Antimicrobial Resistance, Biofilm Formation, and Virulence Genes in Enterococcus Species from Small Backyard Chicken Flocks

Othman M. Alzahrani 1,‡, Mahmoud Fayez 2,3,*,†, Amal Alswat 4, Mohamed Alkafafy 4,©, Samy F. Mahmoud 4,©, Theeb Al-Marri 2, Ahmed Almuslem 2, Hassan Ashfaq 5 and Shaymaa Yusuf 6,*,‡

Abstract: Backyard birds are small flocks that are more common in developing countries. They are used for poultry meat and egg production. However, they are also implicated in the maintenance and transmission of several zoonotic diseases, including multidrug-resistant bacteria. Enterococci are one of the most common zoonotic bacteria. They colonize numerous body sites and cause a wide range of serious nosocomial infections in humans. Therefore, the objective of the present study was to investigate the diversity in Enterococcus spp. in healthy birds and to determine the occurrence of multidrug resistance (MDR), multi-locus sequence types, and virulence genes and biofilm formation. From March 2019 to December 2020, cloacal swabs were collected from 15 healthy backyard broiler flocks. A total of 90 enterococci strains were recovered and classified according to the 16S rRNA sequence into Enterococcus faecalis (50%), Enterococcus faecium (33.33%), Enterococcus hirae (13.33%), and Enterococcus avium (3.33%). The isolates exhibited high resistance to tetracycline (55.6%), erythromycin (31.1%), and ampicillin (30%). However, all of the isolates were susceptible to linezolid. Multidrug resistance (MDR) was identified in 30 (33.3%) isolates. The enterococci AMR-associated genes ermB, ermA, tetM, tetL, vanA, cat, and pht5 were identified in 24 (26.6%), 11 (12.2%), 39 (43.3%), 34 (37.7%), 1 (1.1%), 4 (4.4%), and 23 (25.5%) isolates, respectively. Of the 90 enterococci, 21 (23.3%), 27 (30%), and 36 (40%) isolates showed the presence of cylA, gelE, and agg virulence-associated genes, respectively. Seventy-three (81.1%) isolates exhibited biofilm formation. A statistically significant correlation was obtained for biofilm formation versus the MAR index and MDR. Multi-locus sequence typing (MLST) identified eleven and eight different STs for E. faecalis and E. faecium, respectively. Seven different rep-family plasmid genes (rep1–2, rep3, rep5–6, rep9, and rep11) were detected in the MDR enterococci. Two-thirds (20/30; 66.6%) of the enterococci were positive for one or two rep-families. In conclusion, the results show that healthy backyard chickens could act as a reservoir for MDR and virulent Enterococcus spp. Thus, an effective antimicrobial stewardship program and further studies using a One Health approach are required to investigate the role of backyard chickens as vectors for AMR transmission to humans.

Keywords: antimicrobial resistance; Enterococcus; backyard chickens; virulence genes; multidrug resistance; antimicrobial-resistance genes
1. Introduction

Enterococci are Gram-positive bacteria that belong to the commensal microbiota of humans, animals, and poultry [1]. They are ubiquitous in nature and can be found in soils, freshwater, and plants [2]. Enterococcus spp. are important opportunistic human pathogens that are responsible for a wide range of serious nosocomial infections, including bacteremia, urinary tract infections, endocarditis, and intra-abdominal infections [3–5].

In veterinary medicine, enterococci are particularly significant as the causative agent of different infections, such as mastitis in cattle, bacteremia in dogs and pigs [6,7], and septicemia, endocarditis, amyloid arthropathy, and spondylitis in poultry [8,9].

Antibiotic resistance is an emerging world health threat. Many bacteria have developed resistance to frequently used antibiotics due to the unregulated use of antimicrobials in humans, agriculture, animals, poultry husbandry, and aquaculture in many developing countries [10,11].

One of the major concerns regarding opportunistic pathogens is their frequent antimicrobial-resistance (AMR) profile. Enterococci are intrinsically resistant to commonly used antibiotic classes, such as cephalosporins, β-lactams, sulfonamides, and are resistant at variable levels to aminoglycosides. Moreover, they are able to acquire resistance to clinically relevant drugs via horizontal transfer [12,13]. Enterococci are thought to play a key role in the acquisition, conservation, and transmission of AMR genes to other bacteria [14].

Enterococci pathogenesis is attributed to a variety of virulence factors. The most important adhesion factors that play a role in biofilm formation are Asa (aggregation substance), Esp (extracellular surface protein), EfaA (E. faecalis antigen A), Ace (adhesin of collagen from E. faecalis), and Ebp (endocarditis and biofilm-associated pili). Enterococci secrete the pathogenic factors CylA (cytolysin) and GelE (gelatinase), which are responsible for the exacerbation of infection. The expression of these factors is essential for biofilm formation, attachment, invasion, and the secretion of toxins [1,15–17].

Several molecular typing methods have been used to type enterococci, including pulsed-field gel electrophoresis, random amplification of polymorphic DNA, repetitive sequence-based PCR, ribotyping, and multi-locus sequence typing (MLST) [18–23]. MLST is a preferable tool for several pathogens, especially when epidemiological, geographical, and evolutionary studies need to be carried out [24,25]. Two MLST schemes have been developed for typing E. faecalis and E. faecium based on differences in the sequences of seven housekeeping genes (gdh, gud, pstS, gki, aroE, xpt, and yiqL) for E. faecalis and (adk, atpA, ddl, gud, gdh, purK, and pstS) for E. faecium [22,23].

One of the major public health concerns related to enterococci is their frequent antimicrobial resistance (AMR). Human infection by AMR enterococci occurs mainly by consuming contaminated meat or meat products from poultry and other livestock [26–28], and contamination can occur during slaughtering and evisceration [29].

Enterococci have a high capacity to acquire antimicrobial resistance either by point mutation or by the horizontal transfer of genetic elements [30–32]. Conjugation is thought to be the most common way of exchanging genetic elements, either by conjugal transposons or by horizontal transfer of plasmids [33].

In general, enterococcal plasmids are classified into two groups: a conjugative group consisting of pheromone-responsive and non-pheromone-responsive plasmids and a non-conjugative group consisting of small rolling-circle replicating (RCR) and mosaic plasmids [34].

Inc18 plasmids and pMG1-type plasmids are classified as conjugative non-pheromone-responsive plasmids and a non-conjugative group consisting of small rolling-circle replicating (RCR) and mosaic plasmids [34].

Backyard birds are small flocks that are common in developing countries. They are also very popular in the USA, where birds are raised for meat production. Direct contact between
poultry and humans is frequent; thus, these backyard flocks are considered a vehicle for disease transmission. Moreover, backyard poultry could be an emerging predisposing cause for MDR pathogenic bacteria, which can disseminate among humans [38]. In Saudi Arabia, many studies highlighted the prevalence of MDR enterococci in hospitals and communities [39,40]. The first vancomycin-resistant Enterococcus was detected in 1992 [41]; however, information concerning enterococci in backyard chicken is scarce.

Consequently, this study aimed to investigate the antimicrobial resistance, virulence determinants, and biofilm formation, and to characterize the plasmid content and multilocus sequence types in Enterococcus isolates from healthy chickens in backyard farms to highlight their zoonotic importance.

2. Results

2.1. Bacterial Isolation and Identification

Ninety Enterococcus isolates were isolated from 15 backyard chicken flocks and were biochemically identified into four species. The predominant species were Enterococcus faecalis (E. faecalis) (50%), followed by Enterococcus faecium (E. faecium) (33.33%), Enterococcus hirae (E. hirae) (13.33%), and Enterococcus avium (E. avium) (3.33%). Genetically, the 16S rRNA sequences showed more than a 99% homology with the relevant enterococci in the NCBI database. They were deposited in the NCBI sequence database with GenBank accession numbers OL691094-OL691103, OL677341-OL677350, OL691538-OL691543. On the basis of the 16S rRNA sequence analysis, 45 E. faecalis, 30 E. faecium, 12 E. hirae, and 3 E. avium isolates were clustered with the reference enterococci (E. faecalis NR_040789.1, E. faecium NR_042054.1, E. hirae NR_037082.1, and E. avium NR_028748.1), with a similarity level of 100% Supplementary Figure S1.

2.2. Antimicrobial Sensitivity

The antimicrobial-resistance profile of the 90 Enterococcus isolates is shown in Figure 1. Sixteen isolates (17.7%) were susceptible to all antibiotics. Resistance to one or more antimicrobials was determined in 74 (82.22%) enterococci. The MIC values and the resistance levels of 90 enterococci to 10 different antimicrobials are shown in Table 1 and Supplementary Table S1. Resistance to tetracycline (55.6%), erythromycin (31.1%), ampicillin (30%), ciprofloxacin (21.1%), and nitrofurantoin (17.8%) were the most frequent. Conversely, none of the isolates were resistant to linezolid, and four isolates (3.3%) showed resistance to vancomycin. E. faecalis showed a high frequency of resistance to tetracycline (62.2%), rifampin (24.4%), and nitrofurantoin (22.2%), while E. faecium exhibited a high resistance to tetracycline (50%), ampicillin (30.3%), and ciprofloxacin (23.3%).

