Antimicrobial Susceptibility of Enterococcus Isolates from Cattle and Pigs in Portugal: Linezolid Resistance Genes optrA and poxtA

Joana Gião 1,2, Célia Leão 1,3, Teresa Albuquerque 1, Lurdes Clemente 1,4 and Ana Amaro 1,*

Abstract: Enterococci are part of the commensal gut microbiota of mammals, with Enterococcus faecalis and Enterococcus faecium being the most clinically relevant species. This study assesses the prevalence and diversity of enterococcal species in cattle (n = 201) and pig (n = 249) cecal samples collected in 2017. Antimicrobial susceptibility profiles of E. faecium (n = 48) and E. faecalis (n = 84) were assessed by agar and microdilution methods. Resistance genes were screened through PCR and nine strains were analyzed by Whole Genome Sequencing. A wide range of enterococci species was found colonizing the intestines of pigs and cattle. Overall, the prevalence of resistance to critically important antibiotics was low (except for erythromycin), and no glycopeptide-resistant isolates were identified. Two daptomycin-resistant E. faecalis ST58 and ST93 were found. Linezolid-resistant strains of E. faecalis (n = 3) and E. faecium (n = 1) were detected. Moreover, oxazolidinone resistance determinants optrA (n = 8) and poxtA (n = 2) were found in E. faecalis (ST16, ST58, ST207, ST474, ST1178) and E. faecium (ST22, ST2138). Multiple variants of optrA were found in different genetic contexts, either in the chromosome or plasmids. We highlight the importance of animals as reservoirs of resistance genes to critically important antibiotics.

Keywords: Enterococcus spp.; pigs; cattle; Linezolid resistance; optrA gene; WGS

1. Introduction

The Enterococcus genus comprises over 50 species of ubiquitous Gram-positive bacteria found in the environment and the gastrointestinal tract of various hosts, including humans and other mammals, birds, and invertebrates, as part of their normal microbiome [1,2]. The presence and diversity of enterococci species can significantly vary according to host species, age, diet, gastrointestinal tract region, environmental stress, and season [3–5]. Enterococcus faecalis and Enterococcus faecium represent up to 1% of the adult gut microbiota in humans [4] and are the two most relevant species associated with multidrug resistance and nosocomial infections.

Although members of the Enterococcus genus are considered commensal bacteria, they can also become opportunistic pathogens in favorable environmental conditions. Hospital-acquired enterococcal infections became a cause of global concern due to their increasing prevalence and resistance to several classes of antibiotics [6]. These bacteria are also known for efficiently recruiting and exchanging antibiotic resistance determinants. Various enterococci strains acquired resistance to many last-resort antibiotics, such as vancomycin, daptomycin, linezolid and tigecycline [7]. Glycopeptides, lipopeptides, oxazolidinones
and glyclycyclines have been placed in category A (Avoid) of the European Medicines Agency (EMA) categorization of antibiotics in the European Union (EU), currently not being approved for veterinary use [8].

Studies suggest that *E. faecium* isolates from animals may act as donors of antibiotic resistance determinants to human-adapted bacteria after ingestion of products of animal origin [9]. There is also evidence that *E. faecalis* strains from animals may be considered a hazard to humans [9]. For instance, the emergence of vancomycin-resistant enterococci (VRE) due to the overuse of the vancomycin analog avoparcin as a growth promoter in farm animals is suspected of having contributed to human VRE outbreaks in some countries [10].

Under the One Health concept, EU countries have implemented surveillance programs monitoring resistance to critically important antibiotics of commensal bacteria from farm animals [11]. In the case of enterococci, these antibiotics include last-resort antibiotics such as glycopeptides, linezolid, and daptomycin. Nevertheless, enterococci surveillance is not mandatory, and thus in several countries, no antimicrobial resistance surveillance program is routinely applied to *Enterococcus* spp. in farm animals yearly, including Portugal.

Resistance to glycopeptides in enterococci has been mostly associated with the *vanA* and *vanB* gene clusters that allow for the synthesis of alternative cell wall precursors with low binding affinity to vancomycin [12]. Linezolid resistance can be linked with mutations in the V domain of the 23S rRNA and the rplC/rplD genes coding for the L3/L4 ribosomal proteins or with the acquisition of oxazolidinone resistance genes such as *cfr* [13], which encodes a 23S rRNA modifying methyltransferase, and *optrA* [14] and *poxtA* [15], two genes encoding ABC-F proteins that presumably protect the ribosomal target from binding to the antibiotic. The underlying mechanisms conferring reduced susceptibility in enterococci are not entirely understood regarding daptomycin. Non-susceptibility to daptomycin has been connected to mutations in multiple genes. Most of them involved cell envelope stress response, metabolism of important cell membrane phospholipids, or peptidoglycan biosynthesis [7,16].

The usage of glycopeptides in farm animals has been unauthorized in the EU for over two decades. However, vancomycin-resistant enterococci carrying the *van* operon (particularly the *vanA* gene) were still being recovered from samples of food-producing animals years after the ban [9,17–20].

Resistance to the last-resort antibiotics linezolid and daptomycin has been reported in enterococci strains from European countries including Portugal [7]. In 2018, the overall prevalence of resistance to last-resort antibiotics among enterococci from humans in European countries (such as Denmark, Poland, Spain, Ireland, France, and Portugal) was very low (1%) [7]. Because those antibiotics are not approved for veterinary use in the EU, reports of resistant strains are more frequent in humans. Nevertheless, linezolid resistant enterococci from food-producing animals have been described in Europe [21–23]. Very few linezolid resistant *E. faecium* and *E. faecalis* isolates from broilers, cattle, and pigs were detected in countries such as Belgium, Croatia, France, Spain, and The Netherlands [22]. *Enterococcus* carrying the *poxtA* and *optrA* genes have been found in samples from a swine farm in Spain [24] and various food-producing animals in Belgium [25]. An emergence of linezolid-resistant human clinical *Enterococcus* isolates harboring these genes was also registered in European countries [25–28].

Regarding daptomycin, non-susceptibility is rare in humans and can emerge with or without prior exposure to the antibiotic [29]. Daptomycin non-susceptible enterococci have been reported in samples of food-producing animals from Lithuania and Denmark [30,31].

Here, we aim to investigate the diversity and frequency of gut colonization of cattle and pigs by *Enterococcus* and evaluate the antimicrobial susceptibility patterns of *E. faecalis* and *E. faecium* strains. Moreover, some antimicrobial resistance determinants to critically important antibiotics, including vancomycin and linezolid were also searched. To our knowledge, this study reports, for the first time in Portugal, the occurrence of daptomycin non-susceptible enterococci and the linezolid resistance-encoding genes *optrA* and *poxtA* from food-producing animals.
2. Results

2.1. Enterococcus Isolation and Species Diversity

A total of 314 presumptive Enterococcus spp. were isolated among 450 bovine and swine cecal samples. The genus-specific PCR assay confirmed that 292 isolates belonged to the Enterococcus genus, 138 were recovered from cattle and 154 from pigs, with recovery rates averaging around 69% for bovine and 62% for swine samples.

The distribution and diversity of Enterococcus species identified from the cecal samples of healthy bovines and swine are illustrated in Figure 1. The isolates without identification to the species level \( n = 13 \) remained classified as Enterococcus spp.

![Figure 1. Distribution of Enterococcus species in cattle and pigs.](image)

Among the 292 Enterococcus spp. strains, the multiplex PCR assays identified 85.6% of the isolates as Enterococcus hirae \( n = 107 \), Enterococcus faecalis \( n = 84 \), Enterococcus faecium \( n = 48 \), Enterococcus casseliflavus \( n = 7 \) and Enterococcus durans \( n = 4 \). Sanger sequencing of the 16S rRNA gene allowed the identification of Enterococcus hirae \( n = 6 \), Enterococcus asini \( n = 3 \) and Enterococcus thailandicus \( n = 2 \). Additionally, the API® 20 Strep system also identified E. hirae \( n = 12 \), E. casseliflavus \( n = 1 \) and E. durans \( n = 2 \).

The most frequent species of Enterococcus found in pigs were E. faecalis (42.9%), E. faecium (23.4%), and E. hirae (22.8%), representing nearly 90% of the isolates (Figure 1). Other species of Enterococcus, namely E. durans \( n = 4 \), E. casseliflavus \( n = 4 \), E. thailandicus \( n = 2 \), and E. asini \( n = 3 \), were also found. E. hirae was the most abundant species recovered from bovine cecal samples, comprising 65.2% of the isolates (Figure 1). Other species recovered from cattle included E. faecalis \( n = 18 \), E. faecium \( n = 12 \), E. casseliflavus \( n = 4 \), E. durans \( n = 2 \) and E. mundtii \( n = 3 \).

2.2. Antimicrobial Susceptibility Testing by Agar Dilution

The antimicrobial susceptibility profiles of 84 E. faecalis and 48 E. faecium isolates from cattle \( n = 30 \) and pigs \( n = 102 \) were established. Important parameters comprising the MICs\(_{50}\), MICs\(_{90}\) and the frequencies of decreased susceptibility are summarized in Table 1.
Table 1. Antimicrobial susceptibility of *E. faecalis* and *E. faecium* isolates (*n* = 132) using the agar dilution technique.

