugue *pico*, Heraeus Instruments, Germany). The supernatants (~170 µL) were transferred to a new eppendorf and stored at -20 ºC. A dilution of 1:10 of each DNA suspension was performed to be used in Polymerase Chain Reactions (PCR).

The effectiveness of the DNA extraction was verified by PCR amplification of internal fragment of native 16S rDNA gene in each isolate, as previously described (131). The PCR mixture was prepared in a final volume of 50 µL for each sample: 25.75 µL of sterile MilliQ water, 10 µL of 5X Green GoTaq® Flexi Buffer (Promega, Madison, Wisconsin, USA), 4 µL of dNTPs mixture [2 mM of each dNTP] (Bioron, Bonsai Technologies, Römerberg, Germany), 3 µL of $MgCl_2$ [25 mM] (Promega, Madison, Wisconsin, USA), 1 µL of each primer [20 ρmol/µL] (Annex 4, Table 4), 0.25 µL of GoTaq® G2 Flexi DNA polymerase enzyme [5U/µL] (Promega, Madison, Wisconsin, USA) and 5 µL of total DNA (dilution 1:10). The amplification reaction was carried out in a PCR apparatus (MiniAmp™ Thermal Cycler, ThermoFisher Scientific) with the following conditions: initial denaturation at 94 ºC for 5 minutes, 30 amplification cycles (denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds and extension at 72 ºC for one minute), final extension at 72 ºC for 10 minutes, and kept at 16 ºC until storage at 4 ºC. *E. faecalis* ATCC 29212 was used as positive control.

PCR products (10 µL) were run in 1% agarose gel (Seakem LE, Lonza, Rockland, ME, USA) in TAE 1X buffer (Annex 2, Table 2) with 0.025 µL/mL of GreenSafe (NZYTech - Genes & Enzymes, Lisbon, Portugal), at 4 V/cm for 60 minutes. The GeneRuler 1 kb Plus DNA Ladder with 6X TriTrack DNA Loading Dye (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used as molecular weight marker. The PCR products were visualized under UV light in a Gel Doc XR apparatus (Bio-Rad Laboratories, Hercules, California, USA).

2.3.2 *Enterococcus* spp. speciation

Enterococcus species were identified by PCR amplification of internal fragments of the species specific *ddl* gene, which encodes a D-alanine-D-alanine ligase, for *E. faecium* and *E. faecalis*, and based on the *sodA* gene, a superoxide dismutase, for *E.*

casseliflavus, *E. gallinarum*, *E. raffinosus*, *E. hirae* and *E. durans*, as previously described (132,133). Four master mixes with species specific primer sets were prepared (Annex 4, Table 4): Multiplex PCR 1, including primers for the identification of *E. faecium* and *E. faecalis*; Multiplex PCR 2, including primers for the identification of *E. casseliflavus* and *E. gallinarum*; Multiplex PCR 3, including primers for the identification of *E. raffinosus* and *E. hirae*; and an uniplex PCR for the identification of *E. durans*.

For multiplex PCR 1, master mix included 25.75 µL of sterile MilliQ water, 10 µL of 5X Green GoTaq® Flexi Buffer, 4 µL of dNTPs mixture [2 mM of each dNTP], 3 µL of $MgCl_2$ [25 mM], 0.5 µL of each primer (Efaecalis_E1, Efaecalis_E2, Efaecium_F1 and Efaecium_F2) [20 ρmol/µL] (Annex 4, Table 4), 0.25 µL of GoTaq® G2 Flexi DNA polymerase enzyme [5U/µL] and 5 µL of total DNA (dilution 1:10). The amplification reaction conditions were as follows: initial denaturation at 95 ºC for 5 minutes, 30 amplification cycles (denaturation at 95 ºC for 30 seconds, annealing at 50 ºC for one minute and extension at 72 ºC for one minute), final extension at 72 ºC for 10 minutes, and kept at 16 ºC until storage at 4 ºC. ATCC 29212 as used as positive control for *E. faecalis* and ATCC 6057 for *E. faecium*. PCR products (10 µL) were run in a 1.5% agarose gel with TAE 1X buffer and with 0.025 µL/mL of GreenSafe, at 4 V/cm for 60 minutes. GeneRuler 1 kb Plus DNA Ladder with 6X TriTrack DNA Loading Dye was used as molecular weight marker. PCR products were visualized under UV light in a Gel Doc XR apparatus.

For multiplex PCR 2 and 3 and for the uniplex PCR for *E. durans*, the master mix was prepared in a final volume of 25 µL with 11.625 µL of sterile MilliQ water, 2.5 µL of 10X Reaction buffer (NZYTech - Genes & Enzymes, Lisbon, Portugal), 1.25 µL of $MgCL_2$ [50 mM] (NZYTech - Genes & Enzymes, Lisbon Portugal), 5 µL of dNTPs mixture [2 mM of each dNTP], and 0.5 µL of each primer [20 ρmol/µL] (except for *E. gallinarum*, for which it was added 0.75 µL of each primer), 0.125 µL of NZY taq II DNA polymerase [5U/µL] and 2.5 µL of total DNA (1:10). The amplification reaction was as follows: initial denaturation at 95 ºC for 4 minutes, 30 amplification cycles (denaturation at 95 ºC for 30 seconds, annealing at 55 ºC for one minute and extension at 72 ºC for one minute), final extension at 72 ºC for 7 minutes, and kept at 16 ºC until storage at 4 ºC. As positive controls it was included: *E. casseliflavus* ATCC 25788, *E. gallinarum* ATCC 49673, *E. raffinosus* ATCC 49427, *E. hirae* ATCC 8043 and *E. durans*

ATCC 19432. PCR products were run in 2.5% agarose gel in TAE 1X buffer with 0.025 µL/mL of GreenSafe, at 4 V/cm for 60 minutes, but the visualization conditions, were the same as the conditions described above for the *Enterococcus* speciation Multiplex PCR 1 (section 2.3.2 *Enterococcus* speciation).

2.3.3 Vancomycin resistance genes detection

2.3.3.1 *vanA* and *vanB* genes

The presence of *vanA* and *vanB* genes, that are responsible for resistance to vancomycin, was detected by multiplex PCR in all isolates, as previously described (132,134). The PCR mixture was prepared for a final volume of 50 µL for each sample: 24.75 µL of sterile MilliQ water, 10 µL of 5X Green GoTaq® Flexi Buffer, 4 µL of dNTPs mixture [2 mM of each dNTP], 3 µL of $MgCl_2$ [25 mM], 0.75 µL of each primer [20 ρmol/µL] (Annex 4, Table 4), 0.25 µL of GoTaq® G2 Flexi DNA polymerase enzyme and 5 µL of total DNA (1:10). The amplification reaction conditions were as follows: initial denaturation at 94 ºC for 2 minutes, 30 amplification cycles (denaturation at 94 ºC for 30 seconds, annealing at 54 ºC for one minute and extension at 72 ºC for one minute), final extension at 72 ºC for 10 minutes, and kept at 16 ºC until storage at 4 ºC. *E. faecium* clinical strains available in the lab, VRE3 and VRE2, were used as positive controls for *vanA* and *vanB* genes, respectively. PCR products gel electrophoresis run and visualization conditions were the same as described above for the *Enterococcus* speciation Multiplex PCR 1 (section 2.3.2 *Enterococcus* speciation).

2.3.3.2 *vanC1* and *vanC2/3* genes

Screening of *vanC1* or *vanC2/3* resistance genes was performed in all *vanA* or *vanB* negative isolates, by multiplex PCR as previously described (132). The PCR mix, the program settings and the visualization of the amplification products were the same as the conditions described above for the *vanA*/ *vanB* genes screening, except for the primers which in this case only 0.5 µL of each primer [20 ρmol/µL] was used instead of 0.75 µL

(Annex 4, Table 4). DSMZ 20628 and ATCC 49996 were used as positive controls for *vanC1* and *vanC2/3*, respectively.

Given the clinical significance of *E. faecium* and *E. faecalis* in both humans and animals, as described in Introduction section, additional detailed characterization of isolates and resistance mechanisms will be focused on *E. faecium* and *E. faecalis* harboring *vanC* genes isolates.

2.3.4 Linezolid resistance genes detection

In order to assess the presence of linezolid resistance genes on *E. faecium* and *E. faecalis* carrying *vanC* genes, it was performed a multiplex PCR for *cfr*, *optrA* and *poxtA* genes (135), in a final volume of 50 µL for each sample: 23.25 µL of sterile MilliQ water, 10 µL of 5X Green GoTaq® Flexi Buffer, 4 µL of dNTPs mixture [2 mM of each dNTP], 3 µL of $MgCl_2$ [25 mM], 0.75 µL of each primer [20 ρmol/µL] (Annex 4, Table 4), 0.25 µL of GoTaq® G2 Flexi DNA polymerase enzyme and 5 µL of total DNA (1:10). The amplification reaction conditions were initial denaturation at 96 ºC for 2 minutes, 30 amplification cycles (denaturation at 96 ºC, annealing at 50 ºC and extension at 72 ºC, each for 30 seconds), final extension at 72 ºC for 5 minutes. PCR products gel electrophoresis run and visualization conditions were the same as described above for the *Enterococcus* speciation Multiplex PCR 1 (section 2.3.2 *Enterococcus* speciation). Clinical strains available in the lab were used as positive controls, *E. faecium* CNR 16-424 for *optrA* and *Staphylococcus epidermidis* LNZD9 for *cfrA*.

2.3.5 Screening of virulence determinants

To understand the pathogenic potential of the *E. faecium* and *E. faecalis* isolates harboring *vanC* recovered in this study, virulence determinants commonly associated to *Enterococcus* capacity of adherence, colonization and invasiveness were screened by PCR. Two duplex PCRs were carried out to detect virulence genes *asa1* and *gelE,* and

esp and *hyl;* and uniplex PCR reactions for *cylA, ace* genes and insertion sequence 16 (IS16), as described (136,137). Duplex PCRs mixture was prepared in a volume of 50 µL, as follows: 24.75 µL of sterile MilliQ water, 10 µL of 5X Green GoTaq® Flexi Buffer, 4 µL of dNTPs mixture [2 mM of each dNTP], 3 µL of MgCl2 [25 mM], 0.75 µL of each primer (Annex 4, Table 4), 0.25 µL of GoTaq® G2 Flexi DNA polymerase enzyme and 5 µL of total DNA (1:10). For the uniplex PCR, mixture conditions were the same as duplex PCRs, except for sterile MilliQ water that was adjusted to 25.75 µL, and primers volume adjusted to 1 µL (of each primer) (Annex 4, Table 4).

The amplification conditions were described: denaturation at 95 ºC for 10 minutes, 30 amplification cycles (94 ºC for 30 seconds, annealing at 56 ºC for 45 seconds and extension at 72 ºC for one minute), final extension at 72 ºC for 10 minutes. Except for *ace* gene and IS16, in which the annealing temperatures were adjusted to 58 ºC and 53 ºC, respectively. PCR products (10 µL) were run in a 1% agarose gel for uniplex and 2% agarose gel for multiplex, in TAE 1X buffer with 0.025 µL/mL of GreenSafe, at 4 V/cm for 60 minutes. *E. faecium* ATCC29212 was used as positive control of *asa1*, *gelE, cytA* and *ace*, and a clinical strain available in the lab were used as positive control, Efm16 for *hyl* and *esp*.

2.4 Phenotypic characterization of isolates

2.4.1 Antibiotic susceptibility testing

All *E. faecium* and *E. faecalis* isolates harboring *vanC* genes, were tested for antimicrobial susceptibility trough disk diffusion method (Kirby-Bauer) for 16 antibiotics (Oxoid™, Thermo Fisher Scientific, Basingstoke, United Kingdom): ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (30 µg), levofloxacin (5 µg), linezolid (10 µg), linezolid (30 µg), nitrofurantoin (100 µg), quinupristin-dalfopristin (15 µg), teicoplanin (30 µg), tetracycline (15 µg), tigecycline (30 µg), trimethoprim (5 µg), trimethoprim-sulfamethoxazole (25 µg) and vancomycin (30 µg), according to the EUCAST (European Committee on Antimicrobial Susceptibility

Testing) guidelines (2022) (138) or CLSI (Clinical and Laboratory Standards Institute) guidelines (2021) (139) whenever EUCAST breakpoints are not available.

Each inoculum was prepared by suspending three to four bacterial colonies grown in Tryptic Soy Agar (TSA, Becton Dickinson, Sparks, MD, USA) (Annex 1, Table 1), in a sterile saline solution of 0.85% NaCl (Merck KGaA, Darmstadt, Germany) and adjusted to a turbidity of 0.5 McFarland ($1x10^8$ CFU/ml; 0.08 - 1 OD_{620nm}) in a densitometer DEN-1B (Sai Biosan, Riga, Latvia). The bacterial suspensions were inoculated on Mueller-Hinton II agar (MHA, BBLTM, Becton Dickinson, Sparks, MD, USA) with sterile cotton swabs and antibiotic discs were placed on top of the inoculum with a manual dispenser (six antibiotic discs per plate). After incubation at 35º C ± 2º C for 16 to 18 hours, the halo formed around the discs was measured (in mm) for each antibiotic and the isolates were considered susceptible (S), intermediate (I) or resistant (R) according to EUCAST and CLSI guidelines as indicated above. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality control as indicated in EUCAST and CLSI, respectively.

2.4.2 Determination of minimal inhibitory concentration (MIC) of glycopeptides and linezolid

Minimal inhibitory concentration (MIC) was determined by epsilon test (E-test) (BioMérieux, Marcy-l'Étoile, France) according to manufacturer instructions, for vancomycin and teicoplanin in all isolates (*E. faecium* and *E. faecalis* harboring *vanC*) and for linezolid in isolates previously classified as resistant by disc diffusion. Briefly, bacterial suspensions on 0.85% NaCl adjusted to 0.5 McFarland were inoculated on Mueller-Hinton II agar with sterile cotton swabs. Then, a strip impregnated with gradient antibiotic concentrations was plated on top of the agar with the help of a tweezer and incubated at 35 ºC ± 2 ºC for 24 hours. After incubation, plates were inspected and the antibiotic concentration in the strip at the intersected point between the point end of the inhibition ellipse and the side of the strip, was recorded as the MIC. According to the EUCAST guidelines (2022) (138), vancomycin and linezolid resistance breakpoints were

defined as: a MIC ≤ 4 µg/ml was considered susceptible, and a MIC > 4 µg/ml was considered resistant. For teicoplanin, a MIC ≤ 2 µg/ml was considered susceptible and MIC > 2 µg/ml was considered resistant. *E. faecalis* ATCC 29212 was used as quality control.