Table 1. Frequency of antimicrobial resistance and MIC values to 10 different antimicrobials for E. faecalis, E. faecium, E. hirae and E. avium isolated from backyard chickens.

| Antimicrobial Agents | E. faecalis (n = 45) | E. faecium (n = 30) | E. hirae (n = 12) | E. avium (n = 3) |
|---------------------|---------------------|---------------------|-------------------|-----------------|
| %R | MIC50 | MIC90 | %R | MIC50 | MIC90 | %R | MIC50 | MIC90 | %R | MIC50 | MIC90 |
| Ampicillin | 15.6 | 2 | 32 | 33.3 | 4 | 64 | 66.7 | 16 | 32 | 66.7 | 32 | 32 |
| Rifampin | 24.4 | 1 | 16 | 10 | 1 | 1 | 0 | 0.5 | 1 | 0 | 1 | 1 |
| Ciprofloxacin | 13.3 | 1 | 8 | 23.3 | 1 | 16 | 33.3 | 1 | 8 | 66.7 | 4 | 8 |
| Fosfomycin | 17.8 | 32 | 256 | 13.3 | 32 | 256 | 8.3 | 32 | 256 | 0 | 32 | 64 |
| Nitrofurantoin | 22.2 | 16 | 256 | 16.7 | 16 | 256 | 8.3 | 16 | 32 | 0 | 32 | 32 |
| Linezolid | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 |
| Vancomycin | 3.3 | 2 | 4 | 3.3 | 2 | 4 | 0 | 2 | 4 | 0 | 2 | 4 |
| Chloramphenicol | 8.9 | 4 | 8 | 6.7 | 4 | 8 | 0 | 4 | 8 | 0 | 4 | 8 |
| Tetracycline | 62.2 | 32 | 64 | 50 | 4 | 32 | 41.7 | 4 | 32 | 66.7 | 16 | 32 |
| Erythromycin | 31.1 | 0.5 | 32 | 16.7 | 16 | 256 | 33.3 | 0.5 | 16 | 0 | 0.5 | 0.5 |
Of the 74 resistant enterococci, 9 isolates (10%) were resistant to one antimicrobial agent, 35 (38.9%) showed resistance to two antimicrobials, and the remaining 30 (33.3%) showed MDR. The MDR enterococci isolates were distributed into 15 \textit{E. faecalis} (33.3%), 8 \textit{E. faecium} (26.7%), and 5 \textit{E. hirae} (41.6%) isolates. The mean MAR index was 0.22 for \textit{E. faecalis} (range: 0.1 to 0.5), 0.3 for \textit{E. avium}, 0.23 for \textit{E. faecium} (range: from 0.1 to 0.4), and 0.25 for \textit{E. hirae}. (range: from 0.1 to 0.4). Table 2 shows the resistance profile of the 90 enterococcus isolates.

Table 2. Antimicrobial-resistance profile of Enterococcus isolates (n = 90).

| Resistance Profile | Number of Isolates | %Isolates |
|--------------------|--------------------|-----------|
| 16                 |                    | 17.8      |
| FOS                | 3                  | 3.3       |
| TCY                | 4                  | 4.4       |
| ERY                | 2                  | 2.2       |
| CIP FOS            | 1                  | 1.1       |
| TCY RIF            | 2                  | 2.2       |
| TCY FOS            | 2                  | 2.2       |
| TCY CIP            | 3                  | 3.3       |
| NIT TCY            | 5                  | 5.6       |
| AMP CIP            | 5                  | 5.6       |
| AMP CHL            | 1                  | 1.1       |
| AMP TCY            | 4                  | 4.4       |
| AMP NIT            | 2                  | 2.2       |
| ERY RIF            | 2                  | 2.2       |
| ERY CHL            | 2                  | 2.2       |
| ERY TCY            | 3                  | 3.3       |
| ERY NIT            | 2                  | 2.2       |
| VAN TCY            | 1                  | 1.1       |
| TCY CHL RIF       | 1                  | 1.1       |
| NIT TCY RIF       | 2                  | 2.2       |
| AMP TCY FOS       | 3                  | 3.3       |
Table 2. Cont.

| Resistance Profile | Number of Isolates | %Isolates |
|--------------------|--------------------|-----------|
| AMP TCY CIP        | 3                  | 3.3       |
| ERY TCY RIF        | 2                  | 2.2       |
| ERY TCY FOS        | 1                  | 1.1       |
| ERY TCY CIP        | 3                  | 3.3       |
| ERY NIT RIF        | 2                  | 2.2       |
| ERY NIT TCY        | 1                  | 1.1       |
| ERY PEN TCY        | 6                  | 6.7       |
| AMP TCY CHL CIP    | 1                  | 1.1       |
| AMP NIT CIP FOS    | 1                  | 1.1       |
| ERY CHL CIP FOS    | 1                  | 1.1       |
| VAN NIT TCY RIF    | 1                  | 1.1       |
| VAN ERY TCY RIF    | 1                  | 1.1       |
| AMP TCY CIP FOS RIF| 1                  | 1.1       |

FOS = Fosfomycin; TCY = tetracycline; ERY = erythromycin; CIP = ciprofloxacin; VAN = vancomycin; PEN = penicillin; NIT = nitrofurantoin; LZD = linezolid; CHL = chloramphenicol; RIF = rifampicin.

2.3. Antimicrobial-Resistance Genes

Nine antimicrobial-resistance genes were detected among the enterococci. Of the vancomycin-resistant isolates, 33.3% (1/3) contained vanA, while vanB was not detected in the three isolates. Twenty-four (86%) phenotypically erythromycin-resistant isolates were positive for erythromycin-resistant genes; the ermB gene was detected in all isolates, whereas the ermA gene was only detected in 11 isolates. Four tetracycline resistance genes (tetM, tetA, tetB, and tetL) were found in 94% (47/50) of tetracycline-resistant isolates. The prevalent resistance genes were tetM and tetL, accounting for 78% and 68% of the isolates, respectively; however, tetA and tetB were detected in 2% of the isolates. The cat gene was detected in 66% of the chloramphenicol-resistance isolates and the pbp5 gene for ampicillin resistance was detected in 22 enterococci isolates. Table 3 shows the distribution of antimicrobial-resistance genes among different enterococci.

Table 3. Distribution of antimicrobial-resistance genes among the isolated enterococci.

| E. faecalis (N = 45) | E. faecium (N = 30) | E. hirae (N = 12) | E. avium (N = 3) | Total Enterococci (N = 90) |
|----------------------|---------------------|-------------------|------------------|--------------------------|
| vanA                 | 1                   | 0                 | 0                | 1                        |
| vanB                 | 0                   | 0                 | 0                | 0                        |
| ermA                 | 5                   | 4                 | 2                | 11                       |
| ermB                 | 13                  | 9                 | 2                | 24                       |
| pbp5                 | 7                   | 8                 | 6                | 23                       |
| tetA                 | 1                   | 0                 | 0                | 1                        |
| tetB                 | 0                   | 0                 | 0                | 1                        |
| tetM                 | 26                  | 8                 | 3                | 39                       |
| tetL                 | 15                  | 12                | 5                | 34                       |
| optrA                | 0                   | 0                 | 0                | 0                        |
| Cat                  | 3                   | 1                 | 0                | 4                        |

2.4. Biofilm Formation

Overall, 73 Enterococcus isolates (81.1%) were biofilm producers, among which 39 were E. faecalis, 24 were E. faecium, 8 were E. hirae, and 2 were E. avium. The isolates were further classified into four categories based on the OD of the bacterial biofilm: 17 non-biofilm producers (18.9%), 16 weak biofilm producers (17.8%), 29 medium biofilm producers (32.2%), and 28 strong biofilm producers (31.1%). E. faecalis showed a significantly higher biofilm formation (p < 0.0001), and 13 E. faecalis isolates (28.9%) exhibited strong biofilm formation.

Figure 2 shows the biofilm formation strength of the Enterococcus species. A statistically significant pairwise correlation (p < 0.001) was obtained for biofilm formation versus MAR
index \((r = 0.807)\) and MDR \((r = 0.639)\). Table 4 shows the bacterial biofilm OD MAR index mean values in the four categories.

| Biofilm Category          | Mean Biofilm OD | Mean MAR Index | MDR | Gelatinase | Cytolysin | Agg | gelE |
|---------------------------|----------------|----------------|-----|------------|-----------|-----|------|
| Non-biofilm producers     | 0.29           | 0.017          | 0   | 0          | 0         | 0   | 0    |
| Weak biofilm producers    | 0.47           | 0.13           | 0   | 3          | 0         | 2   | 2    |
| Medium biofilm producers  | 0.9            | 0.22           | 8   | 3          | 3         | 8   | 7    |
| Strong biofilm producers  | 1.65           | 0.3            | 22  | 9          | 9         | 23  | 16   |

### 2.5. Gelatinase and Cytolysin Activity

Fifteen *Enterococcus* isolates (16.7%) were gelatinase producing, and 12 exhibited cytolysin activity (13.3%) (Figure 3). *E. faecalis* showed significantly higher gelatinase and cytolysin activities \((p < 0.0001)\). A statistically significant pairwise correlation \((p < 0.001)\) was found between biofilm formation versus gelatinase activity \((r = 0.245)\) and cytolysin activity \((r = 0.386)\) (Figure 4).

Figure 2. OD_{570} values indicate the amounts of bacterial biofilm among the Enterococcus species \((n = 90)\): non-biofilm producers (0.29–0.31); weak producers (0.4–0.55); medium producers (0.7–1.1); and strong producers (1.4–1.7).

Table 4. Distribution of mean MAR index and virulence genes among different biofilm categories.

Figure 3. Distribution of *gelE* (gelatinase), *agg* (aggregation substance), and *cylA* (activator of cytolysin) genes, and gelatinase and cytolysin activity in the 90 *Enterococcus* isolates.
was found between biofilm formation versus gelatinase activity \( (r = 0.245) \) and cytolysin activity \( (r = 0.386) \) (Figure 4).

Figure 3. **Distribution of** \( gelE \) (gelatinase), \( agg \) (aggregation substance), and \( cylA \) (activator of cytolysin) genes, and gelatinase and cytolysin activity in the 90 \( Enterococcus \) isolates.

Figure 4. Correlation matrix of phenotypic (biofilm formation ability, gelatinase activity, hemolytic activity, and antibiotic resistance) and genotypic (\( agg, gelE, cylA, \) and antibiotic-resistance genes) features exhibiting a significant \( (p < 0.05) \) correlation. White spaces are not significantly correlated. Significant positive correlation is represented by blue circles, whereas significant negative correlation is represented by red circles. The numerical value of the Phi correlation coefficient is represented by the size and strength of the color.

### 2.6. Virulence Genes

Figure 3 shows the distribution of virulence genes in all of the isolates and can be summarized as follows: \( agg \) in 21 \( E. faecalis \) (46.7%), 10 \( E. faecium \) (33.33%), and 5 \( E. hirae \) (41.66%); \( gelE \) in 18 \( E. faecalis \) (40%), 5 \( E. faecium \) (16.7%), 3 \( E. hirae \) (25%), and 1 \( E. avium \) (33.3%); \( cylA \) in 13 \( E. faecalis \) (28.9%), 6 \( E. faecium \) (20%), and 2 \( E. hirae \) (16.6%). The virulence genes were significantly higher in \( E. faecalis \) isolates \( (p < 0.0001) \). Simultaneously, nine \( E. faecalis \) (20%), two \( E. faecium \) (6.7%), and one \( E. hirae \) (8.3%) were positive for the three tested genes. The distribution of virulence genes among different biofilm categories is shown in Table 4. A significant correlation \( (p < 0.0001) \) was found between \( gelE \) and gelatinase \( (r = 0.521) \), \( cylA \) and cytolysin \( (r = 0.7) \), \( agg \) versus MDR \( (r = 0.0577) \), and \( gelE \) versus MDR \( (r = 0.514) \) (Figure 4).