| Antimicrobial | Criteria (a) | *E. faecalis* (n = 84) | *E. faecium* (n = 48) |
|---------------|-------------|------------------------|-----------------------|
|               | (T)ECOFF (b) | Cattle (n = 18)         | Pigs (n = 66)         | Cattle (n = 12) | Pigs (n = 36) |
| **Vancomycin** |             |                        |                       |               |               |
| MIC<sub>50</sub> | ≤1          | ≤1                     | ≤1                    | ≤1            | ≤1            |
| MIC<sub>90</sub> | 4           | 2                      | 4                     | 1             | 0             |
| % DS          | 0           | 0                      | 0                     | 0             | 0             |
| **Teicoplanin** |             |                        |                       |               |               |
| MIC<sub>50</sub> | ≤0.5        | ≤0.5                   | ≤0.5                  | ≤0.5         | ≤0.5         |
| MIC<sub>90</sub> | 2           | 2                      | 2                     | 1             | 0             |
| % DS          | 0           | 0                      | 0                     | 0             | 0             |
| **Tetracycline** |             |                        |                       |               |               |
| MIC<sub>50</sub> | ≤1          | 128                    | 16                    | 128           | 128           |
| MIC<sub>90</sub> | 64          | 128                    | 4                     | 58            | 78            |
| % DS          | 44<sup>(c)</sup> | 98<sup>(c)</sup>    |                       |               |               |
| **Ciprofloxacin** |             |                        |                       |               |               |
| MIC<sub>50</sub> | 4           | 1                      | 2                     | 4             | 2             |
| MIC<sub>90</sub> | 2           | 4                      | 8                     | 4             | 2             |
| % DS          | 0           | 9                      | 0                     | 0             | 0             |
| **Erythromycin** |             |                        |                       |               |               |
| MIC<sub>50</sub> | ≤1          | >128                   | ≤8                    | ≤8            | ≤8            |
| MIC<sub>90</sub> | >128        | 128                    | 32                    | 128           | 128           |
| % DS          | 17<sup>(c)</sup> | 86<sup>(c)</sup>   |                       |               |               |
| **Linezolid** |             |                        |                       |               |               |
| MIC<sub>50</sub> | 2           | 1                      | 2                     | 2             | 2             |
| MIC<sub>90</sub> | 2           | 2                      | 4                     | 2             | 2             |
| % DS          | ND          | -                      | -                     | 0             | 0             |
| **Gentamicin** |             |                        |                       |               |               |
| MIC<sub>50</sub> | ≤8          | ≤8                     | ≤8                    | ≤8            | ≤8            |
| MIC<sub>90</sub> | 64          | 128                    | 32                    | 128           | 128           |
| % DS          | 0           | 11                     | 0                     | 0             | 0             |
| **Ampicillin** |             |                        |                       |               |               |
| MIC<sub>50</sub> | ≤0.5        | 1                      | 1                     | 1             | 1             |
| MIC<sub>90</sub> | 2           | 2                      | 8                     | 1             | 8             |
| % DS          | 0           | 0                      | 0                     | 0             | 0             |
| **Chloramphenicol** |             |                        |                       |               |               |
| MIC<sub>50</sub> | ≤4          | 8                      | ≤4                    | ≤4            | ≤4            |
| MIC<sub>90</sub> | 32          | 64                     | 32                    | 16            | 16            |
| % DS          | 0<sup>(c)</sup> | 27<sup>(c)</sup>   |                       |               |               |

DS, Decreased susceptibility according to the epidemiological breakpoints; ND, Not determined. <sup>(a)</sup> MIC<sub>50</sub>/90 (µg/mL), % resistance (R) is based on the summed isolate numbers; <sup>(b)</sup> Tentative epidemiological cut-off values established by EUCAST; <sup>(c)</sup> *p*-value ≤ 0.05.

Overall, this study showed moderate to high decreased susceptibility rates to tetracycline (44–98%). Isolates with decreased susceptibility to erythromycin were significantly more prevalent in pigs (*p*-value ≤ 0.05) for both *E. faecium* and *E. faecalis* strains. No isolates were resistant to glycopeptides, namely vancomycin and teicoplanin. Regarding *E. faecium*, decreased susceptibility to erythromycin was high (58%) and found exclusively in isolates from pigs. Decreased susceptibility to chloramphenicol and ampicillin was rarely observed in isolates from both animal species. Moreover, no isolates displayed decreased susceptibility to ciprofloxacin, gentamicin, and linezolid. Concerning *E. faecalis* isolates, decreased susceptibility to chloramphenicol and tetracycline was prevalent in pigs (*p*-value ≤ 0.05), followed by erythromycin (17–86%). Among seven isolates of non-wild-type gentamicin, six were also resistant to ciprofloxacin. One *E. faecalis* isolated from pigs displayed resistance to linezolid (MIC = 8 µg/mL). Resistance to ampicillin was not found.

Major differences (over three dilution steps) between MIC<sub>50</sub> and MIC<sub>90</sub> values were found for tetracycline in *E. faecium* isolates of bovine origin, gentamicin in *E. faecalis* isolates from pigs, and erythromycin in *E. faecalis* from cattle.
Multidrug resistance (MDR) was noticed in 27.4% of *E. faecalis* and 4.2% of *E. faecium* isolates from pigs (Figure 2). The most prevalent MDR profile in *E. faecalis* strains was tetracycline-erythromycin-chloramphenicol. Two different multidrug resistance profiles were found among *E. faecium* strains, one of them unique to this species. Seven MDR patterns were identified in *E. faecalis* isolates. Resistance to both tetracycline and erythromycin was present in all. Full susceptibility was observed in 27.0% of *E. faecium* and 11.9% of *E. faecalis* and more frequent in *E. faecalis* isolates from bovines than swine (p-value ≤ 0.05).

![Diversity of multidrug resistance profiles found in (a) *E. faecalis* (n = 84) and (b) *E. faecium* (n = 48) strains from swine using the agar dilution susceptibility test. MDR, Multidrug-resistant; TET, Tetracycline; ERY, Erythromycin; LZD, Linezolid; AMP, Ampicillin; CLO, Chloramphenicol; CIP, Ciprofloxacin; GEN, Gentamicin.](image)

**Figure 2.** Diversity of multidrug resistance profiles found in (a) *E. faecalis* (n = 84) and (b) *E. faecium* (n = 48) strains from swine using the agar dilution susceptibility test. MDR, Multidrug-resistant; TET, Tetracycline; ERY, Erythromycin; LZD, Linezolid; AMP, Ampicillin; CLO, Chloramphenicol; CIP, Ciprofloxacin; GEN, Gentamicin.

2.3. PCR Screening of Antimicrobial Resistance Determinants

Ninety isolates with linezolid MIC values of 1–8 µg/mL were subjected to the PCR assays targeting the *optrA* gene. The *optrA* gene was detected in six strains of *E. faecalis* and two *E. faecium*, all sourced from pigs.

Among the isolates exhibiting decreased susceptibility to chloramphenicol (n = 20), none harbored the *cfr* gene.

All isolates were negative for the detection of *vanA* and *vanB* genes, corroborating the vancomycin susceptibility profile observed.

2.4. Antimicrobial Susceptibility Testing of *optrA* Positive Strains by Microdilution

All isolates harboring the *optrA* gene (n = 8) were further subjected to antimicrobial susceptibility testing using commercially available EUVENC microplates to confirm linezolid MIC values obtained by agar dilution (Figure 3). An additional set of 13 isolates (nine *E. faecalis* and four *E. faecium*) was also tested.

Overall, the results obtained using the EUVENC microplates were like those obtained by the agar dilution technique (data not shown). For some antibiotics, such as linezolid and chloramphenicol, a few strains exhibited MICs one dilution step higher in the EUVENC microplates compared to the agar dilution technique.

Notably, three isolates (*E. faecium* INIAV004, *E. faecalis* INIAV168 and *E. faecalis* INIAV171) displayed linezolid MIC = 4 µg/mL using the agar dilution technique and MIC = 8 µg/mL in the EUVENC plates. All isolates carrying the *optrA* gene displayed decreased susceptibility to chloramphenicol (MIC > 32 µg/mL) in the EUVENC microplates.
Two *E. faecalis* isolates from pigs (INIAV005 and INIAV175) showed decreased susceptibility to daptomycin with MICs = 8 µg/mL and 16 µg/mL, respectively.

2.5. Genomic Characterization of Isolates

The genomic content of nine strains, including MLSTs, acquired resistance genes, and virulence genes are shown in Appendix A Table A1. The strains belonged to eight different sequence types, including ST16, ST58, ST93, ST207, ST474 and ST1178 for *E. faecalis* and ST22 for *E. faecium* INIAV004. In addition, a novel *E. faecium* sequence type was observed in isolate INIAV173, submitted to PubMLST and assigned as ST2138.

A great variety of virulence factors were found in all strains of *E. faecalis*, which included the *elrA*, *srtA*, *ace*, *agg*, *ccf10*, *cob1*, *cad*, *camE*, *cylA*, *cylL*, *cylM*, *ebpA*, *ebpC*, *efaAfs*, *hylA* and *tpx* genes; *acm* and *efaAfm* virulence genes were detected in *E. faecium* strains.

Other than INIAV005, all isolates carried *repLIS43*. Other plasmid replicons found were *repLIS1*, *rep9a*, *rep9b* and *rep6* in *E. faecalis* strains and in *rep1*, *rep2*, *rep11c*, *rep18b*, *rep29*, *repLIS15* and *rep33* in *E. faecium* strains.

The following antimicrobial resistance genes were detected: *erm*(A) and *erm*(B) (conferring the macrolide-lincosamide-streptogramin B resistance profile), *ant*(9)-Ia, *aac(6’)-aph(2’’)* and *aph(3’)-III* (aminoglycoside resistance genes), *tet*(M) and *tet*(L) (encoding resistance to tetracyclines), *dftrG* (a trimethoprim resistant determinant), *lmu*(B) and *Iso*(E) (which confer the lincosamide and pleuromutilin-lincosamide-streptogramin A phenotypes, respectively), *caA1tA*, *catB*, *fexA* and *fexB* (encoding phenicol resistance), *poxtA* (conferring decreased susceptibility to phenicols, oxazolidinones and tetracyclines) and *optrA* (a gene that can confer resistance to oxazolidinones and phenicols).

Genomic analyses of the *E. faecium* isolate INIAV004 also revealed the presence of several mutations of the *ppb5* gene encoding a low-affinity species-specific class B penicillin-binding protein PBP5. *Enterococcus faecalis* INIAV169, INIAV170 and INIAV174 showed mutations in the *gyrA* and *parC* genes, both encoding resistance to quinolones.

2.5.1. Molecular Characterization of Linezolid Resistance Mechanisms

The *optrA*-harboring strains, variants [31–33], and linezolid MICs are mentioned in Table 2.
Table 2. OptrA variants and linezolid MICs detected among *E. faecium* (*n* = 2) and *E. faecalis* (*n* = 5) isolates from pigs.

| Strain   | Species | EUVENC MIC *(µg/mL)* | Interpretation *(a)* | OptrA Variant | Amino Acid Substitutions |
|----------|---------|----------------------|----------------------|----------------|------------------------|
| INIAV173 | *E. faecium* | 4 | S | DVD *(b)* | OptrA_28 *(b)* | Y176D, A350V, G393D |
| INIAV004 | *E. faecium* | 8 | R | WT | OptrA_1 | V19 |
| INIAV168 | *E. faecalis* | 8 | R | DP | OptrA_8 | V22, Y176D, T481P |
| INIAV169 | *E. faecalis* | 4 | S | EDD | OptrA_7 | V34, K3E, Y176D, G393D |
| INIAV170 | *E. faecalis* | 8 | R | EDD | OptrA_7 | V34, K3E, Y176D, G393D |
| INIAV171 | *E. faecalis* | 2 | S | EDD | OptrA_7 | V34, K3E, Y176D, G393D |

MIC, Minimum Inhibitory Concentration; S, Susceptible; R, Resistant. *(a)* According to clinical breakpoints provided by EUCAST. *(b)* Tentative variant name following the criteria set in the references abovementioned.