2.4.3 Detection of High-Level Aminoglycoside Resistance (HLAR)

The detection of high-level aminoglycoside resistance was performed by Agar dilution method, according to the CLSI guidelines (139), on BHI agar plates supplemented with gentamicin (500 µg/mL) or streptomycin (2000 µg/mL). In brief, 10 µL of a 0.5 McFarland bacterial suspension in 0.85% NaCl was spotted onto agar surface of antibiotic supplemented plates, which were incubated at 35 ºC ± 2 ºC for 24 hours. If no growth was observed after 24 hours, the plates were re-incubated for an additional 24 hours. In case of >1 colony grow in a spotted area, the isolate was considered as resistant, HLAR. *E. faecalis* ATCC 51299 was used as positive control for HLAR (both gentamicin and streptomycin) and *E. faecalis* ATCC 29212 as a negative control.

2.5 Whole-genome sequencing (WGS)

2.5.1 Total DNA extraction for WGS

A total of 23 *E. faecium* isolates were selected for whole genome sequencing (WGS), based on antimicrobial resistance profile and including a wide variability of resistant patterns. Initially, bacterial isolates were cultivated overnight in Tryptic Soy Broth (TSB) at 35°C ± 2º C with agitation (Incubator shaker Innova™ 4300), and total DNA was extracted using the DNeasy Blood and Tissue kit according to the manufacturer protocol (Qiagen, Hilden, Germany). DNA purity was verified by the A260/A280 and A260/A230 ratios assessment using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Portugal). Further DNA quantification was performed in the Qubit apparatus (Qubit® 2.0 Fluorometer, Invitrogen, Thermo Fisher Scientific, USA) with Qubit double

stranded DNA (dsDNA) high-sensitivity (HS) assay kit (Invitrogen/Thermo Fisher Scientific, Karlsruhe, Germany). DNA integrity was verified on agarose gel electrophoresis.

A volume of 10 µl (10 ng/µl) of total DNA was sent for sequencing in an outsourced facility, according to sequencing facility requirements. Genomic libraries were created using the Nextera XT DNA sample preparation kit (Illumina, Little Chesterford, UK) followed by a 150 bp paired-end sequencing with an estimated coverage of 100X.

2.5.2 Resistance, virulence, and plasmid content analysis

Raw data were assembled using the INNUca v3.1 pipeline (140) available at GitHub (https://github.com/B-UMMI/INNUca), which allow reads quality analysis and *de novo* genome assembly. Briefly, the fastq integrity was checked and the expected coverage calculated based on the expected genome size as input. True coverage was then assessed via Bowtie2 v2.2.9 alignment mode. The reads quality was initially analyzed and reported using FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), followed by Trimmomatic v0.38 (141) for trimming and quality improvement. Genome assembly was performed by *de novo* approach with SPAdes v3.11.0 (142) and then refined using Pilon v1.23 (143). Finally, the MLST 2 (https://github.com/tseemann/mlst) was used to predict *in situ* multilocus sequence type (MLST). Novel identified gene allele sequences or MLST profiles were submitted to PubMLST website (http://pubmlst.org) for new allele or sequence types (ST) assignments, respectively.

After contigs assembling, genome data was screened for antimicrobial resistance, virulence and plasmid detection, following two different approaches. In the first approach genome data analysis was performed using the Center for Genomic Epidemiology (CGE) resources freely available at http://www.genomicepidemiology.org. Genome contigs were interrogated for the presence of antimicrobial resistance genes or mutations responsible for resistant phenotypes through Resfinder 4.1 and Linezolid Resistant Enterococci Finder (LRE-finder) 1.0 databases. Virulence determinants were identified through VirulenceFinder 2.0. In order to complete data analysis, a homemade database

with additional two alleles of *asa1* virulence factor and an insertion sequence (IS16), important in *Enterococcus* spp., was used trough the MyDbFinder 2.0 tool also available at CGE. Plasmids identification was performed trough the detection of the replicon (*rep*) genes associated to each Gram-positive plasmid family (Inc18, Rep1, Rep3, Rep_Trans, RepA_N) through PlasmidFinder 2.1 database. Screening was performed by blasting genes present in the database against the genome contigs obtained for each isolate. All gene searches were performed using the identity and coverage default parameters (identity (ID) threshold of 90% and minimum coverage length of 60%), with the exception of PlasmidFinder that has a defined default ID threshold of 95%.

In the second approach, data analysis was performed trough a command line-based program. ABRicate (https://github.com/tseemann/abricate), which perform a mass screening of genome contigs, was used to assess antimicrobial resistance, virulence genes and plasmids, in all the isolates trough a batch mode. The screening was performed using the following databases and settings: ResFinder database (144) the Comprehensive Antibiotic Resistance Database (CARD) platform (145), and National Center for Biotechnology Information (NCBI AMRFinderPlus), for antimicrobial resistance, while virulence determinants detection was performed with Virulence Factor Database (VFDB). Finally, the screening for plasmids was performed using PlasmidFinder. The parameters used in all screenings were defined as minimum identity of 90% and minimum coverage of 60%.

2.5.3 Clonal population analysis

Clonal population structure and prevalent clonal lineages were determined based on MLST assessed trough INNUca pipeline, and on the core genome MLST (cgMLST). cgMLST allele calling was performed using chewBBACA v2.5 pipeline available at GitHub (https://github.com/B-UMMI/chewBBACA), based on a previously established cgMLST scheme for *E. faecium* (146), which includes a total of 1423 loci. An allele number is attributed to each locus to define the corresponding cgMLST profile. For the visualization of relationships between isolates, a minimum spanning tree was constructed on Phyloviz 2.0 (https://online2.phyloviz.net/index), where metadate was further

associated. Isolates with ≤ 20 cgMLST allele differences were considered clonally related and belonging to the same clonal type (CT) (146).

Similar cgMLST analysis was performed to define clonal identity and integration of the livestock and environmental *E. faecium* isolates identified in this study, into the *E. faecium* population causing infection in Portuguese hospitals. A collection of 132 *E. faecium* genomes available at Laboratory of Molecular Genetics (ITQB-NOVA) (118,119) were included for analysis and comparison. All infection isolates were recovered in eight Portuguese hospitals geographically located in the Lisbon and Oporto regions, between 2019 and 2021 (118,119).

2.6 Statistical analysis

Nominal categorical variables as species prevalence, resistance patterns and sample collections, were compared using the Fisher's exact test, with *P* values < 0.05 being considered statistically significant. All statistical analyses were performed using GraphPad Prism software version 9.4.

3. Results and discussion

3.1 *Enterococcus* spp. isolation and species diversity

A total of 470 presumptive *Enterococcus* spp. were isolated from 294 samples (135 samples from livestock (pigs, n= 80; cows n= 55) and 159 samples from the environment (public buses, n= 89; passenger hands, n= 18; urban environment, n= 52)). *Enterococcus* spp. were identified in all samples, except in 28 environmental samples (specifically in 20 samples from the urban environment, seven from the hands of passengers and one from the buses collection). Different enterococci prevalence rates were detected in each collection, with the higher rates identified among pigs and buses (31%, each), followed by cows (26%), urban environment (9%) and hands of bus passengers (3%).

Between one and four *Enterococcus* species were identified per sample, with higher variability detected among bus' samples. The distribution and diversity of *Enterococcus* species identified are displayed in Figure 6. Globally, 82% of the isolates were identified at the species level as *Enterococcus faecalis* (n= 140), *Enterococcus casseliflavus* (n= 95), *Enterococcus gallinarum* (n= 64), *Enterococcus faecium* (n= 59), *Enterococcus hirae* (n= 25) and *Enterococcus durans* (n= 2). None of the isolates were identified as *Enterococcus raffinosus*. The isolates that could not be classified at the species level (n= 85), due to the limited range of species-specific primers available at PCR assays, remained identified at the genus level as *Enterococcus* spp. (Figure 6).

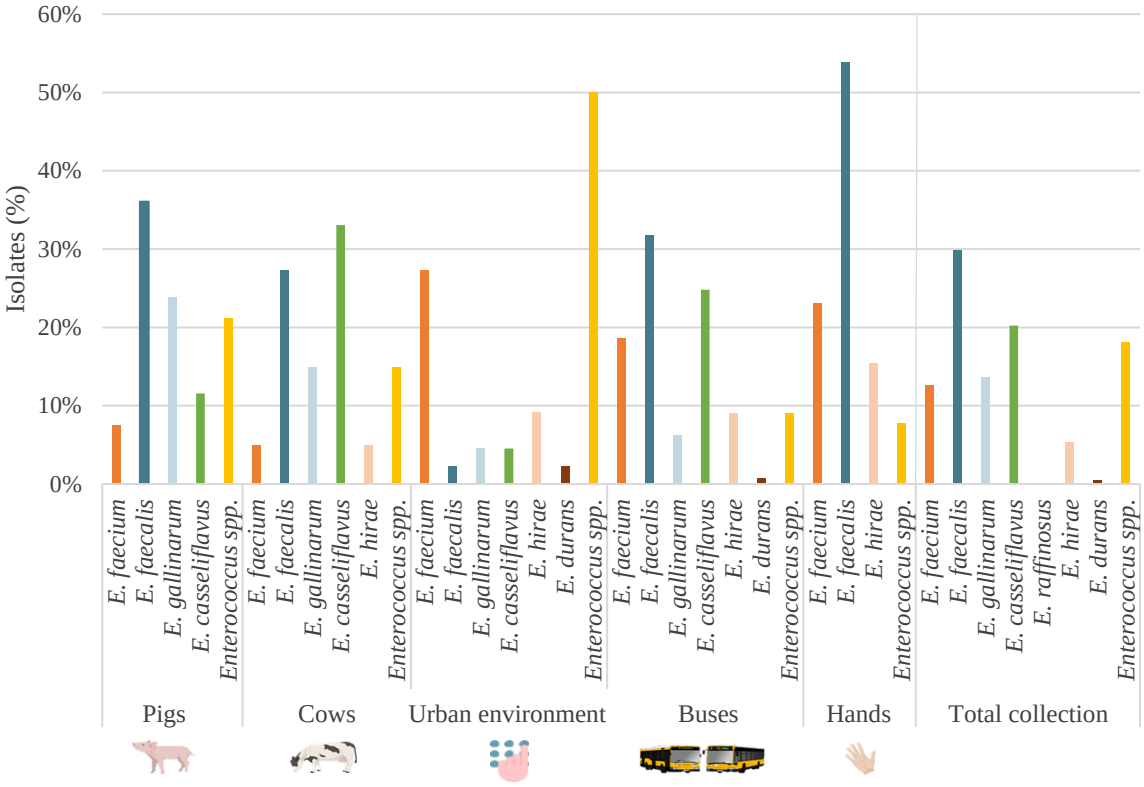


Figure 6. Prevalence of the different *Enterococcus* species in each collection screened and in the total collection.

The distribution and prevalence of *Enterococcus* species could be geographically highly variable and depending on various factors, including genetic variability of bacterial isolates associated to the presence of certain virulence factors, environmental factors or host dynamism (147). In this study, *E. faecalis* and *E. faecium* were the only species identified in all collections, although at different rates depending on the samples source. Globally, *E. faecalis* was the most prevalent species (30%) identified with rates ranging from 54% among the hands of bus passengers, followed by 36% in pigs' samples to the lowest value among urban environment (2%). *E. faecium* was most prevalent among environmental samples (urban environment (27%), hands of bus passengers (23%) and buses (19%)) compared to livestock (pigs (7%) and cows (5%)) ($p < 0.0001$). Our results are in line with a recent One-Health continuum surveillance of *Enterococcus* spp. that

reveal *E. faecium* and *E. faecalis* as the predominant species associated with human associated environments (28).

E. casseliflavus and *E. gallinarum* were mostly found among livestock (41%) (p= 0.0002) which is in agreement to the fact that these species are reported as common colonizers of cattle and swine (20,28,113). *E. casseliflavus* is also associated to vegetation and forage crops (28), which easily come into contact with animals and could explain the higher prevalence of *E. casseliflavus* among livestock, namely in cows.

On the other hand, *E. hirae* was isolated mainly in environment (76%) and at a less extent in healthy cows (24%). Likewise, two unique *E. durans* isolates were identified in the urban environment and in a bus sample.

The most frequent species of *Enterococcus* identified in pigs were *E. faecalis* (36%), *E. gallinarum* (24%) and *E. casseliflavus* (12%), while healthy cows were colonized by *E. faecalis* (27%), *E. casseliflavus* (33%) and *E. gallinarum* (15%). Similar carriage rates were also reported in other countries (148). In Portugal, a recent report identified *E. faecalis* as the most prevalent specie isolated from pigs and to a less extent in cows (149), as in our study. Although *E. faecium* were already reported in a Portuguese pig farm associated to carriage in pigs, humans and dust (150), in our study, *E. faecium* was the less prevalent *Enterococcus* species detected in pigs. Since we performed the screening in nasal swabs, this could be suggested as a possible justification for the small prevalence of *E. faecium* found in our study, even tough, others studies that also used swine nasal swabs were able to identify enterococci carriage in pigs, but with different rates (52). These findings suggest that although the nares are not the common habitat for *Enterococcus* species, nasal sampling might be valuable for enterococci monitoring in animals.

Environmental samples were mostly colonized by enterococci commonly associated to human carriage and infection, or nosocomial environments. Therefore, buses' surfaces were mainly contaminated by *E. faecalis* (32%), *E. casseliflavus* (25%), and by *E. faecium* (19%), which could be transmitted to the passenger hands that contained essentially *E. faecalis* (54%) and *E. faecium* (23%). Similar pathogen transmission evidence was previously identified for MRSA, that was also identified in both bus and passengers' hands, in the same samples included in this study (129). Similar

to our work, a Turkish investigation used bus samples to determine the role of public transport in the transmission of microorganisms, and also identified *Enterococcus* spp., however they were not identified at the species level (151).

Regarding the urban environment near hospitals, the most frequent species isolated were *E. faecium* (27%) and *E. hirae* (9%). The specific locals sampled, as surfaces of parking meters, stairwells, ATMs, bus stops, public phones and treadmills stops, are at risk to be contaminated by hands of individuals that coming from hospitals, as visitors, patients or health care workers. Hands of individuals with a straight contact with the nosocomial environment have a high probability of being contaminated by VRE as previously reported (152), and therefore be a transmission vehicle of these pathogens outside of the hospitals. This highlights the importance of the hands' hygiene measures to prevent VRE transmission.

3.2 Characterization of vancomycin resistance genes

The presence of four of the most common and clinically relevant vancomycin resistance genes (*vanA, vanB, vanC1* and *vanC2/3*), were tested by PCR amplification in the 470 *Enterococcus* spp. isolates. None of the genes *vanA* and *vanB* was detected, but *vanC* genes were amplified in 186 out of 470 (40%) isolates, namely the *vanC1* that was detected in 80 (17%) isolates and *vanC2/3* detected in 106 (23%) (Figure 7).