### 2.7. MLST of \( E. faecalis \) and \( E. faecium \)

MLST allelic profiles for \( E. faecalis \) and \( E. faecium \) are presented in Tables 5 and 6. A total of 11 STs were found among the \( E. faecalis \) isolates. The most prevalent STs were ST16 \( (n = 10) \) and ST302 \( (n = 8) \), followed by ST179 \( (n = 6) \), ST480 \( (n = 5) \), and ST752 \( (n = 3) \) (Table 5). On the basis of the eBurst analysis and the phylogenetic analysis of the concatenated MLST sequences, seven STs were clustered into three groups: the first contained ST16, ST179, and ST302; the second ST81 and ST725; and the third ST176 and ST177; the remaining four STs (ST21, ST32, ST41, and ST480) were identified as singletons (Figure 5).

Eight STs were identified among the \( E. faecium \) isolates. The most abundant ST was ST194 \( (n = 8) \), followed by ST157 \( (n = 5) \), ST82 \( (n = 5) \), and ST9 \( (n = 4) \) (Table 6). The eBurst analysis shows that the registered isolates belong to five major clonal complexes (CC) in the order of their size: CC17, CC9, CC22, CC5, and CC94. Accordingly, ST9, ST157, ST82, ST194, and ST12 identified in this work are part of CC9, and ST16, ST18, and ST360 are part of the globally dispersed clonal lineage CC17 (Figure 6).
Table 5. Multi-locus sequence types and allele numbers for 45 *E. faecalis* strains isolated from backyard chickens.

| ST NO | gdh | gyd | pstS | gki | aroE | xpt | yqiL | Biofilm | MDR | Gelatinase | Cytolysin |
|-------|-----|-----|------|-----|------|-----|------|---------|-----|-------------|-----------|
| 16    | 10  | 5   | 1    | 3   | 7    | 7   | 6    | 10      | 6   | 2           | 3         |
| 21    | 3   | 1   | 7    | 9   | 1    | 1   | 1    | 1       | 0   | 0           | 0         |
| 32    | 1   | 8   | 7    | 9   | 5    | 4   | 1    | 1       | 0   | 0           | 0         |
| 41    | 5   | 1   | 7    | 11  | 21   | 1   | 4    | 1       | 3   | 0           | 0         |
| 81    | 1   | 27  | 2    | 16  | 28   | 26  | 2    | 1       | 1   | 1           | 1         |
| 176   | 2   | 15  | 7    | 3   | 37   | 39  | 15   | 11      | 2   | 0           | 1         |
| 177   | 1   | 15  | 2    | 37  | 37   | 39  | 15   | 11      | 0   | 0           | 0         |
| 179   | 6   | 5   | 1    | 1   | 3    | 7   | 1    | 6       | 5   | 2           | 3         |
| 302   | 8   | 5   | 1    | 1   | 3    | 7   | 7    | 60      | 8   | 4           | 5         |
| 480   | 5   | 1   | 1    | 22  | 22   | 7   | 17   | 6       | 5   | 2           | 0         |
| 752   | 3   | 27  | 2    | 16  | 28   | 26  | 83   | 1       | 3   | 0           | 0         |

Table 6. Multi-locus sequence types and allele numbers for 30 *E. faecium* strains isolated from backyard chickens.

| ST NO | atpA | ddl | gdh | purK | gyd | pstS | adk | Biofilm | MDR | Gelatinase | Cytolysin |
|-------|------|-----|-----|------|-----|------|-----|---------|-----|-------------|-----------|
| 12    | 1    | 5   | 2   | 6    | 6   | 1    | 7   | 1       | 0   | 0           | 0         |
| 360   | 2    | 5   | 2   | 6    | 6   | 1    | 1   | 1       | 0   | 0           | 0         |
| 16    | 3    | 1   | 2   | 1    | 1   | 1    | 1   | 1       | 2   | 1           | 0         |
| 194   | 8    | 1   | 1   | 1    | 1   | 1    | 1   | 1       | 8   | 4           | 2         |
| 157   | 5    | 7   | 1   | 1    | 1   | 5    | 1   | 1       | 5   | 2           | 0         |
| 9     | 4    | 4   | 5   | 1    | 3   | 1    | 1   | 1       | 4   | 0           | 0         |
| 18    | 2    | 15  | 1   | 1    | 1   | 1    | 1   | 1       | 1   | 0           | 1         |
| 82    | 5    | 1   | 36  | 1    | 1   | 1    | 1   | 4       | 1   | 0           | 1         |

Figure 5. Phylogeny of sequence types (ST) of *E. faecalis* isolated from backyard chickens based on neighbor-joining analysis of concatenated sequences of the seven housekeeping genes used for multi-locus sequence typing (MLST).
Figure 6. Phylogeny of sequence types (ST) of *E. faecium* isolated from backyard chickens based on neighbor-joining analysis of concatenated sequences of the seven housekeeping genes used for multi-locus sequence typing (MLST).

2.8. repA Genes (Plasmid Families)

Seven different rep-family plasmid genes (rep1–2, rep3, rep5–6, rep9, and rep11) were detected in the MDR enterococci. Two-thirds (20/30; 66.6%) of the enterococci were positive for one or two rep-families (Table 7). Seven out of fifteen *E. faecalis* (46.6%), two out of eight *E. faecium* (25%), one out of five *E. hirae* (20%), and one *E. avium* did not yield an amplicon for the rep-families. The most prevalent rep-family among *E. faecalis* was rep9 (pCF10), which was found in six isolates, followed by rep6 (pS86), which was found in three isolates; rep1 (pIP501) was found in one isolate. The predominant rep-family among *E. faecium* isolates was rep2 (pEF1071) with five isolates. Positive amplicons for rep6 (pS86) and rep1 (pIP501) were found in two and one isolates, respectively. Four different rep-families were found among *E. hirae*: rep5 (pN315) in three isolates, and each of rep3 (pAW63), rep6 (pS86), and rep11 (pEF1071) in one isolate. Positive amplicons for rep6 (pS86) and rep9 (pCF10) were found in the two *E. avium* isolates.
Table 7. Rep-families, phenotypic and genotypic resistance profile detected among MDR enterococci from backyard chickens.

| Enterococcus spp. | Phenotypic Profile | AMR Genes | Virulence Genes | Gelatinase | Cytolysin | Rep-Family | Plasmid |
|-------------------|-------------------|-----------|-----------------|------------|-----------|------------|---------|
| E. faecalis       | AMP, ERY, TET     | pbp5, tetM, tetL | geIE, agg       | –          | –         | 9          | pCF10   |
| E. faecalis       | AMP, TET, FOS     | pbp5, tetM | geIE, agg, cylA | –          | –         |            |         |
| E. faecalis       | ERY, NIT, TET     | ermA, ermB, tetM | geIE, agg       | +          | –         | 9          | pCF10   |
| E. faecalis       | ERY, CHL, CIP, FOS | ermB       | geIE, agg, cylA | +          | –         |            |         |
| E. faecalis       | VAN, ERY, TET, RIF | vanA, ermB, tetM, tetL | geIE, agg | +          | –         | 9, 1       | pIP501, pCF10 |
| E. faecalis       | ERY, NIT, RIF     | ermA       | geIE, agg, cylA | –          | +         |            |         |
| E. faecalis       | ERY, NIT, RIF     | ermB       | geIE, agg       | –          | –         |            |         |
| E. faecalis       | AMP, TET, CIP, RIF, FOS | pbp5        | geIE, agg, cylA | –          | +         | 6          | pS86    |
| E. faecalis       | TET, CHL, RIF     | tetM, cat  |                | –          | –         |            |         |
| E. faecalis       | AMP, TET, FOS     | pbp5, tetM | geIE, agg       | +          | –         |            |         |
| E. faecalis       | AMP, TET, FOS     | pbp5, tetM, tetL | geIE, agg, cylA | +          | +         | 9          | pCF10   |
| E. faecalis       | ERY, TET, RIF     | ermB, tetM | geIE, agg       | –          | –         | 9          | pCF10   |
| E. faecalis       | ERY, TET, RIF     | ermB       | geIE, agg       | –          | –         | 9          | pCF10   |
| E. faecalis       | ERY, NIT, RIF     | ermB, tetL |                | –          | –         |            |         |
| E. faecalis       | AMP, TET, CIP     | pbp5, tetM, tetL | agg            | –          | –         | 2          | pRE25   |
| E. faecium        | VAN, NIT, TET, RIF | vanA, tetM, tetL | agg            | –          | –         | 2, 1       | pIP501, pRE25 |
| E. faecium        | AMP, TET, CIP, RIF | pbp5, tetM, tetL | geIE, agg      | +          | –         | 2          | pRE25   |
| E. faecium        | ERY, AMP, TET     | ermA, ermB, tetM, tetL | geIE, agg, cylA | –          | +         | 2          | pRE25, pS86 |
| E. faecium        | ERY, TET, CIP     | tetL        | geIE, agg, cylA | –          | –         |            |         |
| E. faecium        | ERY, TET, CIP     | ermB        | agg            | –          | –         |            |         |
| E. faecium        | ERY, TET, CIP     | ermA, ermB | geIE, agg       | +          | –         | 6          | pS86    |
| E. faecium        | ERY, TET, FOS     | ermA, ermB, tetM, tetL | agg, cylA | –          | +         | 2          | pRE25   |
| E. hirae          | ERY, AMP, TET     | tetL        |                | –          | –         |            |         |
| E. hirae          | ERY, AMP, TET     | ermA, ermB, pbp5, tetM, tetL | agg         | –          | –         | 5          | pN315   |
| E. hirae          | ERY, AMP, TET     | ermA, ermB, pbp5, tetL | geIE, agg, cylA | –          | +         | 5, 3       | pN315, pAW63 |
| E. hirae          | AMP, NIT, CIP, FOS | pbp5        | geIE, agg       | –          | –         | 6          | pS86    |
| E. hirae          | ERY, AMP, TET     | ermA, ermB, pbp5, tetM, tetL | agg         | –          | +         | 5, 11      | pN315, pEF1071 |
| E. avium          | AMP, TET, CIP     | pbp5, tetB, tetM, tetL |                | –          | –         | 6          | pS86    |
| E. avium          | AMP, TET, CIP     | pbp5, tetM, tetL | geIE          | –          | –         | 6, 9       | pS86, pCF10 |

3. Discussion

Backyard chickens are considered to be a vector for disseminating several zoonotic diseases, including *Salmonella*, *Campylobacter*, enteropathogenic *E. coli*, and several antibiotic-resistant microorganisms [42–44]. Moreover, enterococci, notably *E. faecium* and *E. faecalis*,
have emerged as major multidrug-resistant zoonotic bacteria due to the widespread use of antibiotics in human and veterinary treatments [45].