A total of four different OptrA variants were identified: WT, DP, EDD, and DVD.

The isolates carrying optrA variants EDD (*E. faecalis* strains INIAV169, INIAV170 and INIAV174) and DVD (*E. faecium* INIAV173) displayed linezolid MICs ≤ 4 µg/mL, while the strains with the WT (*E. faecium* INIAV004) and DP (*E. faecalis* strains INIAV168 and INIAV171) variants consistently exhibited linezolid MICs > 4 µg/mL.

Both *Enterococcus faecium* strains INIAV004 and INIAV173 also harbored the *poxtA* gene with 100% nucleotide sequence identity with the wild-type gene (GenBank accession number MH746818.1). In these two isolates, the *poxtA* and *optrA* genes were in separate contigs.

In isolates with decreased susceptibility to linezolid, mutations in the 23S rRNA were not detected and mutations leading to amino acid changes in proteins L3 and L4 were also not identified through sequence alignment.

2.5.2. Genetic Environment of the optrA Gene

Analyses of the contigs containing the optrA gene in isolates INIAV169, INIAV170, INIAV174, and INIAV173 revealed that in these strains the gene appeared to be in the chromosomal DNA. In the case of isolates INIAV004, INIAV168, INIAV171, the optrA gene was seemingly located in plasmids.

In all genetic backgrounds surrounding the optrA gene, the ermA or an ermA-like gene was present. In the case of *E. faecalis* isolates INIAV168 and INIAV171, the ermA-like gene was detected downstream of optrA while in all other isolates the ermA gene was located upstream of optrA. The fexA gene was also present upstream of optrA in *E. faecalis* strains INIAV168, INIAV169, INIAV170 and INIAV171.

The genetic context of the optrA gene was similar in *E. faecalis* strains INIAV168 and INIAV171. The *impB*, fexA, and ermA genes surrounded optrA in an *impB*-fexA-optrA-ermA arrangement flanked upstream by an ISL3-like element. The respective contigs had nucleotide sequences with 100% identity (query cover 100%) with the previously described plasmid p10-2-2A (GenBank accession number KT862775). These sequences were aligned and are presented in Figure 4.

In the isolates carrying the optrA gene in the chromosome, no transposable elements were found in the vicinity of the gene.
Figure 4. Alignment of the optrA-containing contigs of strains INIAV168 and INIAV171 with the previously described optrA-carrying plasmid p10-2-2 (GenBank accession number KT862775).

3. Discussion

In this study, we analyzed isolates from the caeca of cattle and pigs collected in 2017 under the scope of the surveillance program of antimicrobial resistance in zoonotic and commensal bacteria. Several species of enterococci were found colonizing the intestine of cattle and pigs, such as *E. faecalis*, *E. hirae*, *E. faecium*, *E. durans*, and *E. casseliflavus*.

The predominant species found in cattle was *E. hirae* and in pigs were *E. faecalis*, *E. hirae*, and *E. faecium*. Our results are similar to those found in other studies [35–40], although the relative frequencies of each *Enterococcus* species may vary between studies due to differences in diet, host, and environment-associated factors. Species identification by Sanger sequencing allowed the identification of *E. asini* and *E. thailandicus* in swine, two species seldomly reported in pigs [40], and *E. mundtii*, which is common in cattle [37]. However, thirteen isolates remained as *Enterococcus* spp., either because the species were not included in our PCR assays or due to the low discriminatory power of the 16S rRNA gene when differentiating closely related enterococcal species. Therefore, other techniques such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) or Sanger sequencing of additional genes such as the *sodA* or *tuf* genes could be applied [41].

Overall, the results of antibiotic susceptibility testing obtained are comparable to those found in a previous study assessing the antibiotic susceptibility of enterococci from healthy food-producing animals collected in different European countries from 2004 to 2014 [21].

The frequency of antimicrobial decreased susceptibility was higher for several antibiotic classes in *E. faecalis* compared with *E. faecium* strains, particularly in swine (*p*-value ≤ 0.05). Decreased susceptibility to tetracyclines was widespread. Except for erythromycin, the prevalence of decreased susceptibility to glycopeptides and oxazolidinones, both critically important antibiotics in human medicine, was either low or absent. These results are in accordance with reports showing tetracyclines and macrolides among the most purchased antibiotic classes in Portugal between 2010 and 2018 (ESVAC) [42].

Decreased susceptibility to ampicillin was displayed only by *E. faecium* isolates from swine, as expected since ampicillin is very rare in *E. faecalis* strains. Decreased susceptibility to ampicillin and gentamicin was not observed in any animal species. These antibiotics are frequently used in combination to treat enterococcal infections, and co-resistance to both antimicrobials is uncommon [21].
Regarding gentamicin, MIC\(_{50}\) and MIC\(_{90}\) values observed in *E. faecalis* isolates from pigs may suggest the presence of more than one subpopulation. However, large MIC\(_{50}\) and MIC\(_{90}\) differences between isolates from cattle may not indicate the same since only a few isolates (under 18) were studied [43].

Although decreased susceptibility to chloramphenicol was found in isolates from both *Enterococcus* species, the *cfr* determinant was not detected among these strains, indicating that other phenicol resistance determinants may be present.

In the present study, phenotypic and genotypic resistance to glycopeptides was not observed. Our results contrast with a previous study reporting the *vanA* operon in isolates from 2005 to 2012 collected from food-producing animals in Portugal [44]. Therefore, the absence of vancomycin resistance determinants seen in our isolates is most likely due to the ban on glycopeptides usage in food-producing animals in 1997 [45]. These results are encouraging because they may indicate that this resistance mechanism was finally eradicated in cattle and pigs from Portugal 20 years after banning avoparcin. Nevertheless, national surveillance programs also focused on *Enterococcus* spp. should be implemented on farm animals to confirm these results.

In the present study, the frequencies of MDR in enterococci from farm animals were similar to those described in other European countries [31,46] but lower than those reported in the United States [47], China [48], and Malaysia [49]. MDR isolates were exclusively sourced from swine, while isolates with susceptibility to all antibiotic classes were found more frequently in cattle. The differences between animal species regarding the levels of decreased antibiotic susceptibility and prevalence of MDR strains could reflect the distinct husbandry and antibiotic use practices employed in cattle and pig farming in Portugal.

Regarding antibiotic use for animals in Portugal, overall sales fluctuated, showing a peak in 2016, followed by a decrease in 2017 [42]. In 2020, an overall 19.9% increase in sales was recorded compared to 2019, with tetracyclines, penicillins and macrolides continuing to be the most frequently purchased classes of antibiotics [42].

Globally, we found three *E. faecalis* and one *E. faecium* clinically resistant to linezolid after using the EUVENC microplates. Three *optrA*-carrying enterococci susceptible to linezolid in the agar dilution test were clinically resistant to linezolid (MIC > 4 \(\mu\)g/mL) using the microdilution technique. The one-fold discrepancy observed between methods is common and may occur using different methods due to inherent methodology variations [50].

Antimicrobial resistance profiles predicted by WGS were generally consistent with those displayed in the antibiotic susceptibility tests performed. Interestingly, *E. faecalis* INIAV171 harbored the gene encoding the bifunctional aminoglycoside modifying enzyme AAC(6')-Ie/APH(2')-Ia, which confers resistance to a broad spectrum of aminoglycosides, including high-level gentamicin resistance [51,52], but was susceptible to this antimicrobial agent (MIC = 64 \(\mu\)g/mL). *Enterococcus faecium* INIAV004 was predicted to be resistant to ampicillin but remained susceptible to this antibiotic despite showing several *pbp5* point mutations. This may happen due to the absence of specific amino acid substitutions, such as M485A and E629V, occurring mostly around the active-site region of PBP5 or mutations associated with the addition of a serine at position 466, which are more often responsible for increased MICs to ampicillin in enterococci [53–55]. Other factors, such as regulation, expression, and translational modifications of the *pbp5* gene or other genes, can also interfere with ampicillin MICs [56].
Oxazolidinone resistance determinants optrA and poxtA were found in the present study in enterococci isolates from healthy pigs. These results are most likely associated with the extensive veterinary use of other antimicrobials such as florfenicol and tiamulin [57] since oxazolidines are not approved for veterinary use on farm animals [8,58]. The optrA gene was identified in eight isolates and the presence of this gene did not always confer clinical resistance to linezolid. In addition, the poxtA gene (known to confer decreased susceptibility to phenicols, oxazolidinones, and tetracyclines) was co-carried in E. faecium strains INIAV004 and INIAV173, but INIAV173 remained susceptible to linezolid. Linezolid resistant isolates did not possess the cfr gene or additional mutations of the rplC, rplD and 23S rRNA genes.

Isolates carrying the optrA gene all belonged to different sequence types, except for E. faecalis strains INIAV169 and INIAV170, which belonged to ST474. E. faecalis ST474 and ST207 (assigned to INIAV168) carrying the optrA gene have already been found in human clinical isolates [59–61].

Of all E. faecalis sequence types, ST16 is the most frequently associated with the optrA gene and has been recovered from food-producing animals and several human clinical samples from various countries, [33,60,62–66]. Linezolid resistant E. faecalis INIAV171 ST16 strain is MDR co-harboring acquired antimicrobial resistance determinants associated with resistance to several antibiotic classes, including phenicols, tetracyclines, macrolides, aminoglycosides, lincosamides, pleuromutilins, and trimethoprim. ST16 is considered a zoonotic lineage involved in antimicrobial resistance dissemination [67].

The E. faecium INIAV004 strain belonged to ST22 and carried both the poxtA and optrA genes. Previous studies have reported ST22 strains co-carrying the optrA and poxtA genes sourced from human patients, poultry, swine, and bovines [7,23,68].

Nomenclature for OptrA variants is not uniform among different studies [32–34] creating some difficulties in comparing studies, and thus an extra effort should be made to standardize the designations of these variants.

In this report, strains with the same OptrA variants displayed similar susceptibility to linezolid. MIC values associated with each variant were close to or within the ranges that have been previously reported [32,34,66,69]. Although the same OptrA variants have displayed differences in linezolid MICs across studies, mutations of the optrA gene still appear to influence linezolid resistance levels [66,69]. In enterococci carrying optrA, linezolid MICs also seem to correlate with the genetic context surrounding the gene [69].