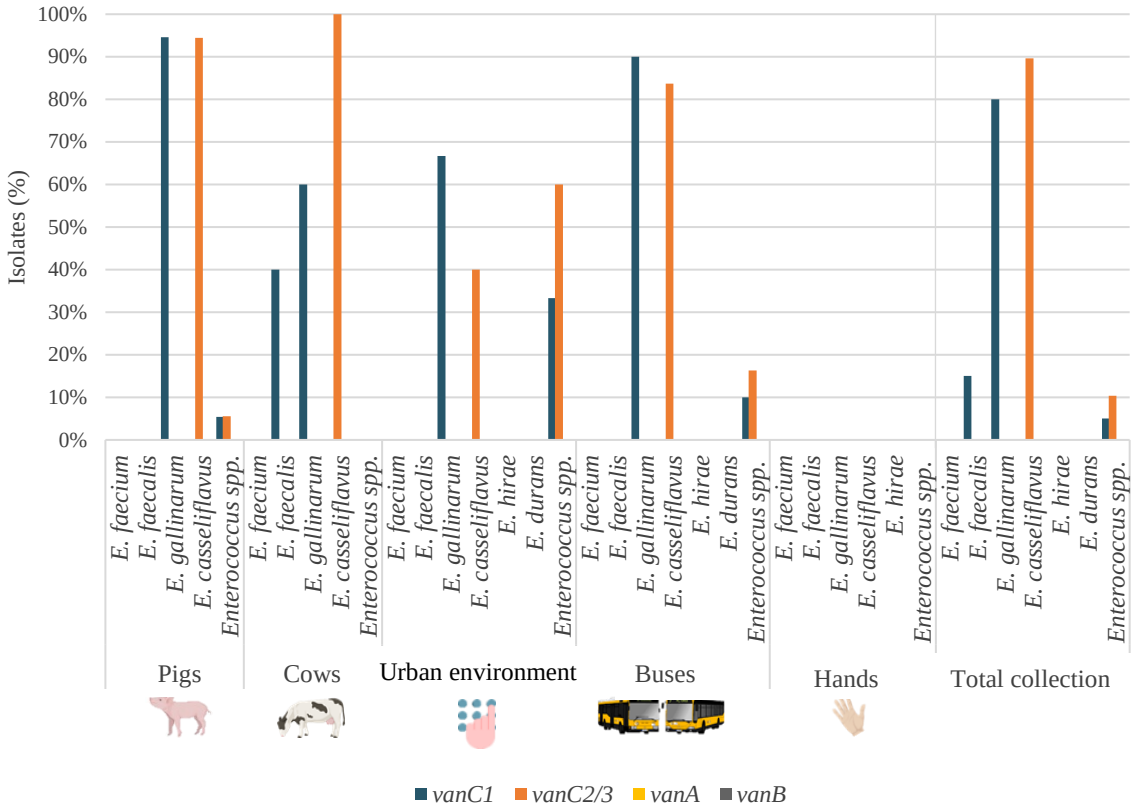


Figure 7. Distribution of *van* genes by *Enterococcus* species and samples collection.

Although none of the *E. faecium* isolates harbored *van* genes, it was identified 12 (40%) *E. faecalis* with *vanC1*, exclusively found among cows' samples (Figure 7).

Moreover, *vanC1* was highly prevalent among *E. gallinarum* in pigs (92%), buses (90%), urban environment (67%) and cows (60%), which was not surprising, since *vanC1* is considered intrinsic to the *E. gallinarum* species (153). Furthermore, *vanC2/3* was exclusively detected among *E. casseliflavus* and *Enterococcus* spp. and seems to be highly associated to *E. casseliflavus* in cows (100%), followed by pigs (94%), buses (84%) and urban environment (40%). However, none of these *van* genes were found in hands samples.

Enterococcus spp. with *vanC1* gene other than *E. gallinarum* have been reported, namely in *E. faecalis* from animal associated environment samples. In Germany, the *vanC1* was detected in two *E. faecalis* isolates susceptible to vancomycin isolated from

pigs manure (154). Another *E. faecalis* isolate from a sheep bulk tank milk in Spain, contained the *vanC1*, but with a vancomycin-resistant phenotype, which were presumably due to horizontal gene transfer between *E. gallinarum* and *E. faecalis* (155). In Japan, it has already been identified *E. faecium* and *E. faecalis* with *vanC2/3* in water samples (156). The detection of *vanC* genes in *Enterococcus* species other than *E. faecalis* and *E. faecium*, was expected, since they are considered to be intrinsic to *E. gallinarum* (*vanC1*), *E. casseliflavus* (*vanC2*) and *E. flavescens* (*vanC3*) (20,75). The *vanC* operon is chromosomally located in regions of transferable elements, highlighting that the chromosomal location of a gene in intrinsically resistant strains does not necessarily avoid the transfer to other species (157).

Although the *vanC* operon remains relatively less abundant among human isolates compared to *vanA* and *vanB* genotypes (75), there has been an increase in reports of *Enterococcus* spp. with *vanC* genes in human colonization. In some Greek hospitals 57% of the *E. gallinarum* isolates, showed *vanC* phenotype (158,159), while a Swiss study identified the *vanC* gene in 98% of the *Enterococcus* isolated from carriage in inpatients, but only one developed bloodstream infection (160). Moreover, an *E. faecium* with *vanC1* isolated from an invasive blood infection was reported in China (161).

3.3 Molecular characterization

As mentioned before in Introduction section, *E. faecalis* and *E. faecium* are considered the most clinically relevant species among the genus *Enterococcus*, being responsible for the overwhelming majority of enterococcal infections in humans, mainly due to their capacity to acquire and disseminate antibiotic resistance and virulence determinants via horizontal gene transfer. Hence, additional characterization of enterococci isolated in the present study will be focused on *E. faecalis* containing *vanC1* gene and particularly in *E. faecium* isolates, which prevalence in human infections have been increasingly reported.

3.3.1 *Enterococcus faecalis* isolates harboring *vanC* genes

3.3.1.1 Antibiotic resistance profile

Antimicrobial resistance was tested for the 12 *E. faecalis* harboring *vanC1*, that were isolated from cows' samples (Annex 5, Table 5). All *E. faecalis* isolates tested were resistant to quinupristin/dalfopristin, while 67% showed resistance to trimethoprim-sulfamethoxazole and 33% to trimethoprim alone. Half of the isolates were resistant to ampicillin and 17% to tigecycline (Figure 8). Erythromycin (100%) and chloramphenicol (8%) were classified with intermediate resistance.

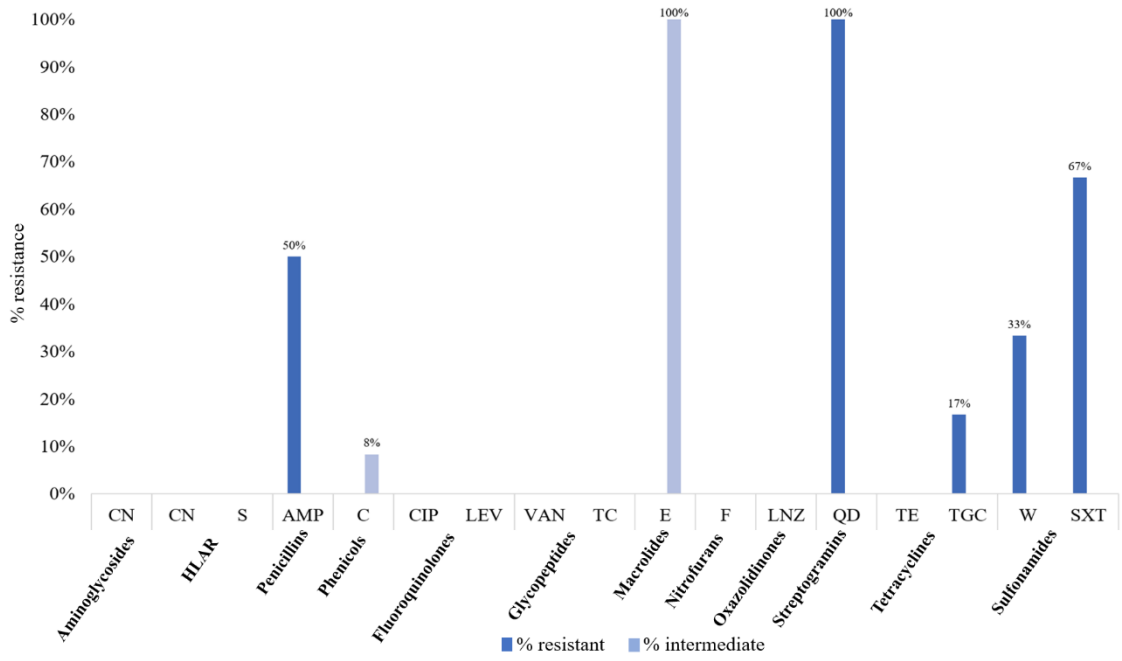


Figure 8. Antibiotic resistance profile of the 12 *E. faecalis* isolates harboring *vanC.*

CN- gentamicin, S- streptomycin, HLAR- high-level aminoglycosides resistance, AMP- ampicillin, C- chloramphenicol, CIP- ciprofloxacin, LEV- levofloxacin, VAN- vancomycin, TC- teicoplanin, E- erythromycin, F- nitrofurantoin, LNZ- linezolid, QD- quinupristin-dalfopristin, TE- tetracycline, TGC- tigecycline, W- trimethoprim, SXT- trimethoprim-sulfamethoxazole.

Although the isolates had the *vanC1* gene, none of them showed phenotypic resistance to vancomycin or teicoplanin, based on disk diffusion method and E-test

(vancomycin MICs VA 2 – 4 µg/mL; teicoplanin MIC 0,064 – 0,5 µg/mL). These isolates were not resistant to linezolid (MIC 2- 3 µg/mL) either, which was also confirmed by non-detection of any of the linezolid resistance genes *cfr*, *optrA* and *poxtA* by PCR.

All *E. faecalis* isolates were susceptible to tetracycline, nitrofurantoin, fluoroquinolones and high-level concentrations of gentamicin and streptomycin (respectively 500 µg/mL and 2000 µg/mL tested by agar dilution).

E. faecalis is considered intrinsically resistant to several antibiotics including aminoglycosides, macrolides, clindamycin, quinupristin-dalfopristin and sulfonamides (162), which in general could be confirmed in our study (except for clindamycin that was not tested). The intermediate resistance to erythromycin observed in all isolates is noteworthy, taking into account that the EUCAST and CLSI breakpoints are designed for clinical purposes.

In a recent study, Makarov et al. identified 12% of resistance to trimethoprim and trimethoprim-sulfamethoxazole in *E. faecalis* isolated from cattle, which is much lower than the rates detected among our isolates (33% and 67%, respectively). Resistance to trimethoprim and sulfonamides can be mediated by the resistance genes *dfr* and *sul*, respectively (163). In addition to these genes, a possible mechanism that may explain the higher resistance observed to trimethoprim-sulfamethoxazole compared to trimethoprim alone, is the horizontal transfer of *folP* gene or parts of it, that can also induce sulfonamide resistance (164,165).

All *E. faecalis* isolates showed phenotypic resistance to quinupristin-dalfopristin, which may be attributable to its intrinsic resistance to the combination of streptogramins given by the expression of the efflux-pump associated to the *lsa* genes (7).

Besides the low prevalence of resistance observed for the most commonly used antibiotics in veterinary medicine, such as β-lactams, tetracyclines, aminoglycosides, macrolides, and sulfonamides (166), half of *E. faecalis* isolates were resistant to ampicillin, which is uncommon (167,168) and of major significance given the widespread use of β-lactams, namely ampicillin, in the treatment of enterococcal infections in humans. Moreover, *E. faecalis* resistance to aminopenicillins is one of the microorganisms-antibiotic resistant combinations under regular surveillance by EARS-

Net (169). Regarding chloramphenicol, although it is not allowed in food producing animals in Portugal, we detected 8% of intermediate resistance in our *E. faecalis*. Moreover, highly variable rates have been reported ranging from 1.9 to 77% in cattle and swine animals, independently of being allowed or not in food producing animals, as in Korea (170,171).

Resistance to tigecycline, one of the last resort antibiotics for the treatment of multidrug-resistant bacterial pathogens, such as VRE and MRSA, is of critical importance to human medicine (172). Although the precise mechanism of how enterococci become resistant to tigecycline remains undetermined, it is known that the increased expression of two tetracycline resistance determinants (*tet(M)* and *tet(L)*) could play a role (63). The identification of 17% of *E. faecalis* with phenotypic resistance to tigecycline among healthy cattle in our study is of major concern and deserves further investigation.

In order to better understand the distribution of the resistance patterns of the *E. faecalis*, resistances were separately plotted according to the sample swabbed site, udder region or anterior nares (Figure 9a and 9b).

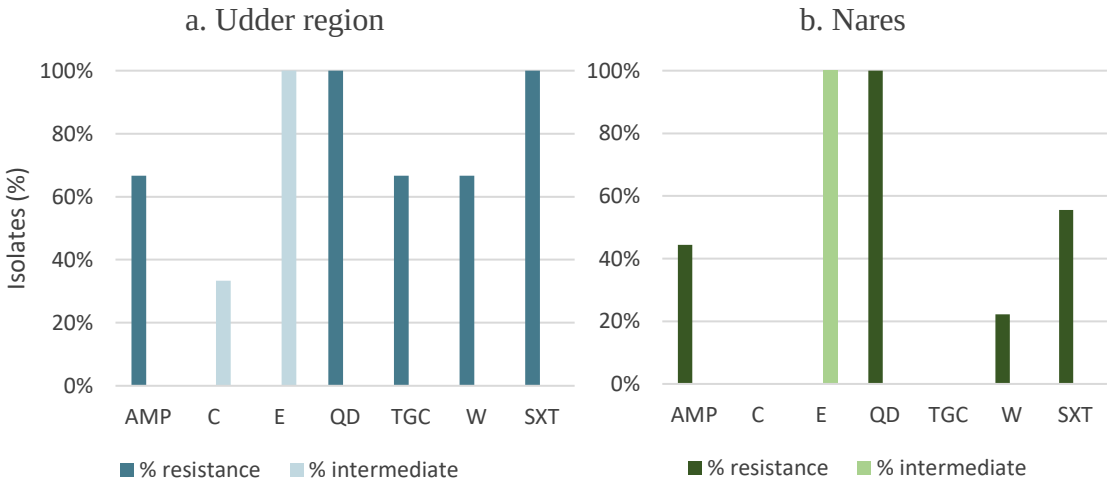


Figure 9. Antibiotic resistance profile of *E. faecalis* harboring *vanC* recovered from a. the udder region and b. from the nares of cows.