In the current study, four Enterococcus species were isolated from healthy backyard chickens. E. faecalis was the most predominant species, which is consistent with the results of previous studies [43,46,47]. In contrast, E. faecium was reported as the prevalent species in poultry [48,49].

Antimicrobial resistance is one of the characteristics of enterococci, and their ability to acquire and spread antibiotic resistance presents a challenge for infection control [50]. In this study, a high proportion of resistance was identified in the isolated enterococci, the majority showing tetracycline resistance, which is accordance with various studies [43,51–53]. Tetracycline-resistance genes (tetM and tetL) were detected in 78% and 68% of the isolates, respectively, while tetA and tetB genes were detected in 2% of the isolates. Tetracycline resistance is most often mediated by tetM and tetL in enterococci from humans, animals, food, and the environment [46,54–57]. Different tetracycline-resistance genes were identified in the Enterococcus species [28,58]. Phenotypic resistance to erythromycin and ampicillin was observed in 31.1% and 30% of isolates. The ermB gene was detected in 86% of isolates, whereas the ermA gene was detected in 11 isolates, which is in accordance with Mlynarczyk et al. [59] who described erm(B) as the most prevalent gene conferring erythromycin resistance in enterococci. The pbp5 gene of ampicillin resistance was identified in 22 isolates, concurrent with [43]. A high level of Enterococcus resistance to both tetracycline and erythromycin was reported in Switzerland [60], the Netherlands [61], France [62], and Portugal [63]. Macrolides, tetracyclines, and penicillins are the major antimicrobials used in integrated broiler companies [64], while in Saudi Arabia, tetracycline and erythromycin are the most frequently used antimicrobials in poultry farms [65]. Furthermore, tetracycline resistance has been described to co-select for erythromycin resistance [53]. The WHO classified macrolides as critically important (the highest priority) and tetracycline as highly important antimicrobials for human medicine [66].

Resistance to vancomycin has generated substantial research interest during the last decade because it is the drug of last resort to treat enterococci infections in humans [67]. In this work, we observed low vancomycin resistance (3.3%) among the isolated enterococci. However, the vanA gene was identified in all phenotypic-resistance isolates. Our values are lower than those reported in other studies [68,69] and higher than the results of da Costa et al. [70]. However, Semedo-Lemsaddek et al. [43] did not detect any vancomycin resistance among their isolates. In the last decade, linezolid-resistant enterococci were detected in the USA, Europe, and Asia [50,71,72]. Remarkably, linezolid-resistant enterococci were not detected in our study.

MDR was frequently detected among the isolated enterococci in this study. The emergence of MDR enterococci has also been reported worldwide, particularly in Korea (26.9%), Spain (87.5%), and Ethiopia (78.2%), and is currently regarded as a growing public health concern [72–74].

Biofilm formation plays a considerable role in enterococcal infections and antibiotic resistance [75,76]. In this study, biofilm formation was observed in 81.1% of the isolates, which is concordant with [75,76]. A positive pairwise correlation was observed between biofilm production and both the MAR index and MDR. Moreover, biofilm production has been linked to antibiotic resistance in enterococci [77]. In addition, the gelE gene was found in all biofilm-producing isolates but not in non-biofilm-producing isolates, suggesting its significance in biofilm development. gelE is necessary for biofilm formation because it stimulates cell aggregation in microcolonies, allowing them to construct a three-dimensional structure [78].

Despite gelE gene detection in 28% of isolates, in vitro gelatinase activity was only detected in 17% of isolates. Similarly, other investigations found that 30%, 56%, and 59% of clinical isolates generated gelatinase, while 90%, 88%, and 92% were gelE positive, respectively [79–81]. Together with our findings, these reports show that while gelE regulated
the fsr locus is necessary for gelatinase activity, it is insufficient since fsrA and fsrB are also required for the gelatinase phenotype.

Enterococcal cytolysin is a hemolytic virulence factor linked to human disease and increased patient mortality [82]. The cylA gene was detected in 21 isolates (23%), concordant with other studies in poultry [64,65]. However, only 57.1% of the isolates expressed hemolysin activity, which is in agreement with the results in [83–85]. These findings may be attributable to environmental factors such as the in vitro and in vivo conditions used to test for phenotypic characteristics, as these could have a significant impact on gene expression [86].

MLST represents an outstanding tool for global and long-term epidemiological studies. In this work, all 45 E. faecalis were divided into 11 STs. The most common STs in the backyard chickens (ST16, ST302, and ST179) have been previously found in poultry, wild birds, and pigs [63,87,88]. Furthermore, ST16, ST21, ST179, and ST480 were reported among E. faecalis hospital isolates in Saudi Arabia [89]. ST16 isolates were previously reported to display major diversity as to the source of isolation and lesions [22]. Among the ST16 E. faecalis isolates, two isolates were resistant to vancomycin. vanA E. faecalis ST116 isolates were previously isolated from turkey meat and non-hospitalized humans [67,90].

MLST genotyping of E. faecium isolates revealed eight different ST types, of which five belonged to CC9 and three belonged to CC17, suggesting an evolutionary link between backyard E. faecium isolates [91]. ST9, ST157, ST194, and CC9 in particular were previously isolated from poultry and poultry meat [92,93]. Although CC17 was reported as a nosocomial clonal complex [94], several studies reported the circulation of E. faecium CC17 in animals [67,93,95]. Backyard chickens possibly acquired the CC17 E. faecium isolates from contaminated environments, or humans visiting the farm. This suggestion is supported by a previous study that demonstrated the transmission of E. faecium of human origin to chickens [96]. Moreover, human-linked E. faecium has been isolated from various water and food sources [97,98].

Plasmids are believed to be plastic structures that change as a result of the ever-changing environment in which they reside [33]. In this work, a recently published scheme for plasmid classification was utilized in order to investigate whether specific plasmid families were involved in AMR in enterococcus from backyard chickens. Ten enterococci did not carry any plasmid of the rep-families. This result is concordant with the studies of Jensen et al. and Cho et al. [37,99], in which approximately one-third of their isolates sets from humans, animals, and environments did not yield any amplicons. However, a lower percentage of negative rep-families was reported elsewhere [100,101], where 4% and 1.3% of E. faecium and E. faecalis did not yield any amplicons, respectively.

Seven different rep-family plasmid genes were identified in this study, a value concordant with several previous studies that detected five to nine rep-family plasmid genes [37,54,102–104] and lower than the study of Cho et al. [99], who identified 12 rep-family plasmid genes.

In this study, rep9 (pCF10) and rep2 (pEF1071) were the predominant plasmid among E. faecalis and E. faecium, respectively. These two rep-families were also reported in earlier studies [37,54,102–104]. The linkage between both Inc18 plasmids, mainly represented by rep2pRE25, and pheromone-responsive plasmids, represented by rep9 pCF10 and both tetracycline and glycopeptide, was previously reported [105–107].

Studies on E. hirae revealed the detection of rep5 (pN315), rep3 (pAW63), and rep11 (pEF1071). These rep-families had not been previously detected among E. faecalis and E. faecium isolates [37,54,102–104]. However, Cho et al. [99] reported these rep-families among E. hirae, E. casseliflavus, E. gallinarum, and E. mundti. This finding may suggest diverse plasmid contents among different species of Enterococcus.

A limitation of this study was the lack of environmental samples from the investigated flocks. However, in a previous study [108], a diversity of Enterococcus species was isolated from farm environments with multiple antibiotic-resistance profiles, indicating the role of
chicken in environmental contamination. Furthermore, this was a small study that could not include additional samples of Enterococcus species taken from this region.

4. Materials and Methods

4.1. Study Area

The study was conducted in Al-Ahsa Governorate, in the eastern region of Saudi Arabia (25°22’44.1” N 49°35’12.5” E) from March 2019 to December 2020. A total of 150 cloacal swabs were collected from apparently healthy broilers in 15 different backyard chicken flocks (10 samples from each flock). Selected backyard flocks size ranged from 20 to 180 (median = 80) chickens per flock. Chickens were fed a balanced diet of protein, carbohydrates, vitamins, and minerals without antibiotic additives. Swabs were collected by random capture of birds and transported at 4 °C to the laboratory for bacteriological examination.

4.2. Bacterial Isolation

Swabs were cultured on BD™ Enterococcosel™ Agar (Heidelberg, Germany) and incubated at 37 °C for 24 h. Colonies exhibiting a black halo were selected and subcultured on 5% sheep blood agar (Oxoid, UK) for purification. Purified isolates were identified based on colony morphology, Gram staining, catalase, and oxidase tests. Further, species-level identification was conducted biochemically using GP identification cards and the automated Vitek 2 compact system (BioMérieux, France).

4.3. DNA Extraction and 16S rRNA Gene Amplification and Sequencing

Biochemically identified isolates were cultured in brain heart infusion broth (Oxoid, UK) at 37 °C for 48 h. Cells were harvested by centrifugation, and the bacterial DNA was extracted and purified using the QIAamp DNA mini-kit (Qiagen SA, Courtaboeuf, France) according to the manufacturer’s instructions. The 16S rRNA gene was amplified and sequenced according to Weisburg et al. [109] and further analyzed using the National Center for Biological Information (NCBI) Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 15 December 2021).

4.4. Antimicrobial Sensitivity Test

Ten antimicrobials, i.e., vancomycin (VAN, ≥4 µg/mL), erythromycin (ERY, ≥0.5 µg/mL), ampicillin (AMP, ≥8 µg/mL), nitrofurantoin (NIT, ≥32 µg/mL), tetracycline (TET, ≥4 µg/mL), linezolid (LZD, ≥2 µg/mL), chloramphenicol (CHL, ≥8 µg/mL), ciprofloxacin (CIP, ≥1 µg/mL), rifampicin (RIF, ≥1 µg/mL), and fosfomycin (FOS, ≥64 µg/mL) (Merck KGaA, Darmstadt, Germany), were selected for enterococcus antimicrobial sensitivity testing. Each antibiotic’s minimum inhibitory concentration (MIC) was established using the CLSI 202 criteria and recommendations [110]. Multidrug resistance (MDR) was considered when isolates were resistant to three or more different antimicrobial classes [111], and the MAR index was calculated using the methodology outlined by Krumperman et al. [112]. Calculation of MIC50 and MIC90 (equivalent to the median MIC value) was performed according to Schwarz et al. [113].