The optrA gene appeared to be carried either in the chromosome or in plasmids and with different genetic environments in the analyzed isolates. We often found fexA and ermA (or ermA-like genes) were located close to the optrA gene, as observed in many isolates with multiple genetic contexts [28,33,69,70]. Both E. faecalis strains INIAV168 and INIAV171 harbored the DP OptrA variant and shared an identical ISL3-impB-fexA-optrA-ermA genetic arrangement. The contigs containing these genes were highly similar to plasmid p10-2-2, which has also been described in the E. faecalis strain 10-2-2 (GenBank accession no KT862775) sourced from swine. Due to the short sequence size of the contigs, we were not able to determine the presence of the two IS1216 elements bracketing the optrA-carrying central region in p10-2-2, which could allow the mobilization of this DNA region [70].

In enterococci isolates harboring the optrA gene in the chromosome, the radC gene has often been reported as a favored site for the insertion of Tn554 family-flanked segments containing the optrA gene, disrupting the radC gene [28,33,34,69,70]. However, we did not find transposons from the Tn554 family (such as Tn558 and Tn6674), nor the associated tnpA, tnpB, and tnpC transposase genes or the radC gene in the vicinity of the optrA gene in isolates carrying this gene in the chromosome. In these strains, no other transposable elements were identified surrounding the optrA gene.
The putative transcriptional regulator \textit{araC} gene frequently found in the upstream region of \textit{optrA} was also not detected in any isolate.

The two daptomycin non-susceptible \textit{E. faecalis} strains from swine reported in our study were susceptible to other antibiotics except for erythromycin and tetracycline. The daptomycin non-susceptible \textit{E. faecalis} INIAV005 belonged to ST58, a sequence type mainly found in pigs \cite{71, 72} and \textit{E. faecalis} INIAV005 belonged to ST93, which has been detected in multiple animal species \cite{73, 74} and in humans \cite{75–77}.

There are no daptomycin formulations approved for animal use in the EU, and cross-resistance between daptomycin and other veterinary-approved antibiotic classes has not been reported. Although non-susceptibility to daptomycin has been associated with exposure to the antibiotic, the development of non-susceptibility without prior daptomycin use has also been documented \cite{29}. Thus, daptomycin non-susceptible enterococci emerged most likely due to spontaneous mutations. Nevertheless, the inappropriate use of this drug and the transmission of daptomycin non-susceptible enterococci from humans cannot be dismissed. The molecular basis associated with daptomycin non-susceptibility in these strains has not been clarified yet, and further investigation should be carried out.

\textit{Enterococcus} spp. are frequently considered food contaminants, although the risk of transmission from animals to humans through the food chain is based on indirect evidence \cite{67, 78}. The food and animal industries seem to have contributed to the spread of multidrug-resistant strains and certain lineages like \textit{E. faecalis} ST16, considered a zoonotic pathogen \cite{67}. In our study, we identified resistance genes (e.g., \textit{optrA}, \textit{poxTA}, \textit{fexA}, \textit{ermA}) \cite{28, 33, 69, 70}, ISs (e.g., IS\textsubscript{1216}) \cite{68}, and STs (e.g., ST16, ST22) \cite{26, 67} like those observed in humans and other animal species. These findings highlight the risk of the spread of antimicrobial resistance between animals and humans in the farm, slaughterhouse, and retail store environments \cite{8}. Moreover, \textit{optrA} genes were found to be co-located with phenicol resistance gene \textit{fexA} and macrolide resistance gene \textit{ermA}, and thus amphenicol use (or macrolide) could also result in cross-selection of linezolid resistant gene \textit{optrA} (and possibly \textit{poxTA}). It is important to stress that increased amphenicols sales have been observed from 2011 to 2020 in European countries, including Portugal \cite{42}. Amphenicols are currently listed as highly important antimicrobials for humans, placed in category C (Caution) of EMA’s categorization of antibiotics in the EU \cite{8}. Nonetheless, their rational use in veterinary settings should be emphasized to prevent the potential spread of resistance.

Our findings underline the risk of frequent and independent acquisition and selection events for antimicrobial resistance on farms through the pressure of antimicrobials usage in animal production.

Only a collaborative, multisectoral and transdisciplinary approach working at the local, regional, national, and global levels can achieve better public health, recognizing the interconnection between people, animals, and their shared environment.

4. Materials and Methods

4.1. Bacterial Isolation and Species Identification

Under the scope of the surveillance program of antimicrobial resistance in zoonotic and commensal bacteria (Commission Decision 2013/652/EU), cecal samples from randomly selected healthy bovines (\(n = 201\)) and swine (\(n = 249\)) were collected in 2017. Briefly, cecal samples were collected after evisceration at the slaughtering line, kept in plastic containers at a temperature of 4–8 °C, and sent to the laboratory for bacteriological analysis within two days.
Upon arrival, samples were inoculated in Heart Infusion Broth with 6% NaCl and incubated at 37 °C for 18 h. The broth cultures were then streaked on the selective medium BBL™ Enterococcosel™ Agar (Becton, Dickinson Company, Wantage, NJ, USA) and incubated under the same conditions. Individual presumptive enterococci colonies were transferred onto Colombia Blood Agar Base (Thermo Scientific™ Oxoid™, Basingstoke, United Kingdom), incubated for 18 to 22 h at 37 °C, and then stored in Tryptone Soy Broth (Thermo Scientific™ Oxoid™, Basingstoke, United Kingdom) with 15% glycerol at −80 °C.

DNA extraction of bacterial isolates followed the boiling lysis procedure, and the concentration and purity of the DNA suspensions were assessed using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific™, ThermoFisher Scientific, Pittsburgh, PA, USA). Confirmation of presumptive Enterococcus colonies was achieved by PCR amplification targeting the 16S rRNA gene as described by Deasy et al. (2000) [79].

The identification of five enterococci species (E. faecium, E. faecalis, E. hirae, E. durans, and E. casseliflavus) was carried out by multiplex PCR using the primer sets designed by Jackson et al. (2004) [80] in optimized thermal cycling conditions (Supplementary Materials Table S1).

Sanger sequencing of the 16S rRNA gene using the primers E1 and E2 described by Jackson et al. (2004) [80] was carried out in isolates not identified by the PCR assays. PCR products were purified using ExoSAP-IT™ (Applied Biosystems™, ThermoFisher Scientific, Pittsburgh, PA, USA) and sequenced at Eurofins Genomics Europe Sequencing GmbH, Konstanz, Germany. The DNA sequences were read with ChromasPro™ v2.1.8.0 (Technelysium Pty Ltd, South Brisbane, Australia) and analyzed with the Basic Local Alignment Search Tool (BLAST) [81]. In addition, consensus DNA sequences were generated using BioEdit v7.2.5.0 (Tom Hall Ibis Therapeutics, Carlsbad, CA, USA) sequence alignment editor and FASTA files were further analyzed with BLAST.

Few isolates for which molecular confirmation of species was not conclusive were further identified using the commercially available API® 20 Strep (bioMérieux, Marcy-l’Étoile, France).

4.2. Antimicrobial Susceptibility Testing

The antibiotic susceptibility of E. faecalis and E. faecium isolates was assessed by the agar dilution technique performed according to standard guidelines (CLSI) [82]. The agar dilution plates contained twofold serial dilutions of nine antibiotics (Glentham Life Sciences, Corsham, UK): vancomycin (1–128 µg/mL), teicoplanin (0.5–64 µg/mL), linezolid (0.5–64 µg/mL), tetracycline (1–128 µg/mL), ampicillin (0.5–64 µg/mL), ciprofloxacin (0.12–16 µg/mL), erythromycin (1–128 µg/mL), gentamicin (8–1024 µg/mL) and chloramphenicol (4–128 µg/mL).

Broth microdilution using Sensititre™ EUVENC plates (Sensititre®, Trek Diagnostic Systems, East Grinstead, United Kingdom) was performed in 21 isolates of E. faecium (n = 6) and E. faecalis (n = 15).

The antibiotic panels were read using a semi-automated Sensititre™ Vizion™ Digital MIC Viewing System (ThermoFisher Scientific, Waltham, MA, USA) and the Thermo Scientific™ Sensititre™ SWIN™ Software System (ThermoFisher Scientific, Waltham, MA, USA). Results were assessed with the epidemiological cut-off (ECOFF) values provided by EUCAST. Linezolid clinical breakpoint (MIC > 4 mg/L) from EUCAST was used for E. faecalis isolates as no ECOFF is available. Isolates non-susceptible to three or more classes of antibiotics were considered multidrug-resistant.

Enterococcus faecalis ATCC 29212 was used as a quality control strain in both procedures.
4.3. PCR Screening of Antibiotic Resistance Genes

Molecular screening of vancomycin and linezolid resistance determinants was performed by PCR using primers previously described [14,83–85], with cycling conditions and reference strains detailed in Supplementary Table S2. All isolates of *E. faecalis* and *E. faecium* were screened for the *vanA* gene, while the *vanB* gene was searched in isolates that exhibited vancomycin MIC $\geq 2$ $\mu$g/mL. Moreover, isolates with linezolid and chloramphenicol MIC $\geq 2$ $\mu$g/mL and MIC $\geq 32$ $\mu$g/mL, respectively, were subjected to a PCR assay targeting the *optrA* and *cfr* genes.

4.4. Whole-Genome Sequencing

Whole-genome sequencing (WGS) was conducted on five *E. faecalis* and two *E. faecium* isolates harboring the *optrA* gene, and on two daptomycin-susceptible *E. faecalis* strains all recovered from swine. DNA extraction was performed using PureLink® Genomic DNA mini kit, Gram-positive bacterial cell lysate protocol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Library preparation and DNA sequencing were performed by Novogene Europe, Cambridge, UK, using Illumina HiSeq sequencing technology (NovaSeq 6000 S2 PE150 XP sequencing mode).

Sequencing data quality was assessed by FastQC [86] and Trimmomatic v0.27 [87] was used with default settings to remove low-quality data and adapter sequences. Pre-processed reads were assembled with SPAdes 3.12.0 [88] and the assembly stats were calculated using QUAST-5.0.2 [89]. Contigs with sizes lower than 500 bp were removed and nucleotide sequences were analyzed using tools available on the Center for Genomic Epidemiology (CGE) website [90].

Bioinformatics tools ResFinder v4.0 (90% threshold for %ID/60% minimum length) [90,91], PlasmidFinder (95% threshold for %ID) [92,93] and VirulenceFinder v2.0 (90% threshold for %ID/60% minimum length) [94] were used to screen for acquired antimicrobial resistance genes, plasmid sequences and virulence genes, respectively. Multi-locus sequence types (MLSTs) were assigned using MLST version 2.0 [95] on CGE [96] and the MLST database on PubMLST [61].

The Basic Local Alignment Search Tool (BLAST) [81] was used to determine *optrA* and *poxT/A* variants.