AMP- ampicillin, C- chloramphenicol, E- erythromycin, QD- quinupristin-dalfopristin, TGC- tigecycline, W- trimethoprim, SXT- trimethoprim-sulfamethoxazole.

E. faecalis recovered from the udder region presents resistance to more antibiotics, namely to tigecycline and intermediate resistance to chloramphenicol, and higher resistance rates when compared to the nares' isolates. This results strongly suggests that the udder region appears to constitute a greater reservoir of antibiotic resistant isolates and thereafter reservoirs for resistance determinants, probably due to a higher contact of the region with fecal material.

3.3.1.2 Virulence factors

A set of seven virulence determinants including *asa*, *esp*, *ace*, *gelE*, IS16, *hyl* and *cylA* were screened by PCR in the 12 *E. faecalis* isolates and the results are displayed in Figure 10. All *E. faecalis* harbored at least two virulence genes, *ace* and *gelE*. In addition, a single isolate was also positive for *asa* gene.

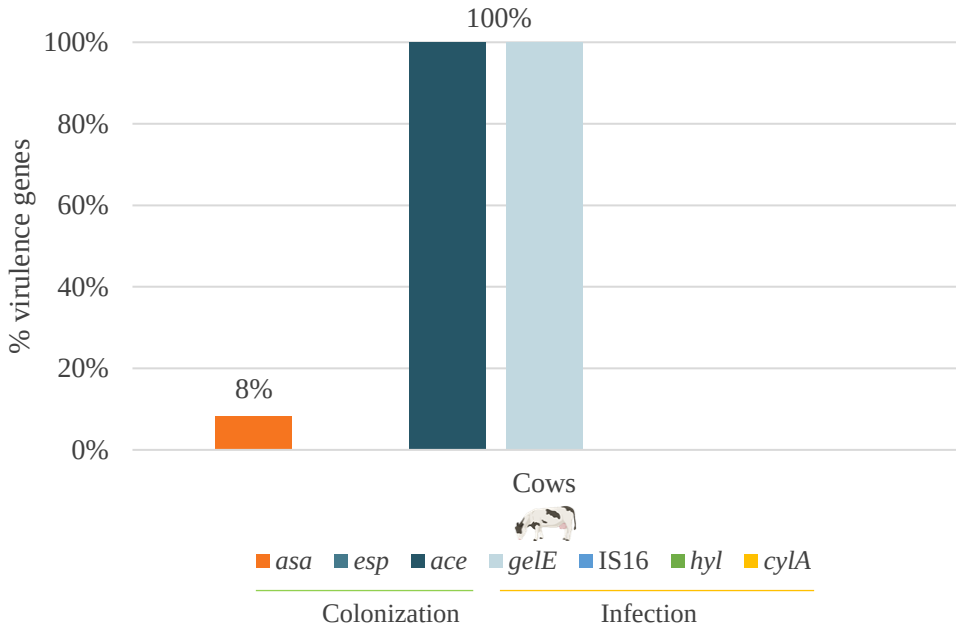


Figure 10. Prevalence of virulence factors in the 12 *E. faecalis* harboring *vanC1*.

Generally, *E. faecalis* is considered more virulent than *E. faecium* (173). These 12 *E. faecalis* in our collection, presented the virulence gene *ace* that promotes

colonization by adhering to host cells. Additionally, was identified the *gelE*, which contributes to biofilm formation, and can damage the hosts tissues promoting bacterial evasion (76). Our data are in agreement with Zou et al., that reported *gelE* as the most prevalent virulence gene in *E. faecalis*, and that *hyl* and *cylA* were not detected (174). The aggregation substance *asa* gene was identified in an *E. faecalis* isolated from the udder region. This virulence factor facilitates adherence of bacterial cells to the epithelium during colonization and mediates the exchange of plasmids that can contain antibiotic and virulence genes (76), which could contribute to the higher resistance rates identified in our study among the *E. faecalis* isolates of this region.

3.3.2 *Enterococcus faecium* isolates

3.3.2.1 Antibiotic resistance

Antimicrobial resistance profile was determined for all 59 *E. faecium* isolates recovered from livestock (pigs n= 11 and cows n=6) and environment (bus n= 27, hands n= 3 and urban environment n=12) (Figure 11) (Annex 5, Table 6). Additionally, antimicrobial resistance determinants were identified in the 23 *E. faecium* isolates selected for WGS (Annex 5, Table 7).

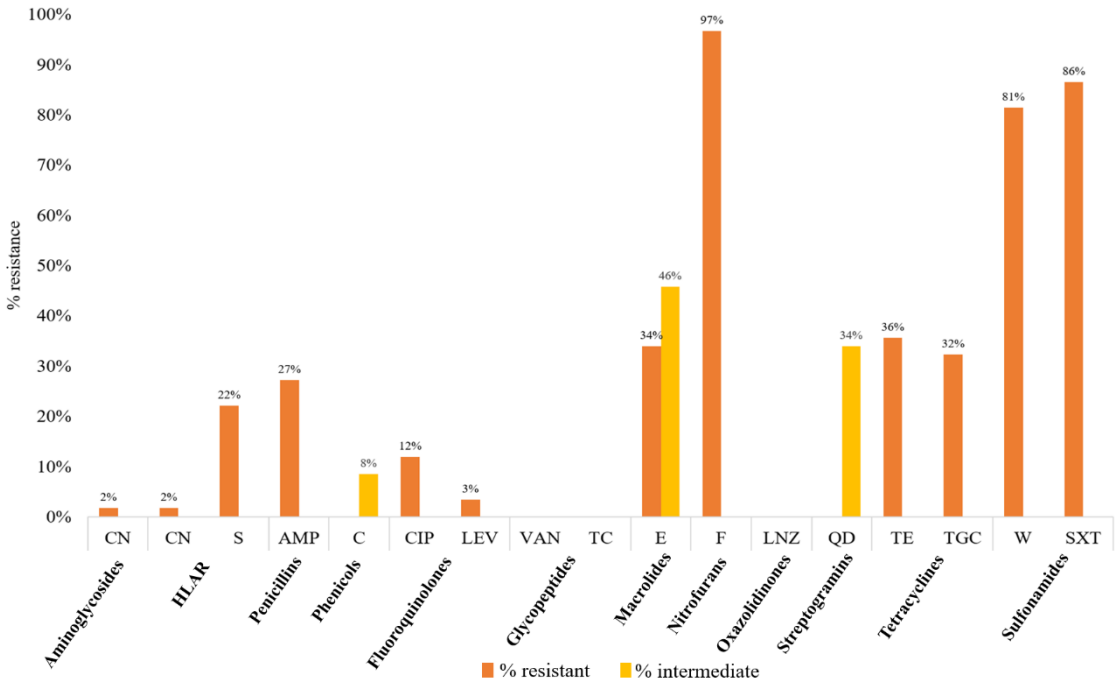


Figure 11. Phenotypic antibiotic resistance profile of the 59 *E. faecium* isolates.

CN- gentamicin, S- streptomycin, HLAR- high-level aminoglycosides resistance, AMP- ampicillin, C- chloramphenicol, CIP- ciprofloxacin, LEV- levofloxacin, VAN- vancomycin, TC- teicoplanin, E- erythromycin, F- nitrofurantoin, LNZ- linezolid, QD- quinupristin-dalfopristin, TE- tetracycline, TGC- tigecycline, W- trimethoprim, SXT- trimethoprim-sulfamethoxazole.

The overwhelming majority of *E. faecium* isolates showed phenotypic resistance to nitrofurantoin (97%), trimethoprim-sulfamethoxazole (86%), trimethoprim (81%) and erythromycin (80% of isolates showed resistance (34%) or intermediate resistance (46%)). In addition, resistance to tetracyclines was observed in one third of the *E. faecium*, including 36% of isolates that were resistant to tetracycline and 32% resistant to tigecycline, while 27% of isolates were resistant to β-lactams (ampicillin). Less resistant rates were detected for ciprofloxacin (12%), levofloxacin (3%), and gentamicin (2%). Intermediate resistance was observed for quinupristin/dalfopristin (34%) and chloramphenicol (8%). None of the isolates showed resistance to glycopeptides, namely vancomycin (MICs 0.38 – 4 µg/mL) and teicoplanin (MICs 0.023 – 1 µg/mL), nor to linezolid (MIC 2 µg/mL).

High- level aminoglycoside resistance (HLAR) was phenotypically detected by agar dilution (Figure 12). Thirteen isolates (22%; pigs n =8, cows n = 2, urban environment n = 1, bus n= 1, hands of passengers n = 1) showed high-level resistance to streptomycin (HLSR) and only one (2%, bus = 1) showed high-level resistance to gentamicin (HLGR).

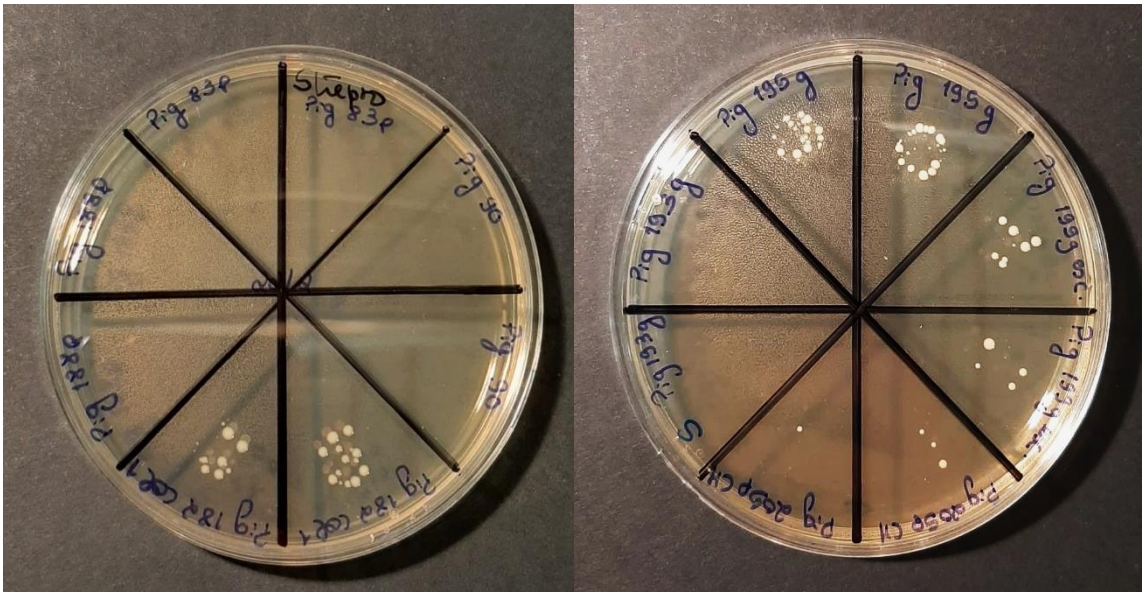


Figure 12. HLAR detected for *E. faecium* isolates by agar dilution method.

Twenty-three *E. faecium* isolates (pigs n= 5, cows n= 4, urban environment n= 4, buses n= 9, and hands of passengers n= 1) were selected for WGS, as previously described (Materials and Methods section) and further antibiotic resistance genotypic characterization was performed.

Genomic characterization identified twenty (20/23, 87%) *E. faecium* as non-susceptible to at least ≥3 antibiotic classes, and were therefore considered multidrug resistant (MDR) (175) (Figure 13). A single isolate, recovered from hands of a bus passenger, showed resistance to 5 antimicrobial classes (aminoglycosides, amphenicols, β- lactam, macrolides and tetracyclines). All isolates were resistant to aminoglycosides and macrolides.

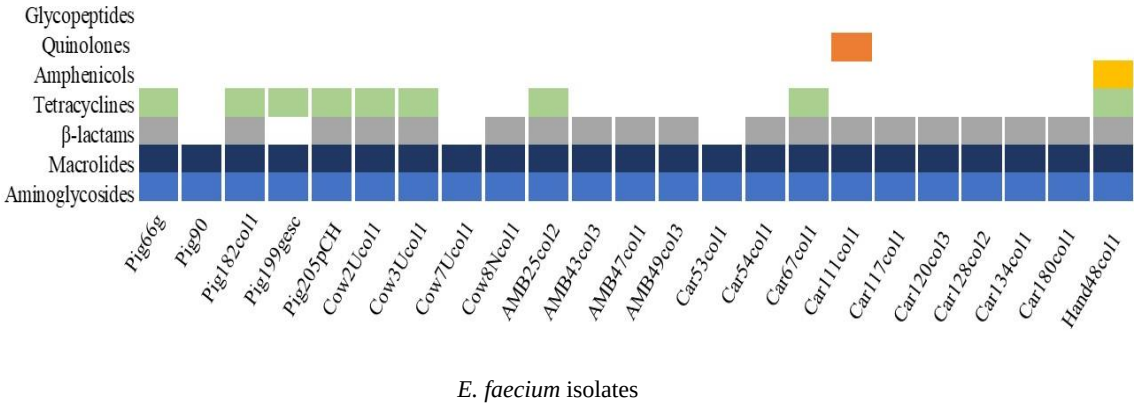


Figure 13. Number of resistant antibiotic classes per isolate.

Colored box indicates that the isolate is resistant to the respective antibiotic class.

The usage of antibiotics affects the intestinal microbiota and its interaction with the host, and select resistance genes in non-pathogenic bacteria, which may be transferred to various pathogenic bacterial species (176). In this study, a high variability of antibiotic resistance determinants (n= 19) were detected among all 23 *E. faecium* isolates mainly associated to aminoglycosides, β-lactams and lincosamides/macrolides and tetracyclines resistance phenotypes (Figure 14). The most prevalent genes detected were *eat(A)*, *msr(C)* and *aac(6')-Ii* presented in all isolates, confering resistance to lincosamides, streptogramins A, and pleuromutilins; macrolides, lincosamides and streptogramin B; and aminoglycosides, respectively.