4.5. Detection of Antimicrobial-Resistance Genes

Antimicrobial-resistance genes associated with vancomycin (vanA, vanB), erythromycin (ermA, ermB), tetracycline (tetA, tetB, tetM, tetL), chloramphenicol (cat), linezolid (optrA), and ampicillin (pbp5) were determined by PCR [11,114–120]. Primers and PCR conditions are presented in Supplementary Table S2.

4.6. Phenotypic Detection of Virulence Factors

4.6.1. Quantitative Biofilm Assay

Antimicrobial Biofilm formation was assessed according to the methods described by Stepanović et al. [121]. Enterococci from an overnight culture were cultivated in trypticase soy broth (TSB) supplemented with 1% glucose and incubated for 24 h at 37 °C. The culture
density was adjusted to an approximate 0.5 McFarland standard. Each culture was diluted in sterile TSB (1:100), and 200 µL from each was transferred to three wells of sterile 96 well polystyrene microtiter plates (Sigma-Aldrich, St. Louis, MO USA). A sterile TSB was used as a negative control, and *E. faecalis* (ATCC 29212) was used as a positive control. The plates were incubated at 37 °C for 48 h, washed with sterile phosphate-buffered solution, air-dried, and stained with 2% crystal violet for 30 min. Subsequently, the wells were gently washed with sterile deionized water and air-dried. The dye bound to the adherent cells was re-solubilized with absolute ethanol (150) µL. Each well’s optical density (OD) was measured at 570 nm in a plate reader (BioTek-800 ST, St. Louis, MO USA). The experiment was performed in triplicate on three different days. Each Enterococcus isolate was classified as a negative, weak, moderate, or strong biofilm producer following the criteria described by Stepanović et al. [121].

### 4.6.2. Gelatinase Activity

Gelatinase activity was assessed by inoculating pure culture on agar plates containing 3% gelatin [122]. After 48 h incubation, plates were flooded with a saturated ammonium sulfate solution. A transparent halo zone surrounding the colonies was considered positive for gelatinase.

### 4.6.3. Cytolysin Activity

For screening hemolysin production, *Enterococcus* isolates were streaked on Columbia agar supplemented with 5% horse blood and incubated at 37 °C for 24 h. A clear (β-hemolysis) or green (α hemolysis) zone around the colonies was defined as positive, whereas the γ-hemolysis was defined as negative activity [123].

### 4.7. Molecular Detection of Virulence Factor Genes

The virulence factor genes, including *gelE* (gelatinase), *agg* (aggregation substance), and *cylA* (activator of cytolysin), were screened by PCR, according to [124,125]. Primers and PCR conditions are tabulated in Supplementary Table S2.

### 4.8. Multi-Locus Sequence Typing

MLST for *E. faecalis* and *E. faecium* was performed by sequencing seven housekeeping genes described by Ruiz-Garbajosa et al. and Homan et al. [22,23]. Different sequences were assigned allele numbers, and different allelic profiles were assigned STs based on the MLST database (http://www.mlst.net/databases/, accessed: 15 December 2021).

### 4.9. PCR for repA Genes (Plasmid Families)

All MDR isolates were screened for rep-like sequences by PCR according to Jensen et al. [37] with primers and PCR conditions listed in Supplementary Table S2.

### 4.10. Statistical Analysis

The Fisher’s exact test or Chi-square test and Spearman’s rank correlation test were used for statistical analyses of the data (Prism 8 GraphPad Software, San Diego, CA, USA). Alignment and phylogenetic reconstructions were performed using the function “build” of ETE3 v3.1.1 [126] as implemented on the GenomeNet (https://www.genome.jp/tools/ete/, accessed: 15 December 2021). The tree was constructed using the Interactive Tree of Life (iTOL) v6.4.3 tool (https://itol.embl.de/, accessed: 15 December 2021) [127].

### 5. Conclusions

This study investigated virulence genes, antibiotic resistance, multi-locus sequence types, plasmid-associated genes, and the biofilm production of enterococci from healthy backyard chickens in Saudi Arabia to highlight the role of backyard chickens as a potential reservoir for MDR and virulent enterococci. Molecular analyses revealed the presence of nosocomial-associated CC17 and a variety of mobile genetic elements among the enterococci.
from backyard chickens, suggesting the possibility of their dissemination in backyard farms and their environments.

High resistance to different antimicrobial classes was identified, suggesting the over-use of antimicrobials in backyard chicken farms. The emergence of MDR and virulent enterococci in backyard chicken farms is a public health concern. Thus, regular surveillance for the occurrence of MDR and virulent enterococci in backyard poultry and its environment is recommended to prevent its spread and to minimize environmental contamination.

Furthermore, proactive antimicrobial agent control measures should be developed to limit the spread of MDR enterococci.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics11030380/s1, Figure S1: Description and categories of the collected variables. Table S1: Description and categories of the collected variables. Table S2: Description and categories of the collected variables.

Author Contributions: Conceptualization, M.F., A.S.A., M.A. and T.A.-M.; methodology, M.F., S.Y., A.A. and O.M.A.; software, M.F., A.A. and S.Y.; validation, M.F., S.Y. and M.A.; formal analysis, M.F., S.F.M. and M.A.; investigation, T.A.-M., A.A., A.S.A. and S.F.M.; resources, M.A., S.F.M., A.A., H.A. and T.A.-M.; data curation, M.F., H.A., M.A. and S.Y.; writing—original draft preparation, M.F., S.Y. and H.A.; writing—review and editing, M.F., S.Y., A.S.A. and H.A.; visualization, S.Y. and H.A.; supervision, M.F.; project administration, O.M.A.; funding acquisition, O.M.A. All authors have read and agreed to the published version of the manuscript.

Funding: Taif University Researchers Supporting Project number (TURSP-2020/262), Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia.

Institutional Review Board Statement: The Taif University Ethics Committee has approved the study protocol (TURSP-2020-262).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank the Al-Ahsa Veterinary Diagnostic Laboratory staff, Saudi Arabia, for technical assistance and sample collection. Authors would also like to thank Taif University Researchers Supporting Program (Project number: TURSP-2020/262), Taif University, Saudi Arabia for their support.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Fisher, K.; Phillips, C. The ecology, epidemiology and virulence of Enterococcus. Microbiology 2009, 155, 1749–1757. [CrossRef] [PubMed]
2. Byappanahalli, M.N.; Nevers, M.B.; Korajkic, A.; Staley, Z.R.; Harwood, V.J. Enterococci in the environment. Microbiol. Mol. Biol. Rev. 2012, 76, 685–706. [CrossRef] [PubMed]
3. García-Solache, M.; Rice, L.B. The Enterococcus: A model of adaptability to its environment. Clin. Microbiol. Rev. 2019, 32, e00058-18. [CrossRef] [PubMed]
4. Sava, I.G.; Heikens, E.; Huebner, J. Pathogenesis and immunity in enterococcal infections. Clin. Microbiol. Infect. 2010, 16, 533–540. [CrossRef] [PubMed]
5. Ammerlaan, H.S.; Harbarth, S.; Buiting, A.G.; Crook, D.W.; Fitzpatrick, F.; Hanberger, H.; Herwaldt, L.A.; van Keulen, P.H.; Kluytmans, J.A.; Kola, A.; et al. Secular trends in nosocomial bloodstream infections: Antibiotic-resistant bacteria increase the total burden of infection. Clin. Infect. Dis. 2013, 56, 798–805. [CrossRef] [PubMed]
6. Cheng, W.N.; Han, S.G. Bovine mastitis: Risk factors, therapeutic strategies, and alternative treatments—A review. Asian-Australas. J. Anim. Sci. 2020, 33, 1699–1713. [CrossRef] [PubMed]
7. Wood, M.W.; Lepold, A.; Tesfamichael, D.; Lasarey, M.R. Risk factors for enterococcal bacteriuria in dogs: A retrospective study. J. Vet. Intern. Med. 2020, 34, 2447–2453. [CrossRef] [PubMed]
8. Robbins, K.M.; Suyemoto, M.M.; Lyman, R.L.; Martin, M.P.; Barnes, H.J.; Borst, L.B. An outbreak and source investigation of enterococcal spondylitis in broilers caused by Enterococcus cecorum. Avian Dis. 2012, 56, 768–773. [CrossRef] [PubMed]
9. Seputiene, V.; Bogdaite, A.; Ruszauskas, M.; Suziedeliene, E. Antibiotic resistance genes and virulence factors in Enterococcus faecium and Enterococcus faecalis from diseased farm animals: Pigs, cattle and poultry. Pol. J. Vet. Sci. 2012, 15, 431–438.
10. Katakevba, A.; Mtambo, M.; Olsen, J.E.; Muhairwa, A.P. Awareness of human health risks associated with the use of antibiotics among livestock keepers and factors that contribute to selection of antibiotic resistance bacteria within livestock in Tanzania. *Livest. Res. Rural. Dev.* 2012, 24, 170.