Linezolid resistant isolates were screened for mutations in the ribosomal proteins L3 and L4 by aligning the respective amino acid sequences with those of reference strains *E. faecalis* ATCC 29212 (GenBank accession number CP008816.1) and *E. faecium* ATCC 8459 (GenBank accession number CP004063.1) using the Molecular Evolutionary Genetics Analysis software (MEGAX) [97].

To identify the genetic platform of the *optrA* gene, contigs containing this gene were annotated using Prokka v1.14.6 [98], followed by analysis with Artemis [99], EasyFig v2.2.5 [100], and BLAST.

4.5. Statistical Analysis

Fisher’s exact test was used with a 95% confidence level to analyze statistical data on Microsoft Excel to verify the association between animal species and antimicrobial susceptibility.

4.6. Accession Numbers

Raw sequence data obtained from all the sequenced isolates were submitted to the European Nucleotide Archive (ENA) under study accession numbers: ERS6142029, ERS11708758, ERS6142031, ERS11708754, ERS11708755, ERS11708756, ERS11708757 ERS11708759 and ERS11708760.
5. Conclusions

Our results underline the impact of the administration of certain antibiotic classes and differences in husbandry and antibiotic use practices in the gut microbiome of clinically healthy cattle and pigs.

The findings of *Enterococcus* spp. strains from pigs resistant to last-resort antimicrobials linezolid and daptomycin are worrying, posing a risk to human health. This is because enterococci in pigs can serve as reservoirs for resistance genes. Moreover, the co-occurrence of resistance mechanisms may perpetuate the emergence and spread of *optrA* and *poxtA* under the selective pressure of amphenicols, even in the absence of oxazolidines usage. Besides clinically relevant lineages like *E. faecalis* ST16 and *E. faecium* ST22, several other lineages, including new STs, were found, suggesting a high diversity among enterococci circulating in pig production in Portugal.

In addition, the emergence of daptomycin non-susceptible *E. faecalis* strains should be carefully monitored, and further research to assess the molecular basis of daptomycin resistance should be performed.

Surveillance programs and research studies to investigate the prevalence and molecular mechanisms of antibiotic resistance in the commensal flora of farm animals are of utmost importance to establishing the risks of the transmission of antibiotic-resistant enterococci from animals to humans and vice versa from a One Health Perspective.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11050615/s1, Table S1: Description of primer sets, annealing temperature, and DNA from control strains used for the molecular species identification of *Enterococcus* spp. Table S2: Description of the primer sets, annealing temperatures and DNA from control strains used for the molecular detection of *vanA*, *vanB*, *optrA*, and *cfr* are available.

Author Contributions: Conceptualization, A.A. and L.C.; methodology, J.G., C.L., T.A., L.C., A.A.; software, J.G., C.L.; validation, J.G., C.L.; formal analysis, J.G., C.L., A.A.; investigation, J.G., C.L., L.C., A.A.; writing—original draft preparation, J.G.; writing—review and editing, L.C, A.A; funding acquisition, L.C., A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the project PTDC/CVT-CVT/28469/2017 “CIAinVET: Food-producing animals as reservoirs of resistance to Critically Important Antibiotics” financed by the Foundation for Science and Technology (FCT), Portugal.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available within the article.

Acknowledgments: The authors would like to thank Patrícia Poeta from UTAD, for the positive control stains for *E. casseliflavus* (AUT 14A) and *E. durans* (AUT 49B) and all technical staff at the laboratory for their assistance and collaboration at every stage of this research project. The authors are grateful to the National Authority for Animal Health, Direção Geral de Alimentação Agrária e Veterinária, for sampling and collecting the biological samples. Part of this research was supported by Cost 219 Action CA18217: European Network for Optimization of Veterinary Antimicrobial Treatment 220 (ENOVAT).

Conflicts of Interest: The authors declare no conflict of interest.
## Appendix A

### Table A1. Genomic characterization of *Enterococcus faecium* (n = 2) and *Enterococcus faecalis* (n = 7) strains from pig cecal samples.

| Isolate   | Species     | Sample Source | Geographic Location | MLST    | EUVENC MDR Profile | Acquired Antimicrobial Resistance Genes and Mutations | Plasmid Replicons | Virulence Genes | Accession Number |
|-----------|-------------|---------------|---------------------|---------|-------------------|-----------------------------------------------------|-------------------|-----------------|------------------|
| INIAV004  | *E. faecium*| Swine         | 39.233, 8.68333     | ST22    | TET-ERY-LZD-CLO   | *poxtA*, *optRA*, *fxrB*, *tet*(L), *erm*(A), *pbp5* (172A), *pbp5*(L77T), *pbp5*(A216S), *pbp5*(F667S), *pbp5*(E204G), *pbp5*(K144Q), *pbp5*(R34Q), *pbp5*(S27G), *pbp5*(E100Q), *pbp5*(A499T), *pbp5*(G66E), *pbp5*(T324A), *pbp5*(A68T), *pbp5*(V24A), *pbp5*(N496K), *pbp5*(E525D), *pbp5*(E85D) | *rep29*, *rep33*, *repUSR43*, *rep1*, *rep2*, *repUSR15* | *acs*, *efaAfm* | ERS6142029       |
| INIAV173  | *E. faecium*| Swine         | 38.9167, 9.26667    | ST2138  | TET-ERY-CLO       | *poxtA*, *optRA*, *fxrB*, *erm*(A), *ant(9)-la*; | *rep1*, *rep11c*, *rep18b*, *rep29*, *repUSR15*, *repUSR43* | *acs*, *efaAfm* | ERS11708758      |
| INIAV005  | *E. faecalis*| Swine       | 38.9485, 9.1967     | ST93    | TET-ERY-DAP       | *aac*4*, *apht*(2")*, *tet*(M), *erm*(B) | *rep9a* | *elrA*, *srtA*, *ace*, *ccF10*, *cOB1*, *cad*, *camE*, *ebpA*, *ebpC*, *efaAfs*, *hylA*, *tpx*, | ERS6142031       |
| INIAV168  | *E. faecalis*| Swine       | 41.4124, 8.5206     | ST207   | TET-ERY-LZD-CLO   | *optRA*, *fxrA*, *tet*(M), *erm*(A), *erm*(B), *clpL* | *repUSR1*, *rep9b*, *repUSR43* | *elrA*, *srtA*, *ace*, *ccF10*, *cOB1*, *cad*, *camE*, *ebpA*, *ebpC*, *efaAfs*, *fxb*, *gcl*, *hylA*, *hylB*, *tpx*, | ERS11708754       |
| INIAV169  | *E. faecalis*| Swine       | 41.4124, 8.5206     | ST474   | TET-ERY-CIP-GEN-CLO | *optRA*, *fxrA*, *cat*, *aac*6*, *apht*(2")*, *apht*(3-)III, *tetr*(M), *erm*(L), *erm*(A), *erm*(B), *dfrG*, *lnuB*, *gmr*, *gmr* (E87G), *parC* (S80I), *isrE* | *rep9a*, *repUSR43* | *elrA*, *srtA*, *ace*, *ccF10*, *cOB1*, *cad*, *camE*, *ebpA*, *ebpC*, *efaAfs*, *fxb*, *gcl*, *hylA*, *hylB*, *tpx* | ERS11708755       |
| INIAV170  | *E. faecalis*| Swine       | 38.7058, 8.97462    | ST474   | TET-ERY-CIP-GEN-CLO | *optRA*, *fxrA*, *cat*, *aac*6*, *apht*(2")*, *apht*(3-)III, *tetr*(M), *erm*(L), *erm*(A), *erm*(B), *dfrG*, *lnuB*, *gmr*, *gmr* (E87G), *parC* (S80I), *isrE* | *rep9a*, *repUSR43* | *elrA*, *srtA*, *ace*, *ccF10*, *cOB1*, *cad*, *camE*, *ebpA*, *ebpC*, *efaAfs*, *fxb*, *gcl*, *hylA*, *hylB*, *tpx* | ERS11708756       |
| INIAV171  | *E. faecalis*| Swine       | 39.4598, 8.6671     | ST16    | TET-ERY-LZD-CLO   | *optRA*, *fxrA*, *cat*, *aac*6*, *apht*(2")*, *apht*(3-)III, *str*, *tetr*(M), *erm*(A), *erm*(B), *str*, *lnuB*, *isrE* | *rep6*, *rep9b*, *repUSR43* | *elrA*, *srtA*, *ace*, *ccF10*, *cOB1*, *cad*, *camE*, *ebpA*, *ebpC*, *efaAfs*, *fxb*, *gcl*, *hylA*, *hylB*, *tpx* | ERS11708757       |
| INIAV174  | *E. faecalis*| Swine       | 39.8167, 9.2667     | ST1178  | TET-ERY-CIP-CLO   | *optRA*, *cat*, *ant(9)-la*, *tetr*(M), *tetr*(L), *erm*(A), *erm*(B), *gmr*, *gmr* (E87G), *parC* (S80I) | *rep9a*, *repUSR43* | *elrA*, *srtA*, *ace*, *ccF10*, *cOB1*, *cad*, *camE*, *ebpA*, *ebpC*, *efaAfs*, *fxb*, *gcl*, *hylA*, *hylB*, *tpx* | ERS11708759       |
| INIAV175  | *E. faecalis*| Swine       | 39.8198, 8.8667     | ST58    | TET-ERY-DAP       | *tetr*(M), *erm*(B), *ant(6)-la*, *dfrG*, *lnuB*, *isrE* | *repUSR43* | *elrA*, *srtA*, *ace*, *ccF10*, *cOB1*, *cad*, *camE*, *ebpA*, *ebpC*, *efaAfs*, *fxb*, *gcl*, *hylA*, *hylB*, *tpx* | ERS11708760       |

MLST, Multilocus Sequence Type; MDR, Multidrug resistance; TET, Tetracycline; ERY, Erythromycin; CIP, Ciprofloxacin; GEN, Gentamicin; LZD, Linezolid; CLO, Chloramphenicol; Linezolid resistance genes are highlighted in bold.
References

1. LPSN—List of Prokaryotic Names with Standing in Nomenclature. Genus Enterococcus. 1997. Available online: https://www.bacterio.net/genus/enterococcus (accessed on 20 December 2021).

2. Staley, C.; Dunny, G.M.; Sadowsky, M.J. Environmental and Animal-Associated Enterococci. In Advances in Applied Microbiology; Elsevier: Amsterdam, The Netherlands, 2014; Volume 87, pp. 147–186. [CrossRef]

3. Devriese, L.A.; Hommez, J.; Wijffels, R.; Haesebrouck, F. Composition of the Enterococcocal and Streptococcocal Intestinal Flora of Poultry. J. Appl. Bacteriol. 1991, 71, 46–50. [CrossRef] [PubMed]

4. Lebretón, F.; Willems, R.; Gilmore, M. Enterococcus Diversity, Origins in Nature, and Gut Colonization. In Enterococci: From Commensals to Leading Causes of Drug Resistant Infection; Gilmore, M.S., Clewell, D.B., Ike, Y., Eds.; Massachusetts Eye and Ear Infirmary: Boston, MA, USA, 2014.