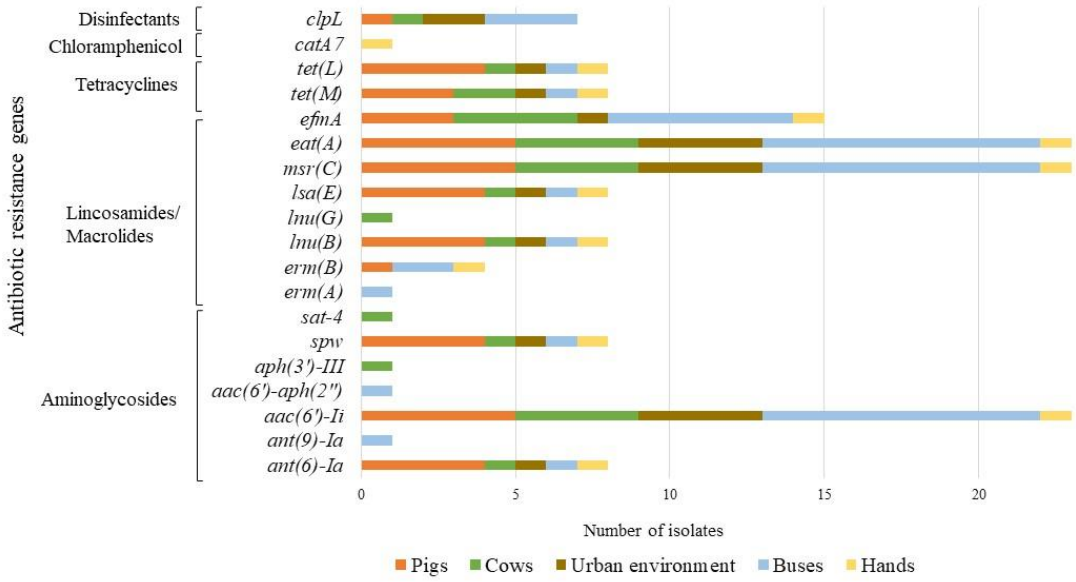


Figure 14. Distribution of antibiotic resistance genes identified among *E. faecium* isolates in each sample collection.

Ten genes (*ant(6)-Ia, aac(6')-Ii, spw, lnu(B), lsa(E), msr(C), eat(A), efmA, tet(M)* and *tet(L)*) were detected simultaneously in the five studied collections. The *erm(B)* gene was identified in three different collections (pigs, buses and in the hands of passengers), while the *clpL* gene was detected in all but the hands of passengers' collections. Other genes seem to be bacterial source collection specific as *aph(3')-III*, *lnu(G)* and *SAT-4* genes that were only identified in cows and *ant(9)-Ia*, *aac(6')-aph(2'')* and *erm(A)* were found exclusively in isolates from buses.

Among these 23 *E. faecium*, no vancomycin resistance genes were found, corroborating the previous results obtained by PCR. Although it was not detected VRE in our samples, it has already been identified VRE in animals, in Portugal (102,112,113,150). However, there is no data regarding VRE in the urban environment, in buses and in the hands of passengers. A single study conducted in Turkey, identified *Enterococcus* spp., in public buses, but did not search for *van* genes (151). Our study contributes to fulfill this gap, with the identification of multidrug resistant *E. faecium*

isolates in buses, passengers and urban environment, although no vancomycin resistance was detected.

The prevalence of linezolid resistance in Gram-positive bacteria is rising in many countries (50). In our study, no resistance to linezolid was identified neither by PCR for the resistance genes (*cfr*, *optrA* and *poxtA*) for all 59 *E. faecium* isolates, nor by 23S rRNA mutations or resistance genes screening on the 23 *E. faecium* genome sequences data, through LRE finder in Center for Genomic Epidemiology website. However, linezolid resistance has already been identified in Portugal in enterococci from different sources, including food-producing animals (149,177).

E. faecium isolates presented higher antimicrobial resistance than *E. faecalis* isolates, as commonly reported (178), which could be partially due to intrinsic resistance to a wide variety of antibiotics as fusidic acid, ceftazidime, cephalosporins, aminoglycosides, macrolides and sulfonamides (162). None of the trimethoprim or sulfonamide resistance genes (*dfr* and *sul*, respectively) were identified in the 23 *E. faecium* isolates. Therefore, the observed phenotypic resistance to trimethoprim (81%) and to the combination trimethoprim- sulfamethoxazole (86%), may be due to intrinsic resistance in *E. faecium* isolates (162). Since the activity of trimethoprim-sulfamethoxazole is uncertain, and the clinical outcome is unpredictable, it is not recommended to treat enterococcal infections with this antibiotic combination (138,164).

It was also observed low resistance rates to gentamicin, to fluoroquinolones (ciprofloxacin and levofloxacin), erythromycin, tetracyclines and nitrofurantoin. Nitrofurantoin is an antibiotic used to treat urinary tract infections, and in comparison to other classes of antimicrobials, acquired resistance to nitrofurantoin is less frequent (179). However, in our study, almost all isolates presented phenotypic resistance to this antibiotic, but no acquired genomic determinant could be identified, which suggest other resistance mechanism. Furthermore, in clinical *E. faecium* isolates it is possible to observe between 56% and 84% resistant to nitrofurantoin (180).

While resistance to penicillins (penicillin or ampicillin) has been common among clinical *E. faecium* isolates (20), we found 27% of phenotypically resistant isolates in our study. Almost all genotyped isolates (n= 19, 83%) showed resistance to β- lactams conferred by mutations in the *pbp5* (Annex 5, Table 7). Moreover, the *clpL* gene usually

linked to β-lactam resistance, in the form of the ClpL heat shock chaperone, was found in *E. faecium* isolated from the four out of the five collections studied (pigs, cows, urban environment and buses), suggesting that this gene is well disseminated (181). This gene is also known to confer resistance to disinfectants (182) and is mainly prevalent in Gram-positive bacteria, for example, in *Streptococcus pneumoniae*, where its expression is also associated to increased penicillin resistance (183). As mentioned before (at 2.1.1.1 Healthy pigs from swine farms, from Materials and methods section), in one of the pigs' farms included in the study, colistin and amoxicillin were still used as prophylaxis in the feed regimen of the animals at the time of sampling, suggesting that this usage of a β-lactam could have been selecting for these resistant strains. Furthermore, enterococci are considered to have intrinsic low-level resistance to β-lactams, but ampicillin remains the treatment of choice for enterococcal infections, generally combined with aminoglycosides for synergistic effects. However, ampicillin- resistant strains have been widely spread in hospitals causing infections and colonization of patients, which in some cases exceeds 70% of high-level ampicillin resistance in *E. faecium* (14). Regarding the incidence of resistance in *E. faecium* from animals, it is variable according to geographic location and animal host species. In some surveillance studies, no resistant *E. faecium* isolates were detected in cattle (184), but a resistance rate of in 30% was reported among poultry isolates in Portugal (123).

Fluoroquinolones have been frequently used to treat *E. faecalis* urinary infections (185). Ciprofloxacin is still the most commonly used quinolone and consequently high resistance rates have been reported. Wang et al. found ciprofloxacin resistance rates ranging between 69.4% and 92%, and to levofloxacin between 84 to 88.4% among both *E. faecium* and *E. faecalis* isolated from patients (180). In our study, phenotypic resistance to quinolones, ciprofloxacin (12%) and levofloxacin (3%) was low compared to isolates from inpatients (180), but comparable to poultry (18.5%) and pets (18%) colonizing *E. faecium* reported in Portugal (123). Although genotypic analysis identified a single isolate (Car111col1) with mutations in *parC* (p.S80I) and *gryA* (p.E87G) genes, responsible for quinolones resistance, the identification of the antimicrobial efflux pump gene *efmA*, also associated to quinolone resistance, in 65% (15/23) of *E. faecium* isolates could justify the additional phenotypic resistance detected.

The high frequency of aminoglycoside resistance reported in enterococci from livestock, suggests the significant use of these antibiotics in veterinary medicine, as for example in the treatment of mastitis in cattle (186). Besides the intrinsic nature of low-level aminoglycosides resistance in *E. faecium*, it can occasionally become HLAR as a result of horizontal transfer of resistance determinants, which is highly concerning. Many European countries have reported clinical enterococci with high rates of high-level gentamicin resistance, ranging from 1 to 48% (187). Over the past two decades, clinical *E. faecium* strains have become more frequently resistant to high levels of aminoglycosides than *E. faecalis* (188,189), a similar scenario was detected in our study. Therefore, in this study, we detected about 24% HLAR in *E. faecium* isolates, which is lower than resistance rates ranging between 34.4% and 41.7% described by others (190,191). In our study, HLSR (22%) was more frequent than HLGR (2%), as previously reported in other studies with animal *E. faecium* isolates (192,193). However, in clinical *E. faecium* strains, HLGR is reported with higher occurrence than HLSR (188,191).

Eight of the 23 genome sequenced *E. faecium* that showed phenotypic HLSR carried the *ant(6)-la* gene associated to this resistance, as reported in other study (194). The single isolate with phenotypic HLGR, presented the *aac(6')-aph(2'')* gene (annex 5, Table 7), which is the most common gene associated with this gentamicin high-level resistance. Therefore, the identification of aminoglycoside high-level resistance genes by genomic analysis corroborated the phenotypic antibiotic resistance detected. Other genes associated with aminoglycoside resistance detected less frequently included the *spw* gene that confers resistance to spectinomycin and was identified in 35% (n= 8) of the isolates; *aph(3')-IIIa* gene that confer resistance to kanamycin; and the *sat-4* gene conferring resistance to streptothricin detected in a single cow isolate that was also resistant to gentamicin and HLSR; and the *ant(9)-la* gene conferring resistance to spectinomycin detected in a bus isolate.

In our study, tetracycline resistance (36%) appears to be mostly associated to the animals' isolates, 73% among pigs and 50% among cows (p= 0,0059), which can be supported by the common use of this antibiotic for prophylactic purposes in livestock (195). Therefore, bacterial species associated with food producing animals are under a significant antibiotic selection pressure, which may compromise the treatment of infections in animals (176). For humans, tetracyclines have been prescribed for

prophylaxis and treatment of community-acquired infections of Gram-negative bacteria, especially respiratory infections (196). Moreover, genotypic characterization identified the tetracycline resistance genes *tet(M)* and *tet(L)* in 39% (8/23) of *E. faecium*. Seven (30%) isolates harbored both genes, while each single gene was detected in two different isolates. *tet(M)* is considered the most frequently mechanism of tetracycline resistance in enterococci from humans, animals, food, and the environment (197), even though in this study *tet(M)* and *tet(L)* were equally detected.

As mentioned before, although no specific resistance mechanism was described so far, for tigecycline resistance in enterococci, tetracycline resistance determinants *tet(M)* and *tet(L)* seems to be involved (63). 32% of *E. faecium* presented phenotypic resistance to tigecycline, out of which only two isolates from a cow and a pig, harbored *tet(L)* and *tet(M)*. These results suggest that other resistance mechanisms could be involved, which deserves further investigation, given the high prevalence of phenotypic resistance detected among *E. faecium* isolated in non-clinical settings in our study.

Macrolides are also widely used in veterinary medicine, as for example, in the treatment of enteric infections (186). Besides being banned in Europe, macrolides as tylosin, spiramycin, and virginiamycin used to be broadly consumed as a growth promotor in pigs and other animals (198). Also, macrolides have been used over many decades to treat common bacterial infections in humans (199). In our study, all *E. faecium* isolates showed resistance to macrolides, probably associated to the presence of *msr(C)*, which is known to be intrinsic in *E. faecium* and was identified in all sequenced isolates. A global prevalence of *msr(C)* gene that confers resistance to macrolides and streptogramin B, was also described by Lee et al. (200). Moreover, the antimicrobial efflux pump gene *efmA*, which confers resistance to macrolides and fluoroquinolones, was also identified in 65% (15/23) of *E. faecium* isolates, including isolates from animals and from environment. Other resistance genes commonly associated to macrolide and lincosamide resistance were detected in our isolates, although with different rates and associated to samples source. While *lnu(B)* and *lsa(E)* were identified in 35% of the isolates each, *erm(B)* and *erm(A)* were less prevalent (n= 4, 17% and n= 1, 4%, respectively). The lincosamide resistance gene *lnu(G)* was exclusively found in a cow isolate. In human clinical practice, lincomycin and clindamycin are widely used to treat

bacterial infections, in particular those caused by anaerobic species, and to treat malaria (199).

Chloramphenicol is an antibiotic used to treat superficial eye infections such as bacterial conjunctivitis and otitis externa, or in the treatment of typhoid and cholera in humans (48). This antibiotic was used in the past in food-producing animals, although it was banned more than 20 years ago in Europe, it is still allowed for the treatment of specific ocular infections in companion animals in Portugal (123). In this study, a single hand isolate showed phenotypic resistance to chloramphenicol associated to the presence the *catA7* gene. Chloramphenicol resistance was already described among *E. faecium* isolates from poultry (13%) and pets (9%), and in a single isolate from human carriage in Portugal, but no genetic determinant was reported in the study (123).

The hands of passengers are represented by a single isolate that showed 12 resistance genes. However, in buses collection was identified the higher number of resistance genes (n= 15), distributed by the 9 representatives isolates. All genes, except *ant(9)-la*, *aac(6')-aph(2'')*, *aph(3')-III*, *erm(A)*, *lnu(G)*, *sat-4* and *catA7*, were identified among the five studied collections.

3.3.2.2 Virulence determinants

A set of seven virulence determinants including *asa*, *esp*, *ace*, *gelE*, *hyl* and IS16, were screened by PCR in all *E. faecium* isolates. Additional virulence determinants were identified by *in silico* genome sequence analysis of the 23 selected isolates for WGS. Regarding each sample collection, a different distribution was observed for each virulence gene (Figure 15).

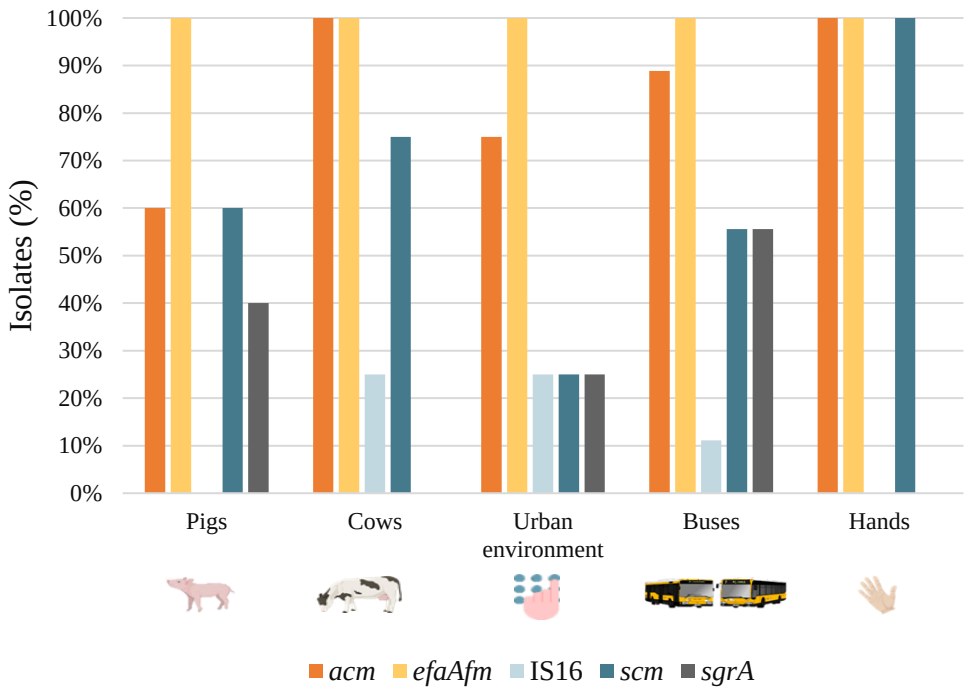


Figure 15. Prevalence of virulence determinants in the 23 *E. faecium* isolates detected by PCR and genomic data analysis, distributed by sample collection.