11. Osman, K.M.; Badr, J.; Orabi, A.; Elbehiry, A.; Saad, A.; Ibrahim, M.D.; Hanafi, M.H. Poultry as a vector for emerging multidrug resistant *Enterococcus* spp.: First report of vancomycin (van) and the chloramphenicol–florfenicol (cat-fex-cfr) resistance genes from pigeon and duck faeces. *Microb. Pathog.* 2019, 128, 195–205. [CrossRef] [PubMed]

12. Hellenbeck, B.L.; Rice, L.B. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence* 2012, 3, 421–433. [CrossRef] [PubMed]

13. Asgin, N.; Otlu, B. Antibiotic Resistance and Molecular Epidemiology of Vancomycin-Resistant Enterococci in a Tertiary Care Hospital in Turkey. *Infect. Drug Resist.* 2020, 13, 191–198. [CrossRef] [PubMed]

14. Werner, G.; Coque, T.M.; Franz, C.M.; Grohmann, E.; Hegstad, K.; Jensen, L.; van Schaik, W.; Weaver, K. Antibiotic resistant enterococci—Tales of a drug resistance gene trafficker. *J. Med. Microbiol.* 2013, 303, 360–379. [CrossRef] [PubMed]

15. Anderson, A.C.; Jonas, D.; Huber, I.; Karygianni, L.; Wolber, J.; Hellwig, E.; Arweiler, N.; Vach, K.; Wittmer, A.; Al-Ahmad, A. *Enterococcus faecalis* from Food, Clinical Specimens, and Oral Sites: Prevalence of Virulence Factors in Association with Biofilm Formation. *Front. Microbiol.* 2015, 6, 1594. [CrossRef] [PubMed]

16. Sandoe, J.A.; Withereden, I.R.; Cove, J.H.; Heritage, J.; Wilcox, M.H. Correlation between enterococcal biofilm formation in vitro and medical-device-related infection potential in vivo. *J. Med. Microbiol.* 2003, 52, 547–550. [CrossRef] [PubMed]

17. Kayaoglu, G.; Orstavik, D. Virulence factors of *Enterococcus faecalis*: Relationship to endodontic disease. *Crit. Rev. Oral Biol. Med.* 2004, 15, 308–320. [CrossRef] [PubMed]

18. Pryce, T.M.; Wilson, R.D.; Kulski, J.K. Identification of enterococci by ribotyping with horseradish-peroxidase-labelled 16S rDNA probes. *J. Microbiol. Methods* 1999, 36, 147–155. [CrossRef]

19. Malathum, K.; Singh, K.V.; Weinstock, G.M.; Murray, B.E. Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *J. Clin. Microbiol.* 1998, 36, 211–215. [CrossRef] [PubMed]

20. Seetulsingh, P.S.; Tomayko, J.F.; Coudron, P.E.; Markowitz, S.M.; Skinner, C.; Singh, K.V.; Murray, B.E. Chromosomal DNA restriction endonuclease digestion patterns of beta-lactamase-producing *Enterococcus faecalis* isolates collected from a single hospital over a 7-year period. *J. Microbiol. Methods* 2004, 34, 1892–1896. [CrossRef]

21. Tomayko, J.F.; Murray, B.E. Analysis of *Enterococcus faecalis* isolates from intercontinental sources by multilocus enzyme electrophoresis and pulsed-field gel electrophoresis. *J. Clin. Microbiol.* 1995, 33, 2903–2907. [CrossRef] [PubMed]

22. Ruiz-Garbajosa, P.; Bonten, M.J.; Robinson, D.A.; Top, J.; Nallapareddy, S.R.; Torres, C.; Coque, T.M.; Cantón, R.; Baquero, F.; Murray, B.E.; et al. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. *J. Clin. Microbiol.* 2006, 44, 2220–2228. [CrossRef] [PubMed]

23. Homo, W.L.; Tribe, D.; Poznanski, S.; Li, M.; Hogg, G.; Spalburg, E.; Van Embden, J.D.; Willems, R.J. Multilocus sequence typing scheme for *Enterococcus faecium*. *J. Clin. Microbiol.* 2002, 40, 1963–1971. [CrossRef] [PubMed]

24. Achtman, M. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. *Annu. Rev. Microbiol.* 2008, 62, 53–70. [CrossRef] [PubMed]

25. Maâtallah, M.; Bakhrouf, A.; Habeeb, M.A.; Turlej-Rogacka, A.; Iversen, A.; Pourel, C.; Sioud, O.; Giske, C.G. Four Genotyping Schemes for Phylogenetic Analysis of Pseudomonas aeruginosa: Comparison of Their Congruence with Multi-Locus Sequence Typing. *PLoS ONE* 2013, 8, e82069. [CrossRef] [PubMed]

26. Aslam, M.; Diarra, M.S.; Checkley, S.; Bohach, V.; Masson, L. Characterization of antimicrobial resistance and virulence genes in *Enterococcus*) spp. isolated from retail meats in Alberta, Canada. *Int. J. Food Microbiol.* 2012, 156, 222–230. [CrossRef] [PubMed]

27. Kim, H.J.; Koo, M. Occurrence, Antimicrobial Resistance and Molecular Diversity of *Enterococcus faecalis* in Processed Pork Meat Products in Korea. *Food Sci. Biotechnol.* 2020, 9, 1283. [CrossRef]

28. Holman, D.B.; Klima, C.L.; Gzyl, K.E.; Zaheer, R.; Service, C.; Jones, T.H.; McAllister, T.A. Antimicrobial Resistance in *Enterococcus* spp. Isolated from a Beef Processing Plant and Retail Ground Beef. *Microbiol. Spectr.* 2021, 9, e0198021. [CrossRef] [PubMed]

29. Moreno, M.F.; Sarantinopoulos, P.; Tsakalidou, E.; De Vuyst, L. The role and application of enterococci in food and health. *Int. J. Food Microbiol.* 2006, 106, 1–24. [CrossRef] [PubMed]

30. Hegstad, K.; Mikalsen, T.; Coque, T.M.; Werner, G.; Sandsjord, A. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. *Clin. Microbiol. Infect.* 2010, 16, 541–554. [CrossRef]

31. Grohmann, E.; Muth, G.; Espinosa, M. Conjugative plasmid transfer in gram-positive bacteria. *Microbiol. Mol. Biol. Rev. MMBR* 2003, 67, 277–301. [CrossRef] [PubMed]

32. Hammerum, A.M.; Flannagan, S.E.; Clewell, D.B.; Jensen, L.B. Indication of transposition of a mobile DNA element containing the vat(D) and erm(B) genes in *Enterococcus faecium*. *Antimicrob. Agents Chemother.* 2001, 45, 3223–3225. [CrossRef] [PubMed]

33. Osborn, M.; Bron, S.; Firth, N.; Holspill, S.; Huddleston, A.; Kiewiet, R.; Meijer, W.; Seegers, J.; Skurray, R.; Terpstra, P.J.; et al. The evolution of bacterial plasmids. In *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*; CRC Press: Boca Raton, FL, USA, 2000; pp. 301–363.

34. Coque, T.M. Evolutionary biology of pathogenic enterococci in *Evolutionary Biology of Bacterial and Fungal Pathogens*; ASM Press: Washington, DC, USA, 2007; pp. 501–521.

35. Gilmore, M.S.; Clewell, D.B.; Courvalin, P.; Dunn, G.M.; Murray, B.E.; Rice, L.B. *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*; ASM Press: Washington, DC, USA, 2002; Volume 10.
36. Khan, S.A. Rolling-circle replication of bacterial plasmids. *Microbiol. Mol. Biol. Rev.* 1997, 61, 442–455. [CrossRef] [PubMed]

37. Jensen, L.B.; Garcia-Migura, L.; Valenzuela, A.J.; Lohr, M.; Hasman, H.; Aarestrup, F.M. A classification system for plasmids from enterococci and other Gram-positive bacteria. *J. Microbiol. Methods* 2010, 80, 25–43. [CrossRef] [PubMed]

38. Kwon, K.H.; Hwang, S.Y.; Moon, B.Y.; Park, Y.K.; Shin, S.; Hwang, C.Y.; Park, Y.H. Occurrence of antimicrobial resistance and virulence genes, and distribution of enterococcal clonal complex 17 from animals and human beings in Korea. *J. Vet. Diagn. Invest.* 2012, 24, 924–931. [CrossRef] [PubMed]

39. Salem-Bekhit, M.M.; Moussa, I.M.; Muharram, M.M.; Alanazy, F.K.; Hefni, H.M. Prevalence and antimicrobial resistance pattern of multidrug-resistant enterococci isolated from clinical specimens. *Indian J. Med. Microbiol.* 2012, 30, 44–51. [CrossRef] [PubMed]

40. Abdallah, M.; Al-Saafin, M. Overview of Prevalence, Characteristics, Risk Factors, Resistance, and Virulence of Vancomycin-Resistant Enterococci in Saudi Arabia. *Microb. Drug Resist.* 2019, 25, 350–358. [CrossRef] [PubMed]

41. Qadri, S.H.; Qunibi, W.Y.; Al-Ballaa, S.R.; Kadhi, Y.; Burdette, J.M. Vancomycin resistant enterococcus: A case report and review of the literature. *Ann. Saudi Med.* 1993, 13, 289–293. [CrossRef] [PubMed]

42. Pohjola, L.; Nykasenoja, S.; Kivisto, R.; Soever, T.; Huovilainen, A.; Hanninen, M.L.; Fredriksson-Ahomaa, M. Zoonotic Public Health Hazards of Antimicrobial Resistance in Enterococcus faecalis and Enterococcus faecium from Humans in the Community, Broilers, and Pigs in Denmark. *Diagn. Microbiol. Infect. Dis.* 2000, 37, 127–137. [CrossRef]

43. Semedo-Lemsaddek, T.; Bettencourt Cota, J.; Ribeiro, T.; Pimentel, A.; Tavares, L.; Bernando, F.; Oliveira, M. Resistance and virulence distribution in enterococci isolated from broilers reared in two farming systems. *Ir. Vet. J.* 2021, 74, 22. [CrossRef] [PubMed]

44. Aarestrup, F.M.; Agerso, Y.; Gerner-Smidt, P.; Madsen, M.; Jensen, L.B. Comparison of antimicrobial resistance phenotypes and genetic relatedness among enterococci isolated from dogs and cats in the United States. *Appl. Environ. Microbiol.* 2004, 70, 327–335. [CrossRef] [PubMed]

45. Tyynismaa, H.; Ishimaru, M.; Endoh, Y.S.; Kojima, A. Antimicrobial susceptibilities of enterococci isolated from faeces of broiler indicator bacteria. *Vet. World* 2021, 14, 2869–2877. [CrossRef] [PubMed]

46. Aarestrup, F.M.; Agerso, Y.; Gerner-Smidt, P.; Madsen, M.; Jensen, L.B. Comparison of antimicrobial resistance phenotypes and genetic relatedness among enterococci isolated from dogs and cats in the United States. *Appl. Environ. Microbiol.* 2004, 70, 327–335. [CrossRef] [PubMed]

47. Kaukas, A.; Hinton, M.; Linton, A.H. Changes in the faecal enterococcal population of young chickens and its effect on the incidence of resistance to certain antibiotics. *Lett. Appl. Microbiol.* 1986, 2, 5–8. [CrossRef] [PubMed]

48. Diarra, M.S.; Rempel, H.; Champagne, J.; Masson, L.; Pritchard, J.; Topp, E. Distribution of antimicrobial resistance and virulence genes in *Enterococcus* spp. and characterization of isolates from broiler chickens. *Appl. Environ. Microbiol.* 2010, 76, 8033–8043. [CrossRef] [PubMed]

49. Yoshimura, H.; Ishimaru, M.; Endoh, Y.S.; Kojima, A. Antimicrobial susceptibilities of enterococci isolated from faeces of broiler and layer chickens. *Lett. Appl. Microbiol.* 2000, 31, 427–432. [CrossRef] [PubMed]