5. Molina, M.; Cyterski, M.; Maines, J.; Fisher, J.; Johnson, B. Comparison of the Temporal Variability of Enterococcal Clusters in Impacted Streams Using a Multiplex Polymerase Chain Reaction Procedure. In Proceedings of the 2007 Georgia Water Resources Conference, Athens, GA, USA, 27–29 March 2007.

6. Dubin, K.; Pamer, E.G. Enterococci and Their Interactions with the Intestinal Microbiome. Microbiol. Spectr. 2017, 5. [CrossRef] [PubMed]

7. Bender, J.K.; Cattoir, V.; Hegstad, K.; Sadowy, E.; Coque, T.M.; Westh, H.; Hammerum, A.M.; Schaffer, K.; Burns, K.; Murchan, S.; et al. Update on Prevalence and Mechanisms of Resistance to Linezolid, Tigecycline and Daptomycin in Enterococci in Europe: Towards a Common Nomenclature. Drug Resist. Updates 2018, 40, 25–39. [CrossRef]

8. Advice on the Designation of Antimicrobials or Groups of Antimicrobials Reserved for Treatment of Certain Infections in Humans—In Relation to Implementing Measures under Article 37(5) of Regulation (EU) 2019/6 on Veterinary Medicinal Products EMA/CVMP/678496/2021Committee for Veterinary Medicinal Products (CVMP). Available online: https://www.ema.europa.eu/en/documents/regulatory-procedural-guideline/advice-designation-antimicrobials-groups-antimicrobials-reserved-treatment-certain-infections-humans/veterinary-medicinal-products_en.pdf (accessed on 16 February 2022).

9. Hammerum, A.M. Enterococci of Animal Origin and Their Significance for Public Health. Clin. Microbiol. Infect. 2012, 18, 619–625. [CrossRef] [PubMed]

10. Nilsson, O. Vancomycin Resistant Enterococci in Farm Animals—Occurrence and Importance. Infect. Ecol. Epidemiol. 2012, 2, 169. [CrossRef]

11. EU Action on Antimicrobial Resistance. Available online: https://ec.europa.eu/health/antimicrobial-resistance/eu-action-antimicrobial-resistance_en (accessed on 15 December 2021).

12. Ahmad, M.O.; Baptiste, K.E. Vancomycin-Resistant Enterococci: A Review of Antimicrobial Resistance Mechanisms and Perspectives of Human and Animal Health. Microb. Drug Resist. 2018, 24, 590–606. [CrossRef]

13. Bender, J.K.; Fleige, C.; Klare, I.; Fiedler, S.; Mischnik, A.; Mutters, N.T.; Dingle, K.E.; Werner, G. Detection of a Cfr(B) Variant in German Enterococcus faecium Clinical Isolates and the Impact on Linezolid Resistance in Enterococcus spp. PloS ONE 2016, 11, e0167042. [CrossRef]

14. Wang, Y.; Lv, Y.; Cai, J.; Schwarz, S.; Cui, L.; Hu, Z.; Zhang, R.; Li, J.; Zhao, Q.; He, T.; et al. A Novel Gene, OptrA, That Confers Transferable Resistance to Oxazolidinones and Phenolics and Its Presence in Enterococcus faecalis and Enterococcus faecium of Human and Animal Origin. J. Antimicrob. Chemother. 2015, 70, 2182–2190. [CrossRef]

15. Antonelli, A.; D’Andrea, M.M.; Benciani, A.; Galeotti, C.L.; Morroni, G.; Pollini, S.; Varaldo, P.E.; Rossolini, G.M. Characterization of PostA, a Novel Phenicol–Oxazolidinone–Tetracycline Resistance Gene from an MRSA of Clinical Origin. J. Antimicrob. Chemother. 2018, 73, 1763–1769. [CrossRef]

16. Tran, T.T.; Munita, J.M.; Arias, C.A. Mechanisms of Drug Resistance: Daptomycin Resistance: Daptomycin Resistance. Ann. N. Y. Acad. Sci. 2015, 1354, 32–53. [CrossRef] [PubMed]

17. Heuer, O.E.; Pedersen, K.; Andersen, J.S.; Madsen, M. Vancomycin-Resistant Enterococci (VRE) in Broiler Flocks 5 Years after the Avoparcin Ban. Microb. Drug Resist. 2002, 8, 133–138. [CrossRef] [PubMed]

18. Novais, C.; Coque, T.M.; Costa, M.J.; Sousa, J.C.; Baquero, F.; Peixe, L.V. High Occurrence and Persistence of Antibiotic-Resistant Enterococci in Poultry Food Samples in Portugal. J. Antimicrob. Chemother. 2006, 57, 1139–1143. [CrossRef]

19. Serum, M.; Johnsen, P.J.; Aasnes, B.; Rosvoll, T.; Kruse, H.; Sundsfjord, A.; Simonsen, G.S. Prevalence, Persistence, and Molecular Characterization of Glycopeptide-Resistant Enterococci in Norwegian Poultry and Poultry Farmers 3 to 8 Years after the Ban on Avoparcin. Appl. Environ. Microbiol. 2006, 72, 516–521. [CrossRef] [PubMed]

20. Garcia-Migura, L.; Liebana, E.; Jensen, L.B.; Barnes, S.; Pleydell, E. A Longitudinal Study to Assess the Persistence of Vancomycin-Resistant Enterococcus faecium (VREF) on an Intensive Broiler Farm in the United Kingdom. FEMS Microbiol. Lett. 2007, 275, 319–325. [CrossRef] [PubMed]

21. De Jong, A.; Simjee, S.; Garch, F.E.; Moyaert, H.; Rose, M.; Youala, M.; Dry, M. Antimicrobial Susceptibility of Enterococci Recovered from Healthy Cattle, Pigs and Chickens in Nine EU Countries (EASSA Study) to Critically Important Antibiotics. Vet. Microbiol. 2018, 216, 168–175. [CrossRef] [PubMed]

22. European Food Safety Authority; European Centre for Disease Prevention and Control. EU Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2013. EFS2 2015, 13, 4036. [CrossRef]
23. Timmermans, M.; Bogaerts, B.; Vanneste, K.; De Keersmaecker, S.C.J.; Roosens, N.H.C.; Kowalewicz, C.; Simon, G.; Argudín, M.A.; De plano, A.; Hallin, M.; et al. Large Diversity of Linezolid-Resistant Isolates Discovered in Food-Producing Animals through Linezolid Selective Monitoring in Belgium in 2019. *J. Antimicrob. Chemother.* 2021, 77, 49–57. [CrossRef]

24. Ruiz-Ripa, L.; Feßler, A.T.; Hanke, D.; Sanz, S.; Olarte, C.; Eichhorn, I.; Schwarz, S.; Torres, C. Detection of PoxtA- and OptrA-Carrying *E. Faecium* Isolates in Air Samples of a Spanish Swine Farm. *J. Glob. Antimicrob. Resist.* 2020, 22, 28–31. [CrossRef]

25. Moure, Z.; Lara, N.; Marin, M.; Sola-Campoy, P.J.; Bautista, V.; Gómez-Bertomeu, F.; Gómez-Dominguez, C.; Pérez-Vázquez, M.; Arcil, B.; Campos, J.; et al. Interregional Spread in Spain of Linezolid-Resistant *Enterococcus* spp. Isolates Carrying the OptrA and PoxtA Genes. *Int. J. Antimicrob. Agents* 2020, 55, 105977. [CrossRef]

26. Egan, S.A.; Shore, A.C.; O’Connell, B.; Brennan, G.I.; Coleman, D.C. Linezolid Resistance in *Enterococcus* faecium and *Enterococcus faecalis* from Hospitalized Patients in Ireland: High Prevalence of the MDR Genes OptrA and PoxtA in Isolates with Diverse Genetic Backgrounds. *J. Antimicrob. Chemother.* 2020, 75, 1704–1711. [CrossRef] [PubMed]

27. Sassi, M.; Guerin, F.; Zouari, A.; Beyrouthy, R.; Auzou, M.; Fines-Guyon, M.; Potrel, S.; Dejoies, L.; Collet, A.; Boukthir, S.; et al. Emergence of OptrA-Mediated Linezolid Resistance in *Enterococci* from France, 2006–2016. *J. Antimicrob. Chemother.* 2019, 74, 1469–1472. [CrossRef] [PubMed]

28. McHugh, M.P.; Farcell, B.J.; Pettigrew, K.A.; Toner, G.; Khatamzas, E.; Karcher, A.M.; Walker, J.; Weir, R.; Meunier, D.; Hopkins, K.L.; et al. Emergence of OptrA-Mediated Linezolid Resistance in Multiple Lineages and Plasmids of *Enterococcus faecalis* Revealed by Long Read Sequencing. *bioRxiv* 2020. [CrossRef]

29. Kelesidis, T.; Humphries, R.; Uslan, D.Z.; Pegues, D.A. Daptomycin Nonsusceptible Enterococci: An Emerging Challenge for Clinicians. *Clin. Infect. Dis.* 2011, 52, 228–234. [CrossRef] [PubMed]

30. Ruzauskas, M.; Virgailis, M.; Šiugždinien, R. Antimicrobial Resistance of *Escherichia coli* Spp. Isolated from Livestock in Lithuania. *Vet. Arch.* 2009, 79, 439–449.

31. DANMAP—Danish Integrated Antimicrobial Resistance Monitoring and Research Programm, 2019. Resistance in Indicator Pathogens. Available online: https://www.danmap.org (accessed on 3 December 2021).