None of the isolates were positive for *asa*, *esp*, *gelE*, *hyl* and *cylA* by PCR screening, which was confirmed by sequence analysis in a subset of isolates. However, it was identified the *ace* gene by PCR in one hand isolate but could not be confirmed by genomic analysis since the isolate was not includes in the selected isolates for WGS.

The urban environment and buses were the collections that presented more virulence genes, concerning both PCR and sequencing data (Figure 15). The passengers' hands collection, here represented by a single isolate, showed only three virulence genes (*acm*, *efaAfm* and *scm*).

The insertion sequence (IS16) is considering a putative genetic marker of hospital strains, capable of differentiating hospital- associated from human commensal, livestock- and food- associated *E. faecium* strains (136). IS16 was detected in three isolates from different collections: cows (n= 1), urban environment (n= 1) and in buses (n= 1), and additionally identified by PCR in one bus and one urban environment isolate, not selected for sequencing. Moreover, *acm* and *scm* adhesins that were highly prevalent in our *E.*

faecium isolates, are also considered virulence markers among *E. faecium* hospital-strains (136).

The *sgrA* gene codes for a binding LPXTG surface adhesin involved in biofilm formation (86). It was detected in buses (56%), in pigs (40%) and in the urban environment (25%). The *efaA*$_{fm}$ gene that encodes a cell wall adhesin is involved in colonization and plays a crucial role in infective endocarditis (77), and was present in all isolates, which is concerning.

The fact that these virulence determinants commonly found in hospital isolates, are spread among *E. faecium* recovered in all sample collections of this study, in non-clinical settings, reflects a wide dissemination of isolates with infection potential which requires active surveillance.

3.3.2.3 Plasmidome

Plasmids play a significant role in the dissemination of resistance and virulence determinants, by horizontal gene transfer (201). In order to have an overview of the plasmid families present in the *E. faecium* isolates of this study, it was performed an *in silico* analysis of the *rep* genes present in each of the 23 *E. faecium* genomes (Annex 5, Table 7).

Globally, there is a high diversity of plasmids that belong to the five major enterococcal plasmid families, either replicate by a theta mechanism (Rep3, Inc18, RepA_N) or by rolling circle replication (Rep_trans and Rep1) (202). The number of different families detected varied among collections, each having at least three different plasmid families (Figure 16). Plasmids from Inc18 and RepA_N families were present in all five sample collections.

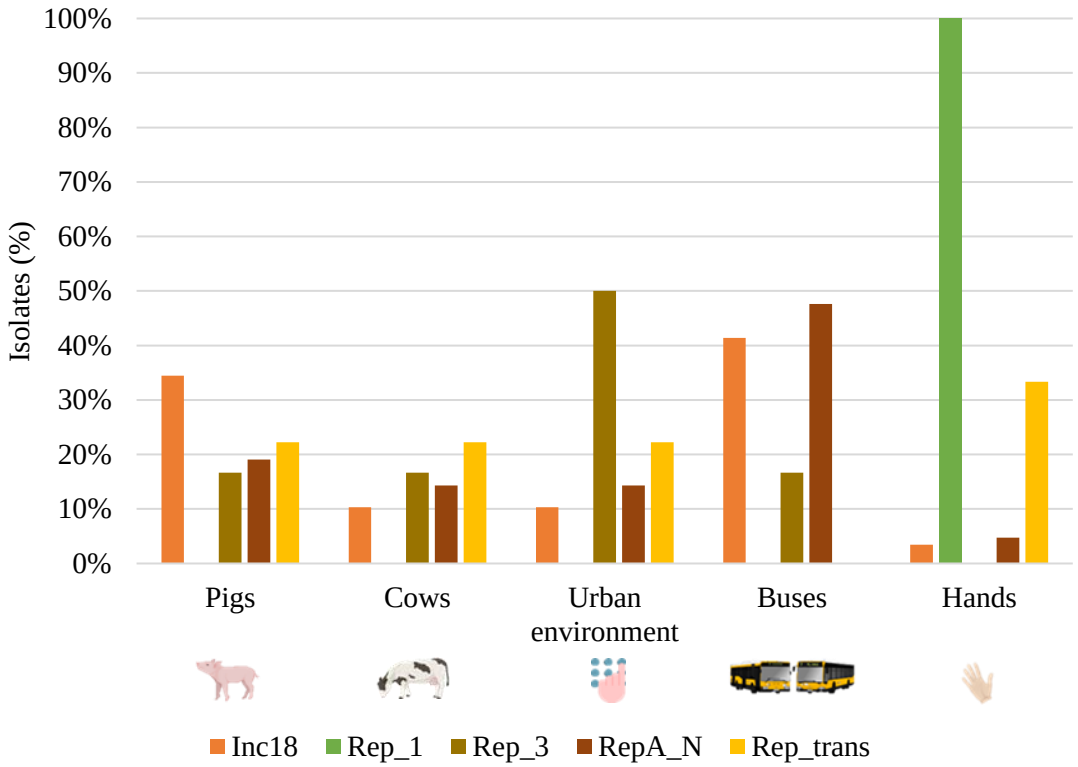


Figure 16. Distribution of plasmids families among 23 *E. faecium* isolates, by sample collection.

Conjugative plasmid transfer is one of the major mechanisms responsible for the spread of antibiotic resistance determinants (203). Inc18 plasmids, which were more prevalent among bus (40%) and in pigs' samples (37%), are known to transport genes encoding resistance to different antibiotics as macrolides, lincosamides and streptogramins, chloramphenicol and vancomycin. Moreover, Inc18 have a broad-host range, which is of major concern, namely among buses that were previously identified as reservoirs of MRSA and may represent a conducive environment to the transfer of plasmids between these pathogenic species (203). Plasmids from the RepA_N family were mainly found in isolates from buses (45%), while 33% were identified in livestock samples. Plasmids from both Inc18 and RepA_N families were reported as predominants in clinical isolates carrying the *vanA* genes in VRE (204). The identification of these plasmid families widespread in *E. faecium* isolates from non-clinical sites in our study, is of major concern regarding its capacity to host antimicrobial resistance genes and becoming a threat to human health.

Rep_1 was only found on the isolate from the hands of the bus passengers, and is probably associated to its antimicrobial resistance pattern, since this family comprises plasmids that are associated with *catA7* gene (201), and the hand isolate was the unique harboring this chloramphenicol resistance gene.

On the other hand, the Rep_3 family was highly prevalent among urban environment (50%), followed by the pigs, cows and buses *E. faecium* isolates (17%, each). In enterococci, Rep_3 plasmids have been found in isolates recovered from hospitalized patients, animals (pigs, cows), food products from animals, frequently associated to the production of bacteriocins (205).

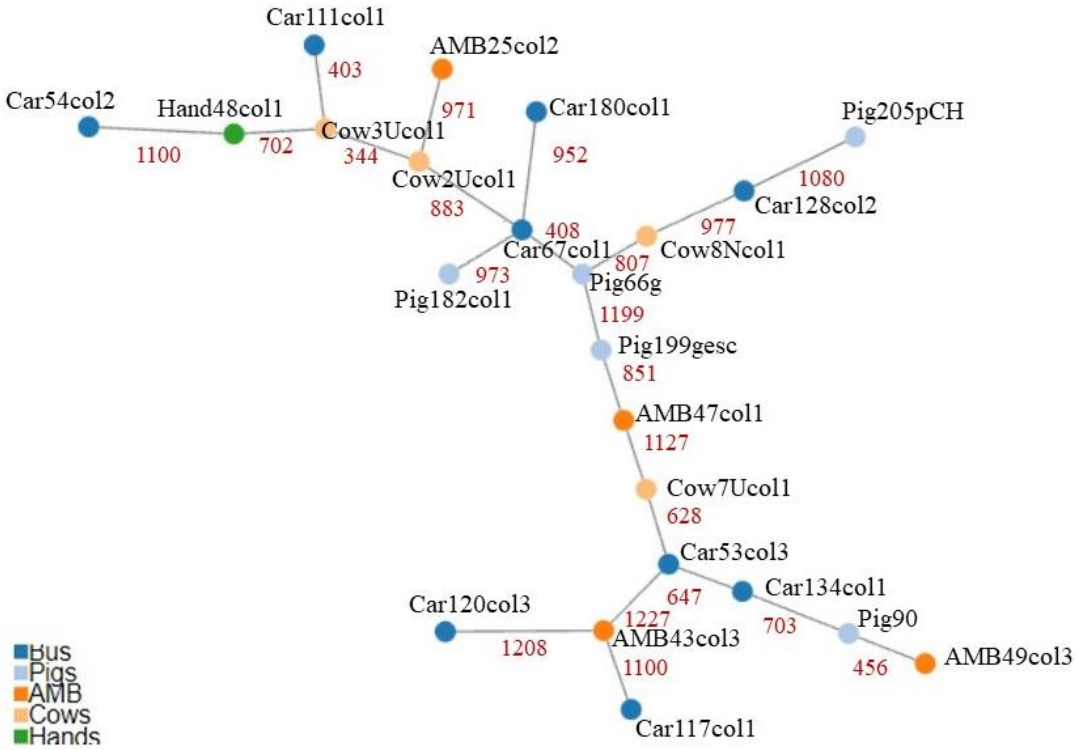
Plasmids from the Rep_trans family were equally found among isolates from pigs, cows and urban environment (22%, each), and in the *E. faecium* from passengers' hands. According to Lanza et al., this family seems widely spread among enterococci (201). Some of these plasmid families (RepA_N, Inc18 and Rep_3) are usually involved in the acquisition and spread of antimicrobial resistance among different genera of Firmicutes (202).

3.3.2.4 Clonal population

Clonal population structure of the 23 *E. faecium* isolates was initially characterized by multilocus sequence typing (MLST) and complemented by a depth analysis trough the core genome multilocus sequence typing (cgMLST) of the genomic data. MLST showed a high diverse *E. faecium* population, with 23 different sequence types (STs) identified, out of which eight corresponded to new STs (ST2203, ST2204, ST2206, ST2207, ST2208, ST2209, ST2210 and ST2211), including also eight new loci. New MLST profiles as well as new alleles were assigned trough the pubMLST database (https://pubmlst.org/). cgMLST analysis considering 1298 loci of the *E. faecium* cgMLST schema (146) that were present in all 23 isolates of our study, confirmed a high variability in the population (Figure 17). All cgMLST isolates profiles showed more than 20 allele differences between them, and were therefore considered to belong to different clonal

types (CT) (146), even among *E. faecium* isolated from the same sample collection setting (Figure 17).

STs associated to two major *E. faecium* clonal complexes (CC; STs sharing at least 5 alleles belonged to the same CC, and the CC founder corresponds to the ST with more related STs) were identified in our collection (Table 1): CC17 which are widespread among infection isolates in hospitals, and CC94 mainly associated to animal isolates, but also identified in humans, in both carriage and infection (102,103,206). The remaining 18 isolates were considered as single STs. CC17 was identified in a single *E. faecium* isolated from a pig, belonging to ST32. Four *E. faecium* isolates recovered from different collections (one from pigs, one from cows, one from the urban environment and other



from buses) belonging to CC94, but assigned to different STs (ST296, ST2206, ST1205 and ST800, respectively).

Figure 17. Minimum spanning tree based on cgMLST analysis of 23 *E. faecium* isolates from livestock and environment. AMB- Urban environment.

Table 1. MLST profile of the 23 *E. faecium* isolates from livestock and environment.

MLST										
id	Collection	*atpA*	*ddl*	*gdh*	*purK*	*gyd*	*pstS*	*adk*	ST	CC
AMB25col2	AMB	9	4	12	157	2	2	5	2208	
AMB43col3	AMB	137	81	14	129	3	208	6	2209	
AMB47col1	AMB	129	13	18	17	10	19	6	1817	
AMB49col3	AMB	24	8	8	8	2	27	6	1205	94
Car111col1	Bus	56	3	1	6	1	7	1	2204	
Car117col1	Bus	137	6	129	158	91	209	85	2211	
Car120col3	Bus	67	54	14	80	88	99	6	2094	
Car128col2	Bus	12	5	1	6	1	42	1	2201	
Car134col1	Bus	13	8	8	8	6	88	6	800	94
Car180col1	Bus	2	7	5	7	1	1	1	27	
Car53col1	Bus	13	8	10	23	6	28	11	76	
Car54col2	Bus	9	2	1	11	1	14	6	69	
Car67col1	Bus	5	3	128	2	2	1	1	2210	
Cow2Ucol1	Cows	5	3	1	6	2	2	1	30	
Cow3Ucol1	Cows	9	3	1	6	1	20	1	2203	
Cow7Ucol1	Cows	13	8	127	8	6	28	6	2206	94
Cow8Ncol1	Cows	2	5	1	156	22	1	1	2207	
Hand48col1	Hands	9	2	1	6	1	7	1	195	
Pig182col1	Pigs	2	40	12	3	1	1	1	639	
Pig199gesc.	Pigs	5	13	18	17	1	19	6	624	
Pig205pCH	Pigs	2	2	6	6	1	11	1	127	
Pig66g	Pigs	3	3	1	2	1	1	1	32	17
Pig90	Pigs	25	8	8	8	10	10	6	296	94

id – isolate identification; ST – sequence type; CC – clonal complex; *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS* and *adk* – seven housekeeping genes included in the MLST schema.

Regarding the isolates from pigs, five different STs were identified: ST32 (CC17), ST127, ST296, ST624 and ST639. ST32 was already isolated from different sources as animals, bovine mastitis (207), pigs and chicken feces and more recently from a bird, from environmental wastewaters and from carriage in healthy humans or hospitalized patients, with some of these isolates carrying the *vanA* (https://pubmlst.org/). A recent report on linezolid resistance surveillance identified a *poxtA*-positive *E. faecium* belonging to ST32 in a rectal swab from healthy human in Portugal (208). Therefore, ST32 seems to be widely disseminated in different hosts, with pathogenic potential to cause infection and capacity to acquire antimicrobial resistance genes, namely to vancomycin and linezolid. The detection of this ST32 (CC17) in our pigs' isolate indicates that a close surveillance in these settings is needed. In addition, other STs identified in *E. faecium* colonizing pigs in our study, were already identified as aetiologic agents of infection as the ST624 that was found in a bovine mastitis; and ST127 identified

in a blood sample from inpatient in Spain (207)(https://pubmlst.org/). These observations highlight the potential of these colonizing isolates to promote disease in animals, and those colonized pigs be at higher risk of infection.