50. Tyson, G.H.; Nyirabahizi, E.; Crarey, E.; Kabera, C.; Lam, C.; Rice-Trujillo, C.; McDermott, P.F.; Tate, H. Prevalence and Antimicrobial Resistance of Enterococci Isolated from Retail Meats in the United States, 2002 to 2014. *Appl. Environ. Microbiol.* 2018, 84, e01902-17. [CrossRef] [PubMed]

51. Boulianne, M.; Arsenault, J.; Daignault, D.; Letellier, A.; Dutil, L. Drug use and antimicrobial resistance among *Escherichia coli* and *Enterococcus* spp. isolates from chicken and turkey flocks slaughtered in Quebec, Canada. *Can. J. Vet. Res.* 2016, 80, 49–59. [PubMed]

52. Obeng, A.S.; Rickard, H.; Ndi, O.; Sexton, M.; Barton, M. Comparison of antimicrobial resistance patterns in enterococci from intensive and free range chickens in Australia. *Avian Pathol.* 2003, 42, 45–54. [CrossRef] [PubMed]

53. Persoons, D.; Dewulf, J.; Smet, A.; Heyndrickx, M.; Martel, A.; Catry, B.; Butaye, P.; Haesebrouck, F. Prevalence and persistence of antimicrobial resistance in broiler indicator bacteria. *Microb. Drug Resist.* 2010, 16, 67–74. [CrossRef] [PubMed]

54. Sadowy, E.; Luszczkiewicz, A. Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiol.* 2014, 14, 66. [CrossRef] [PubMed]

55. Jackson, C.R.; Fedorka-Cray, P.J.; Davis, J.A.; Barnett, J.B.; Brousse, J.H.; Gustafson, J.; Kucher, M. Mechanisms of antimicrobial resistance and genetic relatedness among enterococci isolated from dogs and cats in the United States. *J. Appl. Microbiol.* 2010, 108, 2171–2179. [CrossRef]

56. Huys, G.; D’Haene, K.; Collard, J.M.; Swings, J. Prevalence and molecular characterization of tetracycline resistance in Enterococcus isolates from food. *Appl. Environ. Microbiol.* 2004, 70, 1555–1562. [CrossRef] [PubMed]

57. Cauwerts, K.; Decostere, A.; De Graef, E.M.; Haesebrock, F.; Pasmans, F. High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the erm(B) gene. *Avian Pathol.* 2007, 36, 395–399. [CrossRef] [PubMed]

58. Sadoy, E.; Luszczkiewicz, A. Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiol.* 2014, 14, 66. [CrossRef] [PubMed]

59. Jackson, C.R.; Fedorka-Cray, P.J.; Davis, J.A.; Barnett, J.B.; Brousse, J.H.; Gustafson, J.; Kucher, M. Mechanisms of antimicrobial resistance and genetic relatedness among enterococci isolated from dogs and cats in the United States. *J. Appl. Microbiol.* 2010, 108, 2171–2179. [CrossRef]

60. Huys, G.; D’Haene, K.; Collard, J.M.; Swings, J. Prevalence and molecular characterization of tetracycline resistance in Enterococcus isolates from food. *Appl. Environ. Microbiol.* 2004, 70, 1555–1562. [CrossRef] [PubMed]

61. Frei, A.; Goldenberger, D.; Teuber, M. Antimicrobial susceptibility of intestinal bacteria from Swiss poultry flocks before the ban of antimicrobial growth promoters. *Syst. Appl. Microbiol.* 2001, 24, 116–121. [CrossRef] [PubMed]
62. Petsaris, O.; Miszczak, F.; Gicquel-Brunneau, M.; Perrin-Guyomard, A.; Humbert, F.; Sanders, P.; Leclercq, R. Combined antimicrobial resistance in Enterococcus faecium isolated from chickens. Appl. Environ. Microbiol. 2005, 71, 2796–2799. [CrossRef] [PubMed]

63. 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. 2005, 56, 1139–1143. [CrossRef] [PubMed]

64. Kim, Y.B.; Seo, K.W.; Jeon, H.Y.; Lim, S.K.; Sung, H.W.; Lee, Y.J. Molecular characterization of erythromycin and tetracycline-resistant Enterococcus faecalis isolated from retail chicken meats. Poult. Sci. 2019, 98, 977–983. [CrossRef] [PubMed]

65. Al-Mustafa, Z.H.; Al-Ghamdi, M.S. Use of antibiotics in the poultry industry in Saudi Arabia: Implications for public health. Ann. Saudi Med. 2002, 22, 4–7. [CrossRef] [PubMed]

66. Petsaris, O.; Miszczak, F.; Gicquel-Bruneau, M.; Perrin-Guyomard, A.; Humbert, F.; Sanders, P.; Leclercq, R. Combined antimicrobial resistance in Enterococcus faecium isolated from chickens. Appl. Environ. Microbiol. 2005, 71, 2796–2799. [CrossRef] [PubMed]

67. Mohamed, J.A.; Murray, B.E. Lack of correlation of gelatinase production and biofilm formation in a large collection of Enterococcus faecalis. J. Clin. Microbiol. 2004, 42, 2317–2320. [CrossRef] [PubMed]

68. Colignon, P.; Conly, J.; Andremont, A. Members of the World Health Organization Advisory Group, Bogota meeting on Integrated Surveillance of Antimicrobial Resistance (WHO-AGISAR). Clin. Infect. Dis. 2016, 63, 1087–1093. [CrossRef] [PubMed]

69. Ahmed, 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] [PubMed]

70. da Costa, P.M.; Oliveira, M.; Bica, A.; Vaz-Pires, P.; Bernardo, F. Antimicrobial resistance in Enterococcus spp. and Escherichia coli isolated from poultry feed and feed ingredients. Vet. Microbiol. 2007, 120, 122–131. [CrossRef] [PubMed]

71. Cavaco, L.M.; Korsgaard, H.; Kaas, R.S.; Seyfarth, A.M.; Leekitcharoenphon, P.; Hendriksen, R.S. First detection of linezolid resistance due to the oprA gene in enterococci isolated from food products in Denmark. J. Glob. Antimicrob. Resist. 2017, 9, 128–129. [CrossRef] [PubMed]

72. Yoon, S.; Kim, Y.B.; Seo, K.W.; Ha, J.S.; Noh, E.B.; Lee, Y.J. Characteristics of linezolid-resistant Enterococcus faecalis isolates from broiler breeder farms. Poult. Sci. 2020, 99, 6055–6061. [CrossRef] [PubMed]

73. Castano-Arriba, A.; Gonzalez-Machado, C.; Igrejas, G.; Poeta, P.; Alonso-Calleja, C.; Capita, R. Antibiotic Resistance and Biofilm-Forming Ability in Enterococcal Isolates from Red Meat and Poultry Preparations. Pathogens 2020, 9, 1021. [CrossRef] [PubMed]

74. Bekele, B.; Ashenafi, M. Distribution of drug resistance among enterococci and Salmonella from poultry and cattle in Ethiopia. Trop. Anim. Health Prod. 2010, 42, 857–864. [CrossRef] [PubMed]

75. Collignon, P.; Conly, J.; Andremont, A. Members of the World Health Organization Advisory Group, Bogota meeting on Integrated Surveillance of Antimicrobial Resistance (WHO-AGISAR). Clin. Infect. Dis. 2016, 63, 1087–1093. [CrossRef] [PubMed]

76. El-Zamkan, M.A.; Mohamed, H.M.A. Antimicrobial resistance, virulence genes and biofilm formation in Enterococcus species isolated from milk of sheep and goat with subclinical mastitis. PLoS ONE 2021, 16, e0299584. [CrossRef] [PubMed]

77. Chai, W.L.; Hamimah, H.; Cheng, S.C.; Sallam, A.A.; Abdullah, M. Susceptibility of Enterococcus faecalis multilocus sequence types of antibiotic-resistant Enterococcus faecalis. J. Antimicrob. Chemother. 2015, 70, 1857–1864. [CrossRef] [PubMed]

78. Kim, Y.B.; Seo, K.W.; Jeon, H.Y.; Lim, S.K.; Sung, H.W.; Lee, Y.J. Molecular characterization of erythromycin and tetracycline-resistant Enterococcus faecalis isolated from retail chicken meats. Poult. Sci. 2019, 98, 977–983. [CrossRef] [PubMed]

79. Al-Mustafa, Z.H.; Al-Ghamdi, M.S. Use of antibiotics in the poultry industry in Saudi Arabia: Implications for public health. Ann. Saudi Med. 2002, 22, 4–7. [CrossRef] [PubMed]

80. Roberts, J.C.; Singh, K.V.; Okhuysen, P.C.; Murray, B.E. Molecular epidemiology of the fsr locus and of gelatinase production in Enterococcus faecalis. J. Clin. Microbiol. 2004, 42, 2317–2320. [CrossRef] [PubMed]

81. Mohamed, J.A.; Murray, B.E. Lack of correlation of gelatinase production and biofilm formation in a large collection of Enterococcus faecalis isolates. J. Clin. Microbiol. 2005, 43, 5405–5407. [CrossRef] [PubMed]

82. Cox, C.R.; Coburn, P.S.; Gilmore, M.S. Enterococcal cytolysin: A novel two-component peptide system that serves as a bacterial defense against eukaryotic and prokaryotic cells. Curr. Protein Pept. Sci. 2005, 6, 77–84. [CrossRef] [PubMed]

83. Gholizadeh, P.; Aghazadeh, M.; Ghotsaslou, R.; Ahangarzadeh Rezaee, M.; Pirzadeh, T.; Kose, S.; Ganbarov, K.; Yousefi, M.; Karif, H.S. CRISPR-cas system in the acquisition of virulence genes in dental-root canal and hospital-acquired isolates of Enterococcus faecalis. Virulence 2020, 11, 1257–1267. [CrossRef] [PubMed]

84. Sun, J.; Sundsfjord, A.; Song, X. Enterococcus faecalis from patients with chronic periodontitis: Virulence and antimicrobial resistance traits and determinants. Eur. J. Clin. Microbiol. Infect. Dis. 2012, 31, 267–272. [CrossRef] [PubMed]

85. Sedgley, C.M.; Molander, A.; Flannagan, S.E.; Nagel, A.C.; Appelbe, O.K.; Clewell, D.B.; Dahlen, G. Virulence, phenotype and genotype characteristics of endoendocytic Enterococcus faecalis. Oral Microbiol. Immunol. 2005, 20, 10–19. [CrossRef] [PubMed]