32. Morroni, G.; Brenciani, A.; Simoni, S.; Vignaroli, C.; Mingoia, M.; Giovanetti, E. Commentary: Nationwide Surveillance of Novel Oxa-zolidinidone Resistance Gene OptrA in *Enterococcus* Isolates from China from 2004 to 2014. *Front. Microbiol.* 2017, 8, 1631. [CrossRef]

33. Freitas, A.R.; Tedim, A.P.; Novais, C.; Lanza, V.F.; Peixe, L. Comparative Genomics of Global OptrA-Carrying *Enterococcus Faecalis* Uncovers a Common Chromosomal Hotspot for OptrA Acquisition within a Diversity of Core and Accessory Genomes. *Microb. Genom.* 2020, 6, e000350. [CrossRef]

34. Almeida, L.M.; Lebreton, F.; Gaca, A.; Bispo, P.M.; Saavedra, J.T.; Calumby, L.M.; Nascimento, T.G.; Filsner, P.H.; Moreno, A.M.; et al. Transferable Resistance Gene *sodA* in *Enterococcus faecalis* from Swine in Brazil. *Antimicrob. Agents Chemother.* 2020, 64, e00412-20. [CrossRef]

35. Silva, N.; Igrejas, G.; Gonçalves, A.; Poeta, P. Commensal Gut Bacteria: Distribution of *Enterococcus* Species and Prevalence of *Escherichia coli* Phylogenetic Groups in Animals and Humans in Portugal. *Ann. Microbiol.* 2012, 62, 449–459. [CrossRef]

36. Morroni, G.; Benciani, A.; Simoni, S.; Vignaroli, C.; Mingoa, M.; Giovannetti, E. Commentary: Nationwide Surveillance of Novel Oxa-zolidinidone Resistance Gene OptrA in *Enterococcus* Isolates in China from 2004 to 2014. *Front. Microbiol.* 2017, 8, 1631. [CrossRef]

37. Zaheer, R.; Cook, S.R.; Barbieri, R.; Goji, N.; Cameron, A.; Petkau, A.; Polo, R.O.; Tymensen, L.; Stamm, C.; Song, J.; et al. Transferable Resistance Gene *sodA* in *Enterococcus faecalis* from Swine in Brazil. *Antimicrob. Agents Chemother.* 2020, 64, e000350. [CrossRef] [PubMed]

38. Kühn, I. Comparison of *Enterococcus* Populations in Animals, Humans, and the Environment—A European Study. *Int. J. Food Microbiol.* 2003, 88, 133–145. [CrossRef]

39. Zaheer, R.; Cook, S.R.; Barbieri, R.; Goji, N.; Cameron, A.; Petkau, A.; Polo, R.O.; Tymensen, L.; Stamm, C.; Song, J.; et al. Surveilance of *Enterococcus* spp. Reveals Distinct Species and Antimicrobial Resistance Diversity across a One-Health Continuum. *Sci. Rep.* 2020, 10, 3937. [CrossRef] [PubMed]

40. Novais, C. First Report of the Activity of Linezolid against Portuguese *Enterococci* from Human, Animal and Environmental Sources. *J. Antimicrob. Chemother.* 2005, 51, 1314–1315. [CrossRef] [PubMed]

41. Kelesidis, T.; Humphries, R.; Uslan, D.Z.; Pegues, D.A. Daptomycin Nonsusceptible Enterococci: An Emerging Challenge for Clinicians. *Clin. Infect. Dis.* 2011, 52, 228–234. [CrossRef] [PubMed]

42. European Medicines Agency; European Surveillance of Veterinary Antimicrobial Consumption. Sales of Veterinary Antimicrobial Agents in 31 European Countries in 2019 and 2020 (EMA/S8183/2021). 2021. Available online: https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-31-european-countries-2019-2020-trends-2010-2020-eleventh_en.pdf (accessed on 6 April 2022).

43. Schwarz, S.; Silley, P.; Simjee, S.; Woodford, N.; van Duijkeren, E.; Johnson, A.P.; Gaastra, W. Editorial: Assessing the Antimicrobial Susceptibility of Bacteria Obtained from Animals. *J. Antimicrob. Chemother.* 2010, 65, 601–604. [CrossRef]

44. Marinho, C.M.; Santos, T.; Gonçalves, A.; Poeta, P.; Igrejas, G. A Decade-Long Commitment to Antimicrobial Resistance Surveillance in Portugal. *Front. Microbiol.* 2016, 7, 1650. [CrossRef] [PubMed]
46. Aasmåe, B.; Häkkinen, L.; Kaart, T.; Kalmus, P. Antimicrobial Resistance of Escherichia coli and Enterococcus spp. Isolated from Estonian Cattle and Swine from 2010 to 2015. *Acta Vet. Scand.* 2019, 61, 5. [CrossRef]

47. APHIS–United States Department of Agriculture. Commensal *Enterococcus* on U.S. Swine Sites: Prevalence and Antimicrobial Drug Susceptibility. Available online: https://www.aphis.usda.gov/aphis/ourfocus/animalhealth (accessed on 1 December 2021).

48. Liu, Y.; Liu, K.; Lai, J.; Wu, C.; Shen, J.; Wang, Y. Prevalence and Antimicrobial Resistance of *Enterococcus* Species of Food Animal Origin from Beijing and Shandong Province, China. *J. Appl. Microbiol.* 2013, 114, 555–563. [CrossRef]

49. Tan, S.C.; Chong, C.W.; Teh, C.S.J.; Ooi, P.T.; Thong, K.L. Occurrence of Virulent Multidrug-Resistant *Enterococcus faecalis* and *Enterococcus faecium* in the Pigs, Farmers and Farm Environments in Malaysia. *Peer J.* 2018, 6, e5353. [CrossRef]

50. Humphries, R.M.; Ambler, J.; Mitchell, S.L.; Castanheira, M.; Dingle, T.; Hindler, J.A.; Koeth, L.; Sei, K. CLSI Methods Development and Standardization Working Group Best Practices for Evaluation of Antimicrobial Susceptibility Tests. *J. Clin. Microbiol.* 2018, 56, e01934-17. [CrossRef] [PubMed]

51. Holtenbeck, B.L.; Rice, L.B. Intrinsic and Acquired Resistance Mechanisms in *Enterococcus*. *Virulence* 2012, 3, 421–569. [CrossRef] [PubMed]

52. EUCAST—The European Committee on Antimicrobial Susceptibility Testing, 2020. EUCAST Expert Rules v 3.2 on *Enterococcus* spp. Available online: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Expert_Rules/2020/ExpertRules_V3.2_20190515_Enterococcus_revision_20200224.pdf (accessed on 1 December 2021).

53. Galloway-Peña, J.R.; Rice, L.B.; Murray, B.E. Analysis of PBP5 of Early U.S. Isolates of *Enterococcus faecium*: Sequence Variation Alone Does Not Explain Increasing Ampicillin Resistance over Time. *Antimicrob. Agents Chemother.* 2011, 55, 3272–3277. [CrossRef]

54. Montealegre, M.C.; Roh, J.H.; Rae, M.; Davileva, M.G.; Singh, K.V.; Shamoo, Y.; Murray, B.E. Differential Penicillin-Binding Protein 5 (PBP5) Levels in the *Enterococcus faecium* Clades with Different Levels of Ampicillin Resistance. *Antimicrob. Agents Chemother.* 2017, 61, e02034-16. [CrossRef] [PubMed]

55. Pietta, E.; Montealegre, M.C.; Roh, J.H.; Cocconcelli, P.S.; Murray, B.E. *Enterococcus faecium* PBP5-S/R, the Missing Link between PBP5-S and PBP5-R. *Antimicrob. Agents Chemother.* 2014, 58, 6978–6981. [CrossRef] [PubMed]

56. Wang, Y.; Li, X.; Fu, Y.; Chen, Y.; Wang, Y.; Ye, D.; Wang, C.; Hu, X.; Zhou, L.; Du, J.; et al. Association of Florfenicol Residues with the Abundance of Oxazolidinone Resistance Genes in Livestock Manures. *Microb. Drug Resist.* 2020, 26, 1396–1404. [CrossRef]

57. West, M.; White, J.;单车, W.; Elghanay, S.; Adamcik, J.; Kriedeman, B.; Adamcik, J.; et al. Linezolid-Resistant *Enterococcus faecalis* Isolated from a Hospital in Shanghai. *Infect. Drug Resist.* 2018, 11, 2397–2409. [CrossRef]

58. Wang, Y.; Li, X.; Fu, Y.; Chen, Y.; Wang, Y.; Ye, D.; Wang, C.; Hu, X.; Zhou, L.; Du, J.; et al. Association of Florfenicol Residues with the Abundance of Oxazolidinone Resistance Genes in Livestock Manures. *J. Hazard. Mater.* 2020, 399, 120059. [CrossRef]

59. European Food Safety Authority. Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal *Escherichia coli* and *Enterococcus* spp. from food animals. *EFSA J.* 2008, 141, 1–44.

60. Camara, J.; Camoee, M.; Tubau, F.; Pujol, M.; Ayats, J.; Ardanuy, C.; Domínguez, M.A. Detection of the Novel OpTRa Gene Among Linezolid-Resistant *Enterococci* in Barcelona, Spain. *Microb. Drug Resist.* 2019, 25, 87–93. [CrossRef]

61. Cai, J.; Wang, Y.; Schwarz, S.; Lv, H.; Li, Y.; Liao, K.; Yu, S.; Zhao, K.; Gu, D.; Wang, X.; et al. Enterococcal Isolates Carrying the Novel Oxazolidinone Resistance Gene OpTRa from Hospitals in Zhejiang, Guangdong, and Henan, China, 2010–2014. *Clin. Microbiol. Infect.* 2015, 21, e1–e1095. [CrossRef]

62. Jolley, K.A.; Bray, J.E.; Maiden, M.C.J. Open-Access Bacterial Population Genomics: BIGSdb Software, the PubMLST.Org Website and Their Applications. *Wellcome Open Res.* 2018, 3, 124. [CrossRef] [PubMed]

63. Tamang, M.D.; Moon, D.C.; Kim, S.-R.; Kang, H.Y.; Lee, K.; Nam, H.-M.; Jung, G.-C.; Lee, H.-S.; Jung, S.-C.; Lim, S.-K. Detection of Novel Oxazolidinone and Phenicol Resistant Gene OpTRa in Enterococcal Isolates from Food Animals and Animal Carcasses. *Vet. Microbiol.* 2017, 201, 252–256. [CrossRef] [PubMed]

64. Vorobieva, V.; Roer, L.; Justesen, U.S.; Hansen, F.; Frimodt-Møller, N.; Hasman, H.; Hammerum, A.M. Detection of the OpTRa Gene in a Clinical ST16 *Enterococcus faecalis* Isolate in Denmark. *J. Glob. Antimicrob. Resist.* 2017, 10, 12–13. [CrossRef] [PubMed]

65. Tsilipoundaki, K.; Gerontopoulos, A.; Papagiannitsis, C.; Petinaki, E. First Detection of an OpTRa-Positive, Linezolid-Resistant ST16 *Enterococcus faecalis* from Human in Greece. *New Microbes New Infect.* 2019, 29, 100515. [CrossRef]