Among the four *E. faecium* isolated from the cows, three of them were assigned to new STs (ST2203, ST2206 and ST2207). The remaining isolate belonged to ST30, which was initially recovered in 1995 in a hospitalized patient from a blood sample in Australia (https://pubmlst.org/). Moreover, *E. faecium* isolates from ST30, carrying the *optrA* linezolid resistance gene in plasmids were recently reported in German hospitals (209). In Portugal, ST30 *E. faecium* isolates were reported in a pig, dust and pigs' food in the same farm (112), and in our study was identified in a cow sample, which highlight its capacity of host diversification. Of note is the ST2206, identified for the first time in this study and belongs to the CC94, a clonal complex widely spread among animals but also in humans (102,103,206).

Half of the *E. faecium* isolated from urban environmental surfaces were assigned to new STs (ST2208 and ST2209), while the remaining isolates belonged to ST1817 and ST1205. The ST1817 that in our study was isolated from a bus stop sample, was previously identified in 2020 in a pig *E. faecium* isolate in South Korea (https://pubmlst.org/). ST1205 seems to be associated to environment, since it was firstly identified in 2014 in United Kingdom in a wastewater *E. faecium* isolate (210), with no other reports of this ST.

Three *E. faecium* isolates from bus samples were assigned to new STs (ST2204, ST2210 and ST2211) and the other six, ST27, ST69, ST76, ST800, ST2094 and ST2201 were previously reported. ST27 was identified in both carriage in poultry and pets (dogs) and in human infections associated to *vanA* gene in different European countries (https://pubmlst.org/). Other STs were already identified in human invasive infections as ST2201 (https://pubmlst.org/) and the ST800, that belongs to CC94 commonly associated to the community that can eventually cause sporadic infections in animals and humans (103). ST76 seems to be mostly associated to *E. faecium* from food samples, since it was firstly described in a food outbreak in 1956 in Norway (https://pubmlst.org/), and recently identified in gilthead seabream in Portugal (211). In addition, ST2094 was also identified in animal carriage in Portugal (https://pubmlst.org/). Besides the diversity of STs

identified, none of these was previously detected among environmental surfaces associated to high human contact.

The single isolate from bus passengers' hands belonged to ST195. ST195 seems to be more associated with poultry, since it was isolated from chickens in different countries and from a chicken farmer, with some of these isolates carrying the *vanA* (https://pubmlst.org/). Moreover, ST195 *E. faecium* was also identified in a patient blood sample and presented linezolid resistance (212). Although chickens could be a reservoir for this ST195 clonal lineage, it seems that it could spread and colonize humans in GI tract, bloodstream or contaminate skin, as the case of our *E. faecium* isolate.

The identification of eight new STs in isolates from different sources suggests that besides a high diversity, *E. faecium* is evolving to be adapted and maintained as commensals in the non-clinical settings represented in this study.

In order to define clonal identity and epidemiological link between the livestock and environmental *E. faecium* isolates identified in this study, and *E. faecium* population associated to infection in hospitals, our population was compared to a collection of 132 *E. faecium* isolated from infection in Portuguese hospitals in the Lisbon and Oporto regions between 2019 and 2020 (Conceição, T. et al. unpublished) (118,119).

Globally, MLST analysis identified in our collection, a single-locus variant (SLV) of ST94 (from a bus isolate), five doble-locus variants (DLV) of ST94 (from a cow and a pig isolate), ST2020 (from a bus), ST280 (from a pig) and ST494 (from the udder region of a cow). A single urban environment isolate collected in a stairwell near a hospital in Lisbon, was considered a triple-locus variants (TLV) of the ST94 (Figure 18). Most of the livestock and environment isolates had loci variations of the ST94, that belongs to CC94, which is mostly associated to human community and commensal isolates (103). However, considering the few loci variations of the three STs (ST2020, ST280 and ST494) that belong to the hospital-adapted lineage CC17, it seems that in our study we have strains possibly related to those identified in hospitals, which is of major concern.

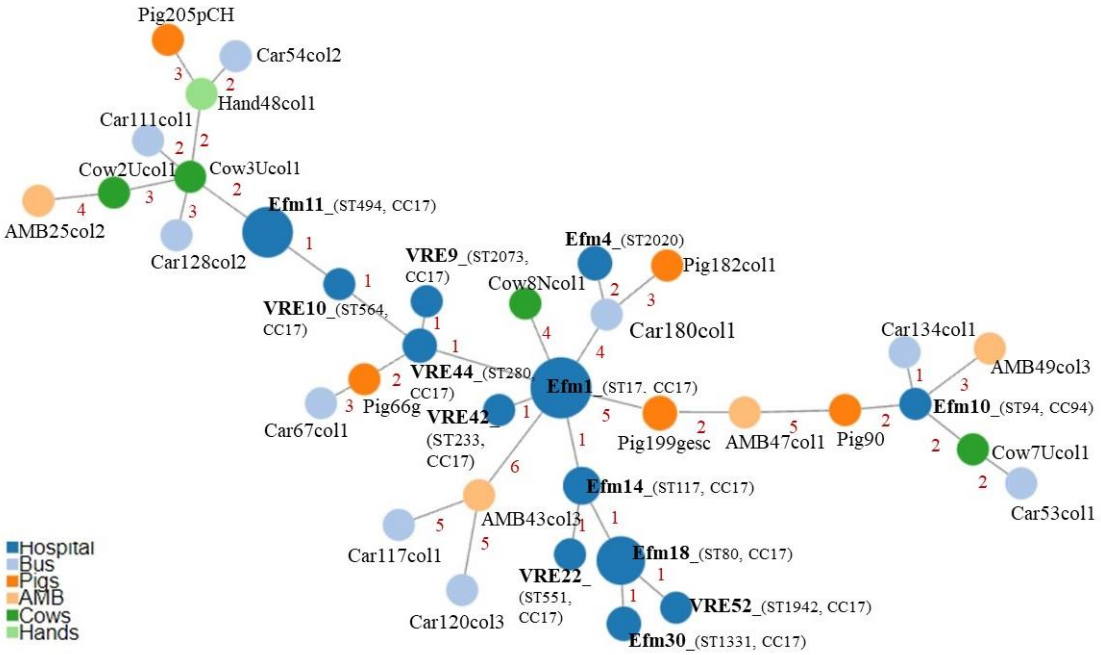


Figure 18. Minimum spanning tree based on MLST analysis of 23 *E. faecium* isolates from livestock and environment and 132 *E. faecium* isolated from infections in Portuguese hospitals between 2019 and 2020. AMB- Urban environment; *E. faecium* from hospital infection representative isolates are labelled in boldface.

A more detailed cgMLST analysis based on the 1159 loci common to all isolates, showed a high population diversity between our isolates and the hospital associated population (Figure 19). None of the isolates from both collections belonged to common clonal types (CTs).

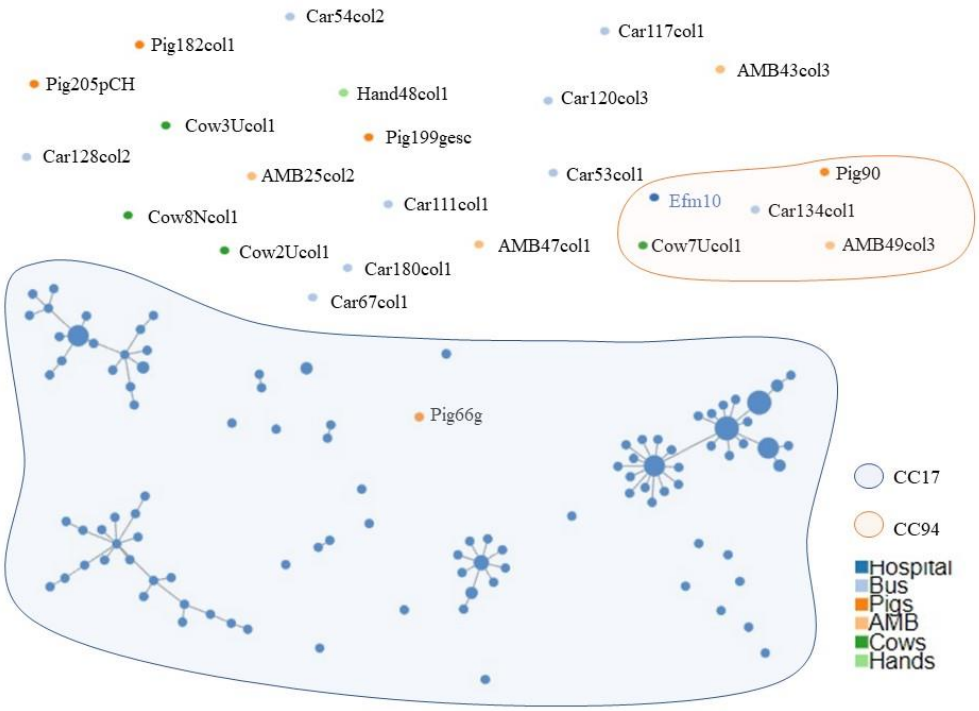


Figure 19. Minimum spanning tree based on cgMLST analysis of 23 *E. faecium* isolates from livestock and environment and 132 *E. faecium* isolated from infections in Portuguese hospitals between 2019 and 2020. The isolates that showed ≤ 20 allele differences between them are linked. AMB- Urban environment; CC – clonal complex.

cgMLST is a typing technique based on 1423 target genes, which facilitates rapid standardized and high-resolution tracing of *E. faecium* outbreaks (146). Differences between isolates are easily identified with this technique. Through cgMLST analysis, no direct epidemiological link between *E. faecium* recovered from this study and the *E. faecium* recovered from infection in Portuguese hospitals was observed. This could be due to the different selective pressure of these isolates or because they are not contemporary and may have evolved during the years between sampling collections. For example, the ST2020, which is a DLV of the ST27 (bus isolate recovered between 2011/2012 in Lisbon) (Figure 18), has probably evolved from this bus isolate.

4. Final considerations

In summary, the identification of VRE reservoirs outside hospitals is of major importance to prevent the dissemination of this major human pathogen. To the best of our knowledge, our study represents the first surveillance of non-clinical settings as the hands of passengers and outdoor urban surfaces near hospitals as possible reservoirs for VRE. Still, this is the first study in Portugal to explore livestock nares and udder region as potential VRE colonization reservoirs. Overall, 90% (266/294) of the total screened samples contained enterococci according to its ubiquous prevalence in nature, but healthy pigs and buses collections showed the highest *Enterococcus* prevalence (31%, each), followed by cows (26%), urban environment (9%) and hands of bus passengers (3%). Six *Enterococcus* species were identified among livestock and environmental samples: *E. faecalis* (30%), *E. casseliflavus* (20%), *E. gallinarum* (14%), *E. faecium* (13%), *E. hirae* (5%) and *E. durans* (< 1%). Moreover, despite the fact that all isolates were susceptible to glycopeptides and no *vanA* or *vanB* were found, 23% of enterococci harbored the *vanC2/3* and 17% carried the *vanC1*, including 12 (40%) *E. faecalis.*

These *E. faecalis* harboring *vanC1*, showed an additional resistance pattern mainly associated to intrinsic resistance, except for ampicillin and tigecycline. While half of these *E. faecalis* isolates from healthy cattle were resistant to ampicillin, 17% showed phenotypic resistance to tigecycline, which is uncommon and of major significance given the widespread use of β-lactams, namely ampicillin, in the treatment of enterococcal infections in humans, and the reserve nature of tigecycline as a last resort antibiotic.

On the other hand, the overwhelming majority of *E. faecium* isolates presented a multidrug resistant pattern including resistance to different antibiotic classes as aminoglycosides, macrolides, β- lactams, tetracyclines, quinolones and amphenicol classes, mainly associated to the presence of 19 different resistant genes and mutations in *gyrA* and *parC*. Also, 24% of *E. faecium* isolates showed high-level aminoglycosides resistance, namely to streptomycin linked to the gene *ant(6)-la* and gentamicin associated to *aac(6')-aph(2'')* gene. Moreover, 32% of isolates showed phenotypic resistance to tigecycline, a last resort antibiotic in human clinic. The detection of different resistance determinants, commonly associated to antibiotics used in human and veterinary clinical

practice, namely to treat enterococcal infections as aminoglycosides, in commensal *E. faecium* isolates widespread in non-clinical settings is worrisome and deserves attention.

Although resistance genes can contribute to enterococcal pathogenicity, there are other factors that should be taken in consideration, such as virulence determinants. Different virulence determinants associated to host cell adherence and invasiveness were identified in our study, namely *asa1*, *gelE* and *ace* detected on *E. faecalis* isolates, and *acm*, *scm*, *sgrA*, *efaAfm* and IS16 detected on *E. faecium*. Part of these virulence factors are known to be hospital associated virulence markers among *E. faecium* which suggests that these commensal strains have a high pathogenic potential and infection capacity, making the hosts at a high infection risk. Moreover, mobile genetic elements as plasmids from Inc18 and Rep_1 families, that are commonly associated to the dissemination of resistance and virulence determinants were widely identified in our *E. faecium* isolates, which potentiates the dissemination of resistance and virulence to other bacteria and promotes the pathogenic potential of these isolates.

The *E. faecium* isolated in this study, showed a high variable population structure with a different ST identified per isolate, and with no specific association to collection source. Furthermore, it was possible to identify five isolates belonging to clonal complexes CC17 and CC94, which are highly associated to hospital infections and human and animal carriage. Although a direct epidemiological link between *E. faecium* recovered from livestock and environment in this study and the *E. faecium* from infection recovered in Portuguese hospitals was not identified through cgMLST analysis, it was possible to identify isolates in our study that by MLST belong to the same clonal background (as single and double locus variants) identified in the hospitals.

The results of this study contributed to full fil the gap on the knowledge about VRE reservoirs outside hospitals. None of the five different settings screened in this study, healthy pigs and cows and environmental surfaces (buses, hands of bus passengers and urban environment near hospitals) seem to constitute a VRE reservoir in Portugal, since no VRE could be detected. However, it was identified a high prevalence of antibiotic resistance and virulence determinants among the most clinically relevant enterococci, *E. faecalis* and *E. faecium*. The maintenance of resistance determinants associated to antibiotics commonly used in human and veterinary clinical practice, and in

particular the detection of resistance to last resort antibiotics, among commensal *E. faecium* in non-clinical setting is of major concern and may jeopardize the treatment of infections in the near future. Moreover, our results suggest that a continuous surveillance on antimicrobial resistance and virulence reservoirs among *E. faecium*, besides VRE, is highly recommended to prevent resistance dissemination, contain the emergence of superbugs and contribute to the worldwide effort on reducing antimicrobial resistance.