86. Finlay, B.B.; Falkow, S. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. MMBR 1997, 61, 136–169. [CrossRef] [PubMed]

87. Stepien-Pyśniak, D.; Hauschild, T.; Nowaczek, A.; Marek, A.; Dec, M. Wild birds as a potential source of known and novel multilocus sequence types of antibiotic-resistant Enterococcus faecalis. J. Wildl. Dis. 2018, 54, 219–228. [CrossRef] [PubMed]

88. Gregersen, R.H.; Petersen, A.; Christensen, H.; Bisgaard, M. Multilocus sequence typing of Enterococcus faecalis isolates demonstrating different lesion types in broiler breeders. Avian Pathol. 2010, 39, 435–440. [CrossRef] [PubMed]
Antibiotics 2022, 11, 380

89. Farman, M.; Yasir, M.; Al-Hindi, R.R.; Farraj, S.A.; Jiman-Fatani, A.A.; Alawi, M.; Azhar, E.I. Genomic analysis of multidrug-resistant clinical Enterococcus faecalis isolates for antimicrobial resistance genes and virulence factors from the western region of Saudi Arabia. Antimicrob. Resist. Infect. Control 2019, 8, 55. [CrossRef] [PubMed]

90. Freitas, A.R.; Novais, C.; Ruiz-Garbajosa, P.; Coque, T.M.; Peixe, L. Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of Enterococcus faecalis from Portugal. J. Antimicrob. Chemother. 2009, 63, 1104–1111. [CrossRef] [PubMed]

91. De Leener, E.; Martel, A.; De Graef, E.M.; Top, J.; Butaye, P.; Haesebrouck, F.; Willems, R.; Decosteere, A. Molecular analysis of human, porcine, and poultry Enterococcus faecium isolates and their erm(B) genes. Appl. Environ. Microbiol. 2005, 71, 2766–2770. [CrossRef]

92. Wist, V.; Morach, M.; Schneeberger, M.; Cernela, N.; Stevens, M.J.A.; Zurfluh, K.; Stephani, R.; Nüesch-Inderbinen, M. Phenotypic and Genotypic Traits of Vancomycin-Resistant Enterococci from Healthy Food-Producing Animals. Microorganisms 2020, 8, 261. [CrossRef]

93. 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, 24. [CrossRef]

94. Lopez, M.; Hormazabal, J.; Maldonado, A.; Saavedra, G.; Baquero, F.; Silva, J.; Torres, C.; Del Campo, R. Clonal dissemination of Enterococcus faecalis ST201 and Enterococcus faecium CC17–ST64 containing Tn5382–vanB2 among 16 hospitals in Chile. Clin. Microbiol. Infect. 2009, 15, 586–588. [CrossRef]

95. Getachew, Y.; Hassan, L.; Zakaria, Z.; Abdul Aziz, S. Genetic variability of vancomycin-resistant Enterococcus faecium and Enterococcus faecalis isolates from chickens, and pigs in Malaysia. Appl. Environ. Microbiol. 2013, 79, 4528–4533. [CrossRef]

96. Sakai, Y.; Tsukahara, T.; Ushida, K. Possibility of vancomycin-resistant enterococci transmission from human to broilers, and possibility of using the vancomycin-resistant gram-positive cocci as a model in a screening study of vancomycin-resistant enterococci infection in the bird chick. Anim. Sci. J. 2006, 77, 538–544. [CrossRef]

97. Blanch, A.; Caplin, J.; Iversen, A.; Kühn, I.; Manero, A.; Taylor, H.; Vilanova, X. Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. J. Appl. Microbiol. 2003, 94, 994–1002. [CrossRef]

98. Novais, C.; Coque, T.M.; Ferreira, H.; Sousa, J.C.; Peixe, L. Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. Appl. Environ. Microbiol. 2005, 71, 3364–3368. [CrossRef]

99. Cho, S.; Barrett, J.B.; Frye, J.G.; Jackson, C.R. Antimicrobial Resistance Gene Detection and Plasmid Typing Among Multidrug-Resistant Enterococci Isolated from Freshwater Environment. Microorganisms 2020, 8, 1338. [CrossRef]

100. Wardal, E.; Gawryszewska, I.; Hryniewicz, W.; Sadowy, E. Abundance and diversity of plasmid-associated genes among clinical isolates of Enterococcus faecalis. Plasmid 2013, 70, 329–342. [CrossRef]

101. Tremblay, C.L.; Charlebois, A.; Masson, L.; Archambault, M. Characterization of hospital-associated lineages of ampicillin-resistant Enterococcus faecalis from clinical cases in dogs and humans. Front. Microbiol. 2013, 4, 245. [CrossRef]

102. Song, X.; Sun, J.; Mikalsen, F.; Paice, L. Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. Front. Microbiol. 2015, 6, 24. [CrossRef]

103. Cho, S.; Barrett, J.B.; Frye, J.G.; Jackson, C.R. Antimicrobial Resistance Gene Detection and Plasmid Typing Among Multidrug-Resistant Enterococci Isolated from Freshwater Environment. Microorganisms 2020, 8, 1338. [CrossRef]

104. Wardal, E.; Gawryszewska, I.; Zabicka, D.; Hryniewicz, W.; Sadowy, E. Diversity of plasmids and Tn1546-type transposons among VanA Enterococcus faecalis in Poland. Eur. J. Clin. Microbiol. Infect. Dis. 2017, 36, 313–328. [CrossRef]

105. Ike, Y.; Flannagan, S.E.; Clewell, D.B. Hyperhemolytic phenomena associated with insertions of Tn916 into the hemolysin determinant of Enterococcus faecalis plasmid pAD1. J. Bacteriol. 1992, 174, 1801–1809. [CrossRef]

106. Zheng, B.; Tomita, H.; Inoue, T.; Ike, Y. Isolation of VanB-type Enterococcus faecalis strains from nosocomial infections: First report of the isolation and identification of the phenomone-responsive plasmids pMG2200, Encoding VanB-type vancomycin resistance and a Bac41-type bacteriocin, and pMG2201, encoding erythromycin resistance and cytolysin (Hly/Bac). Antimicrob. Agents Chemother. 2009, 53, 735–747. [CrossRef]

107. Freitas, A.R.; Coque, T.M.; Novais, C.; Hammerum, A.M.; Lester, C.H.; Zervos, M.J.; Donabedian, S.; Jensen, L.B.; Francia, M.V.; Baquero, F.; et al. Human and swine hosts share vancomycin-resistant Enterococcus faecalis CC17 and CC5 and CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. J. Clin. Microbiol. 2011, 49, 925–931. [CrossRef]

108. Hayes, J.R.; English, L.L.; Carr, L.E.; Wagner, D.D.; Joseph, S.W. Multiple-antibiotic resistance of Enterococcus faecalis and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. J. Clin. Microbiol. 2004, 42, 6005–6011. [CrossRef]

109. Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 1991, 173, 697–703. [CrossRef]

110. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. CLSI Supplement M100, 31st ed.; Clinical and Laboratory Standards Institute: Malvern, PA, USA, 2021.

111. Magiorakos, A.P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; Harbarth, S.; Hindler, J.F.; Kahlmeter, G.; Olsson-Liljequist, B.; et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 2012, 18, 268–281. [CrossRef]
112. Krumperman, P.H. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.* 1983, 46, 165–170. [CrossRef] [PubMed]

113. Schwarz, S.; Silley, P.; Simjee, S.; Woodford, N.; van Duijkeren, E.; Johnson, A.P.; Gaaster, W. Editorial: Assessing the antimicrobial susceptibility of bacteria obtained from animals. *J. Antimicrob. Chemother.* 2010, 65, 601–604. [CrossRef] [PubMed]

114. Nam, S.; Kim, M.J.; Park, C.; Park, J.G.; Maeng, P.J.; Lee, G.C. Detection and genotyping of vancomycin-resistant *Enterococcus* spp. by multiplex polymerase chain reaction in Korean aquatic environmental samples. *Int. J. Hyg. Environ. Health* 2013, 216, 421–427. [CrossRef] [PubMed]

115. Werckenthin, C.; Schwarz, S. Molecular analysis of the translational attenuator of a constitutively expressed *erm(A)* gene from *Staphylococcus intermedius*. *J. Antimicrob. Chemother.* 2000, 46, 785–788. [CrossRef] [PubMed]

116. Jensen, L.B.; Frimodt-Moller, N.; Anæstrup, F.M. Presence of *erm* gene classes in gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiol. Lett.* 1999, 170, 151–158. [CrossRef] [PubMed]

117. Ng, L.K.; Martin, I.; Alifa, M.; Mulvey, M. Multiplex PCR for the detection of tetracycline resistant genes. *Mol. Cell. Probes* 2001, 15, 209–215. [CrossRef] [PubMed]

118. Trzcinski, K.; Cooper, B.S.; Hryniewicz, W.; Dowson, C.G. Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 2000, 46, 785–788. [CrossRef] [PubMed]

119. Wang, S.; Zhang, S.; Liu, Z.; Liu, P.; Shi, Z.; Wei, J.; Shao, D.; Li, B.; Ma, Z. Molecular characterization of enterohemorrhagic *E. coli* O157 isolated from animal fecal and food samples in Eastern China. *Sci. World J.* 2014, 2014, 946394. [CrossRef] [PubMed]

120. Jureen, R.; Top, J.; Mohn, S.C.; Harthug, S.; Langeland, N.; Willems, R.J. Molecular characterization of ampicillin-resistant *Enterococcus faecium* isolates from hospitalized patients in Norway. *J. Clin. Microbiol.* 2003, 41, 2330–2336. [CrossRef] [PubMed]

121. Stepasnovic, S.; Vukovic, D.; Hola, V.; Di Bonaventura, G.; Djukic, S.; Cirkovic, I.; Ruzicka, F. Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS Acta Pathol. Microbiol. Immunol. Scand.* 2007, 115, 891–899. [CrossRef] [PubMed]

122. Lopes Mde, F.; Simoes, A.P.; Tenreiro, R.; Marques, J.J.; Crespo, M.T. Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *Int. J. Food Microbiol.* 2006, 112, 208–214. [CrossRef] [PubMed]

123. Huerta-Cepas, J.; Serra, F.; Bork, P. ETE 3: Reconstruction, Analysis, and Visualization of Phylogenomic Data. *Mol. Biol. Evol.* 2016, 33, 1635–1638. [CrossRef] [PubMed]

124. Letunic, I.; Bork, P. Interactive Tree of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 2021, 49, W293–W296. [CrossRef] [PubMed]