66. Chen, M.; Pan, H.; Lou, Y.; Wu, Z.; Zhang, J.; Huang, Y.; Wu, Q.; Qiu, Y. Epidemiological Characteristics and Genetic Structure of Linezolid-Resistant *Enterococcus faecalis*. *Infect. Drug Resist.* 2018, 11, 2397–2409. [CrossRef]

67. Li, P.; Yang, Y.; Ding, L.; Xu, X.; Lin, D. Molecular Investigations of Linezolid Resistance in Enterococci OpTRa Variants from a Hospital in Shanghai. *Infect. Drug Resist.* 2020, 13, 2711–2716. [CrossRef]

68. Torres, C.; Alonso, C.A.; Ruiz-Ripa, L.; León-Sampedro, R.; Del Campo, R.; Coque, T.M. Antimicrobial Resistance in *Enterococcus* spp. of animal origin. *Microbiol. Spectr.* 2018, 6. [CrossRef]

69. Freitas, A.R.; Tedim, A.P.; Duarte, B.; Elghaieb, H.; Abbassi, M.S.; Hassen, A.; Read, A.; Alves, V.; Novais, C.; Peixe, L. Linezolid-Resistant (Tn 6246:FexB—poxtA) *Enterococcus faecium* Strains Colonizing Humans and Bovines on Different Continents: Similarity without Epidemiological Link. *J. Antimicrob. Chemother.* 2020, 75, 2416–2423. [CrossRef]

70. Cai, J.; Schwarz, S.; Chi, D.; Wang, Z.; Zhang, R.; Wang, Y. Faecal Carriage of OpTRa-Positive Enterococci in Asymptomatic Healthy Humans in Hangzhou, China. *Clin. Microbiol. Infect.* 2019, 25, e1–e630. [CrossRef]
Antibiotics 2022, 11, 615

70. He, T.; Shen, Y.; Schwarz, S.; Cai, J.; Lv, Y.; Li, J.; Feßler, A.T.; Zhang, R.; Wu, C.; Shen, J.; et al. Genetic Environment of the Transferable Oxazolidinone/Phenicol Resistance Gene Optra in Enterococcus faecalis Isolates of Human and Animal Origin. J. Antimicro. Chemother. 2016, 71, 1466–1473. [CrossRef]

71. Zankari, E.; Hasman, H.; Kaas, R.S.; Seyfarth, A.M.; Agero, Y.; Lund, O.; Larsen, M.V.; Aarestrup, F.M. Genotyping Using Whole-Genome Sequencing Is A Realistic Alternative to Surveillance Based on Phenotypic Antimicrobial Susceptibility Testing. J. Antimicro. Chemother. 2013, 68, 771–777. [CrossRef] [PubMed]

72. Sampedro, R.L. Enterococcus faecalis: Nuevas perspectivas sobre la estructura poblacional y el impacto de los elementos genéticos móviles en la evolución. Ph.D. Thesis, Universidad Complutense, Facultad de Farmacia, Madrid, Spain, 2017.

73. Kim, Y.B.; Seo, H.J.; Seo, K.W.; Jeon, H.Y.; Kim, D.K.; Kim, S.W.; Lim, S.-K.; Lee, Y.J. Characteristics of High-Level Ciprofloxacin-Resistant Enterococcus faecalis and Enterococcus faecium from Retail Chicken Meat in Korea. J. Food Prot. 2018, 81, 1357–1363. [CrossRef] [PubMed]

74. Li, J.; Yang, L.; Huang, X.; Wen, Y.; Zhao, Q.; Huang, X.; Xia, J.; Huang, Y.; Cao, S.; Du, S.; et al. Molecular Characterization of Antimicrobial Resistance and Virulence Factors of Enterococcus faecalis from Ducks at Slaughterhouses. Poult. Sci. 2022, 101, 10164. [CrossRef]

75. Poulsen, L.L.; Bisgaard, M.; Son, N.T.; Trung, N.V.; An, H.M.; Dalsgaard, A. Enterococcus faecalis Clones in Poultry and in Humans with Urinary Tract Infections, Vietnam. Emerg. Infect. Dis. 2012, 18, 1096–1100. [CrossRef]

76. Kim, E.B.; Marco, M.L. Nonclinical and Clinical Enterococcus Faecium Strains, but Not Enterococcus faecalis Strains, Have Distinct Structural and Functional Genomic Features. Appl. Environ. Microbiol. 2014, 80, 154–165. [CrossRef]

77. McBride, S.M.; Fischetti, V.A.; LeBlanc, D.J.; Moellering, R.C.; Gilmore, M.S. Genetic Diversity among Enterococcus faecalis. PLoS ONE 2007, 2, e382. [CrossRef]

78. Elghaieb, H.; Freitas, A.R.; Abbassi, M.S.; Novais, C.; Zouari, M.; Hassen, A.; Peixe, L. Dispersal of linezolid-resistant enterococci in retail meat and food-producing animals from Tunisia. J. Antimicro. Chemother. 2019, 74, 2865–2869. [CrossRef]

79. Deasy, B.M.; Rea, M.C.; Fitzgerald, G.F.; Cogan, T.M.; Beresford, T.P. A Rapid PCR Based Method to Distinguish between Lactococcus and Enterococcus. Syst. Appl. Microbiol. 2000, 23, 510–522. [CrossRef]

80. Jackson, C.R.; Fedorka-Cray, P.; Barrett, J.B. Use of a Genus- and Species-Specific Multiplex PCR for Identification of Enterococci. J. Clin. Microbiol. 2004, 42, 3588–3595. [CrossRef]

81. Basic Local Alignment Search Tool. 2014. Available online: https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi (accessed on 10 January 2022).

82. CLSI Standard M07; Chapter 3—Broth and Agar Dilution Susceptibility Testing Process. Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2018; pp. 15–53.

83. Aarestrup, F.M.; Ahrens, P.; Madsen, M.; Pallesen, L.V.; Poulsen, R.L.; Westh, H. Glycopeptide Susceptibility among Danish Enterococcus faecium and Enterococcus faecalis Isolates of Animal and Human Origin and PCR Identification of Genes within the VanA Cluster. Antimicrob. Agents Chemother. 1996, 40, 1938–1940. [CrossRef]

84. Depardieu, F., Perichon, B.; Courvalin, P. Detection of the van Alphabet and Identification of Enterococci and Staphylococci at the Species Level by Multiplex PCR. J. Clin. Microbiol. 2004, 42, 5857–5860. [CrossRef] [PubMed]

85. Kehrenberg, C.; Schwarz, S. Distribution of Flomericol Resistance Genes FexA and Cfr among Chloramphenicol-Resistant Staphylococcus Isolates. Antimicrob. Agents Chemother. 2006, 50, 1156–1163. [CrossRef] [PubMed]

86. Andrews, S. FastQC: A Quality Control Tool for High Throughput Sequence Data. Available online: http://www.bioinformatics. babraham.ac.uk/projects/fastqc (accessed on 6 April 2022).

87. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina Sequence Data. Bioinformatics 2014, 30, 2114–2120. [CrossRef] [PubMed]

88. Nurk, S.; Binkowski, A.; Antipov, D.; Gurevich, A.; Korobeynikov, A.; Lapidus, A.; Pajusi, A.; Sirokin, A.; et al. Assembling Genomes and Mini-Metagenomes from Highly Chimeric Reads. In Research in Computational Molecular Biology: 17th Annual International Conference, RECOMB 2013, Beijing, China, 7–10 April 2013; Deng, M., Jiang, R., Sun, F., Zhang, X., Eds.; Springer: Berlin/Heidelberg, Germany, 2013; pp. 158–170. Available online: http://link.springer.com/chapter/10.1007/978-3-642-37195-0_13 (accessed on 10 January 2022).

89. Mikheenko, A.; Pajusi, A.; Savlevich, V.; Antipov, D.; Gurevich, A. Versatile genome assembly evaluation with QUAST-LG. Bioinformatics 2018, 34, 1142–1150. [CrossRef] [PubMed]

90. Bortolaia, V.; Kaas, R.F.; Ruppe, E.; Roberts, M.C.; Schwarz, S.; Cattoir, V.; Philippeon, A.; Allesoe, R.L.; Rebelo, A.R.; Florensa, A.R. ResFinder 4.0 for predictions of phenotypes from genotypes. J. Antimicrob. Chemother. 2020, 75, 3491–3500. [CrossRef]

91. Zankari, E.; Hasman, H.; Cosentino, S.; Vestergaard, M.; Rasmussen, S.; Lund, O.; Aarestrup, F.M.; Larsen, M.V. Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. 2012, 67, 2640–2644. [CrossRef]

92. Carattoli, A.; Zankari, E.; Garcia-Fernandez, A.; Larsen, M.; Lund, O.; Voldby Villa, L.; Möller, Aarestrup, H.; Hasman, H. PlasmidFinder and pMLST: In silico detection and typing of plasmids. Antimicrob. Agents Chemother. 2014, 58, 3895–3903. [CrossRef]

93. Joensen, K.G.; Scheutz, F.; Lund, O.; Hasman, H.; Kaas, R.S.; Nielsen, E.M.; Aarestrup, F.M. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli. J. Clin. Microbiol. 2014, 52, 1501–1510. [CrossRef]
94. Malberg Tetzchner, A.M.; Johnson, J.R.; Johnston, B.D.; Lund, O.; Scheutz, F.J. In Silico Genotyping of *Escherichia coli* Isolates for Extraintestinal Virulence Genes by Use of Whole-Genome Sequencing Data. *J. Clin. Microbiol.* 2020, 58, e01269-20. [CrossRef]
95. Larsen, M.V.; Cosentino, S.; Rasmussen, S.; Friis, C.; Hasman, H.; Marvig, R.L.; Jelsbak, L.; Sicheritz-Pontén, T.; Ussery, D.W.; Aarestrup, F.M.; et al. Multilocus Sequence Typing of Total Genome Sequenced Bacteria. *J. Clin. Microbiol.* 2012, 50, 1355–1361. [CrossRef]
96. CGE—Center for Genomic Epidemiology. 2020. Available online: https://cge.cbs.dtu.dk/services/ (accessed on 29 December 2020).
97. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* 2018, 35, 1547–1549. [CrossRef] [PubMed]
98. Seemann, T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 2014, 30, 2068–2069. [CrossRef] [PubMed]
99. Carver, T.; Harris, S.R.; Berriman, M.; Parkhill, J.; McQuillan, J.A. Artemis: An integrated platform for visualization and analysis of high-throughput sequence-based experimental data. *Bioinformatics* 2012, 28, 464–469. [CrossRef]
100. Sullivan, M.J.; Petty, N.K.; Beatson, S.A. Easyfig: A genome comparison visualizer. *Bioinformatics* 2011, 27, 1009–1010. [CrossRef] [PubMed]