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6. Annexes

1. Culture media

Table 1 – Culture media and composition (per Liter).

Culture media	Composition
Brain Heart Infusion Agar	Brain infusion solids (12.5 g); Beef heart infusion solids (5.0 g); Proteose peptone (10.0 g); Sodium chloride (5.0 g); Glucose (2.0 g); Disodium phosphate (2.5 g); Agar (10.0 g). pH 7.4 ± 0.2 at 25°C
Brain Heart Infusion Broth	Calf Brains, infusion from 200 g (7.7 g); Beef Heart, infusion from 250 g (9.8 g); Proteose peptone (10.0 g); Dextrose (2.0 g); Sodium Chloride (5.0 g); Disodium phosphate (2.5 g). pH 7.4 ± 0.2 at 25°C
Compass *Enterococcus* Agar	Peptones (27.5 g); Yeast extract (5.0 g); Sodium chloride (5.0 g); Tween 80 (1.0 g); Inibitory mixture (0.3 g); X-glucoside D (1.0 g); Bacteriological agar (14.0 g). pH 7.5 ± 0.2 at 25°C
CHROMagar VRE	Agar (15.0 g); Peptones and yeast extract (20.0 g); Salts (5.0 g); Chromogenic mix (27.3) and supplement selective mix (0.06 g). pH 6.9 ± 0.5 at 25°C
Mueller Hinton II Agar	Beef Extract (2.0 g); Acid Hydrolysate of Casein (17.5 g); Starch (1.5 g); Agar (17.0 g). pH 7.3 ± 01 at 25°C
Tryptic Soy Agar	Pancreatic Digest of Casein (15.0 g); Papaic Digest of Soybean (5.0 g); Sodium Chloride (5.0 g); Agar (15.0 g). pH 7.4 ± 0.2 at 25°C
Tryptic Soy Broth	Pancreatic Digest of Casein (17.0 g); Papaic Digest of Soybean (3.0 g); Dextrose (2.5 g); Sodium Chloride (5.0 g); Dipotassium Phosphate (2.5 g). pH7.3 ± 0.2 at 25°C

2. Buffers

Table 2 – Buffers used for DNA extraction and gel electrophoresis.

Solution	Preparation of stock solution
TE (Tris-EDTA) 10X	10mM Tris pH 7.5; 1 mM EDTA pH 8.0
TAE (Tris- Acetic acid-EDTA) 50X	0.5 M Tris; 0.05 M EDTA pH 8.0; 1 M acetic acid

3. Enzymes

Table 3 – Enzyme used for DNA extraction (cellular lysis).

Enzyme	Composition
Lisozyme	20 mg/ml in sterile purified water. Conserved at -20ºC in 200 µl aliquots

4. Primers List

Table 4 – List of all primers used in this study, including nucleotide sequences, target region, amplicon size and bibliographic references.

Assay	Target region	Primers sequence (5' to 3')	Amplicon size	Reference
DNA integrity	16S rDNA	16S_F: GTGCCAGCAGCCGCGGTAA 16S-R: AGACCCGGGAACGTATTCAC	~800 bp	(131)
Enterococcus speciation	*ddl* gene	Efaecalis_E1: ATCAAGTACAGTTAGTCTT Efaecalis_E2: ACGATTCAAAGCTAACTG	941 bp	(132)
		Efaecium_F1: GCAAGGCTTCTTAGAGA Efaecium_F2: CATCGTGTAAGCTAACTTC	550 bp	
	sodA gene	Ecasseliflavus_CA1: TCCTGAATTAGGTGAAAAAAC Ecasseliflavus_CA2: GCTAGTTTACCGTCTTTAACG	288 bp	(133)
		Egallinarum_GA1: TTACTTGCTGATTTTGATTCG Egallinarum_GA2: TGAATTCTTCTTTGAAATCAG	173 bp	
	sodA gene	Eraffinosus_RF1: GTCACGAACTTGAATGAAGTT Eraffinosus_RF2: AATGGGCTATCTTGATTCGCG	287 bp	(133)
		Ehirae_HI1: CTTTCTGATATGGATGCTGTC Ehirae_HI2: TAAATTCTTCCTTAAATGTTG	187 bp	
	sodA gene	Edurans_DU1: CCTACTGATATTAAGACAGCG Edurans_DU2: TAATCCTAAGATAGGTGTTTG	295 bp	(133)
Vancomycin-resistance genes detection	*vanA* and *vanB* genes	vanA_F: GGAAAACGACAATTGCTATT vanA_R: GTACAATGCGGCCGTTA	731 bp	(132, 134)
		vanB_F: ACTGGCCTACATTCTTACA vanB_R: AGCGTTTAGTTCTTCCGT	175 bp	
	vanC1 and *vanC2/3* genes	vanC1_F: GGTATCAAGGAAACCTC vanC1_R: CTTCCGCCATCATAGCT	822 bp	(132)
		vanC2/3_F: CTCCTACGATTCTCTTG vanC2/3_R: CGAGCAAGACCTTTAAG	439 bp	
Linezolid-resistance genes detection	*cfr* , *optrA* and *poxtA* genes	cfr_F: TGAAGTATAAAGCAGGTTGGGAGTCA cfr_R: ACCATATAATTGACCACAAGCAGC	746 bp	(135)
		optrA_F: TACTTGATGAACCTACTAACCA optrA_R: CCTTGAACTACTGATTCTCGG	422 bp	
		poxtA_F: AAAGCTACCCATAAAATATC poxtA_R: TCATCAAGCTGTTCGAGTTC	533 bp	
Enterococcus virulence factors	IS16	IS16-F: CATGTTCCACGAACCAGAG IS16-R: TCAAAAAGTGGGCTTGGC	547 bp	(136)
	asa1 and *gelE* genes	asa1_1F: GCACGCTATTACGAACTATGA asa1_2: TAAGAAAGAACATCACCACGA	375 bp	(137)
		gel1_1: TATGACAATGCTTTTTGGGAT gel1_2: AGATGCACCCGAAATAATATA	213 bp	
	cylA gene	cyt-1: ACTCGGGGATTGATAGGC cyt-lib: GCTGCTAAAGCTGCGCTT	688 bp	(137)
	esp and *hyl* genes	esp14F: AGATTTCATCTTTGATTCTTGG esp12R: AATTGATTCTTTAGCATCTGG	510 bp	(137)
		hyl-n1: ACAGAAGAGCTGCAGGAAATG hyl-n2: GACTGACGTCCAAGTTTCCAA	276 bp	
	ace gene	ace1_ F: GGAATGACCGAGAACGATGGC ace1_R: GCTTGATGTTGGCCTGCTTCCG	616 bp	(137)

5. Summary tables

Table 5 – Summary of the phenotypic antimicrobial characterization and molecular detection of virulence and *van* genes of the 12 *E. faecalis* with *vanC1* isolated from cows.

		Phenotypic resistance															HLAR		Resistance genes	Virulence genes
Collection	Isolates	AMP	C	CIP	E	CN	LNZ	QD	TC	TE	TIG	VAN	F	LEV	W	SXT	CN	S		
Cows	Cow11Ucol1																		*vanC1*	*asa1, gelE, ace*
	Cow15Ncol1																		*vanC1*	*gelE, ace*
	Cow16Ncol1																		*vanC1*	*gelE, ace*
	Cow16Ucol1																		*vanC1*	*gelE, ace*
	Cow17Ncol1																		*vanC1*	*gelE, ace*
	Cow18Ucol1																		*vanC1*	*gelE, ace*
	Cow20Ncol1																		*vanC1*	*gelE, ace*
	Cow21Ncol1																		*vanC1*	*gelE, ace*
	Cow22Ncol1																		*vanC1*	*gelE, ace*
	Cow23Ncol1																		*vanC1*	*gelE, ace*
	Cow24Ncol1																		*vanC1*	*gelE, ace*
	Cow25Ncol1																		*vanC1*	*gelE, ace*

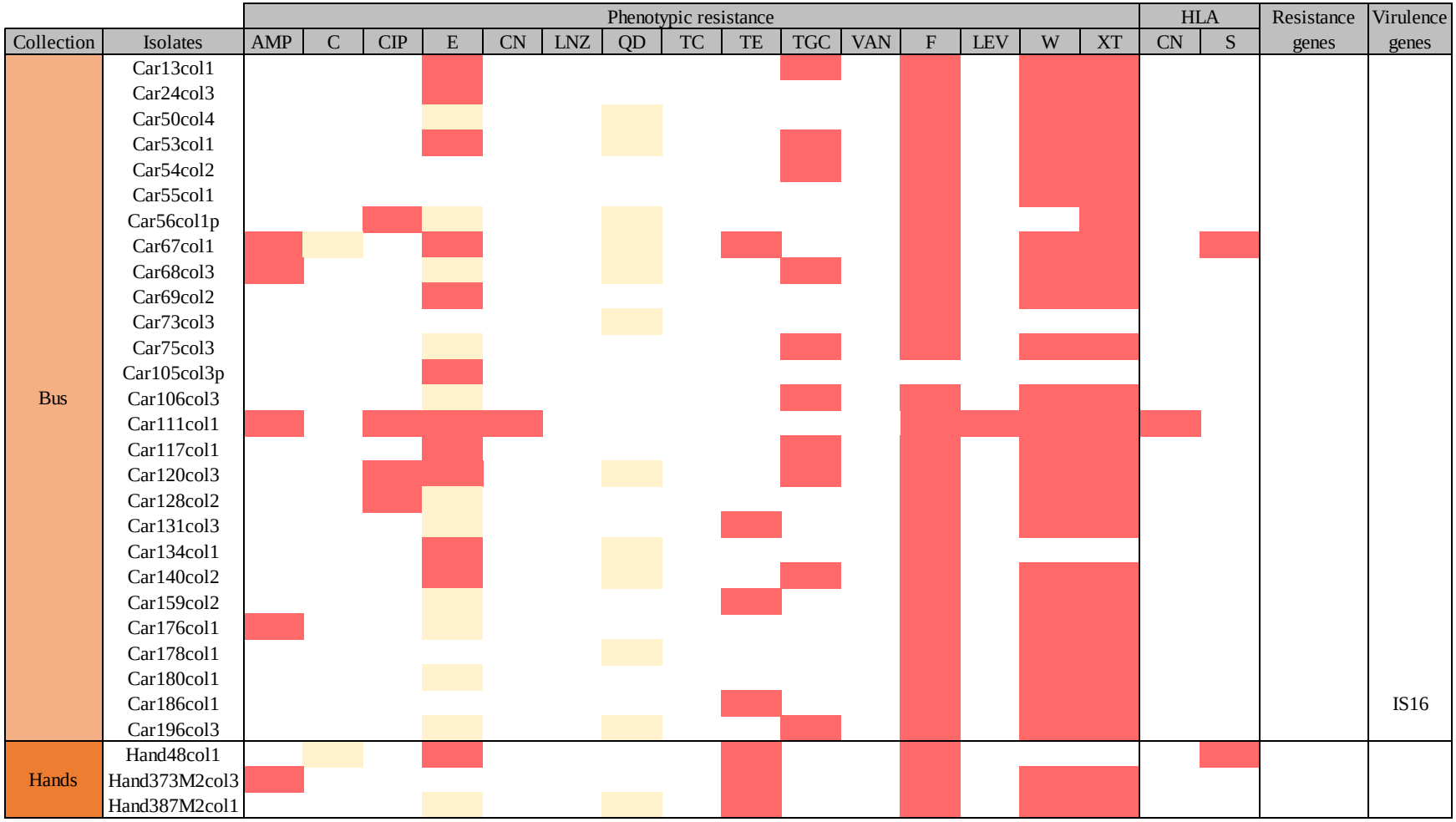
Resistant Intermediate Susceptible

AMP- ampicillin; C- chloramphenicol; CIP- ciprofloxacin; E- erythromycin; CN- gentamicin; LNZ- linezolid; QD- quinupristin-dalfopristin; TC- teicoplanin; TE- tetracycline; TGC- tigecycline; VAN- vancomycin; F- nitrofurantoin; LEV- levofloxacin; W- trimethoprim; SXT- trimethoprim-sulfamethoxazole; HLAR- high-level aminoglycosides resistance; S- streptomycin.

Table 6 – Summary of the phenotypic antimicrobial characterization and molecular detection of virulence and *van* genes of the 59 *E. faecium* isolated from the five collections studied.

		Phenotypic resistance															HLA		Resistance genes	Virulence genes
Collection	Isolates	AMP	C	CIP	E	CN	LNZ	QD	TC	TE	TGC	VAN	F	LEV	W	XT	CN	S		
Pigs	Pig57p																			
	Pig66g																			
	Pig66p																			
	Pig90																			
	Pig182col1																			
	Pig188p																			
	Pig193g																			
	Pig195g																			
	Pig199gec																			
	Pig205pCH																			
	Pig209p																			
Cows	Cow2Ucol1																			IS16
	Cow3Ncol1																			
	Cow3Ucol1																			
	Cow4Ncol3																			
	Cow7Ucol1																			
	Cow8Ncol1																			
AMB	AMB2col2																			
	AMB15col3																			
	AMB16col1																			
	AMB23col1																			IS16
	AMB25col2																			IS16
	AMB35col1																			
	AMB41col3																			
	AMB43col3																			
	AMB46col1																			
	AMB47col1																			
	AMB48col2																			
	AMB49col3																			

Table 6 – Continuation.



		Phenotypic resistance															HLA		Resistance genes	Virulence genes
Collection	Isolates	AMP	C	CIP	E	CN	LNZ	QD	TC	TE	TGC	VAN	F	LEV	W	XT	CN	S		
Bus	Car13col1																			
	Car24col3																			
	Car50col4																			
	Car53col1																			
	Car54col2																			
	Car55col1																			
	Car56col1p																			
	Car67col1																			
	Car68col3																			
	Car69col2																			
	Car73col3																			
	Car75col3																			
	Car105col3p																			
	Car106col3																			
	Car111col1																			
	Car117col1																			
	Car120col3																			
	Car128col2																			
	Car131col3																			
	Car134col1																			
	Car140col2																			
	Car159col2																			
	Car176col1																			
	Car178col1																			
	Car180col1																			
	Car186col1																			IS16
	Car196col3																			
Hands	Hand48col1																			
	Hand373M2col3																			
	Hand387M2col1																			

Table 7- Summary of the genomic data analysis regarding antimicrobial resistance, virulence and plasmids characterization of the 23 *E. faecium* selected for WGS.