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Antibiotic Resistance to Critically Important Antimicrobials and Virulence Genes in *Enterococcus Faecalis* Strains Isolated from Eurasian Griffon (*Gyps fulvus*) Vultures and Their Association with Mobile Genetic Elements

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Antibiotic Resistance to Critically Important Antimicrobials and Virulence Genes in *Enterococcus Faecalis* Strains Isolated from Eurasian Griffon (*Gyps fulvus*) Vultures and Their Association with Mobile Genetic Elements

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Simple Summary

The purpose of this study was to determine the phenotypic resistance of *E. faecalis* isolates from Eurasian griffon vultures to various antimicrobials used in animal health and human medicine, and to characterize the antimicrobial resistance and virulence genes and mobile genetic elements in the genome of a subset of isolates with phenotypic resistance to the critical antimicrobials linezolid, chloramphenicol, ciprofloxacin and gentamicin. Vultures were chosen because of their wide geographic distribution and their scavenging habits, and because they live in close proximity to humans. Most *E. faecalis* isolates (82.1%) exhibited resistance to six antimicrobials, indicating the widespread presence of resistant bacteria in *E. faecalis* isolates in this vulture population. Multidrug resistance was detected in 34% of the isolates. Of special concern is the detection of resistant isolates to antimicrobials linezolid, chloramphenicol, ciprofloxacin and gentamicin, considered clinically important in human medicine. The results of this study show that a significant proportion of *E. faecalis* strains recovered from vultures were multidrug resistant harboring mobile genetic elements (plasmid replicons, transposons and composite transposons) that carried antimicrobial resistance and virulence-associated genes. These findings are cause for concern, since vultures may act as spreaders of these genes to the environment and even to other hosts.

Abstract

The phenotypic resistance of 56 *E. faecalis* isolates from Eurasian griffon vultures was subjected to surveillance testing with the microdilution method using a standardized panel of antimicrobials. Isolates were also characterized by MLST. Additionally, the genome of 19 isolates with phenotypic resistance to linezolid, ciprofloxacin, chloramphenicol and/or high-level resistance to gentamicin were sequenced to determine their antimicrobial resistance (ARGs) and virulence-associated genes and to identify mobile genetic elements (MGEs). Most isolates (82.1%) exhibited non-wild-type phenotypes to six antimicrobial agents, and multidrug resistance (MDR) was detected in 34% of the isolates. Most MDR isolates (53%) belonged to ST16, ST116 and ST35. ARGs were localized on the chromosome as well as on different MGEs previously reported in humans, foods and livestock isolates, suggesting the exchange of antimicrobial-resistant bacteria and/or ARGs to vultures consequence of an anthropogenic pollution. Twenty-two virulence-associated genes encoding cell surface and secreted factors were identified, some of them located on MGEs that also carried ARGs.

The significant proportion of *E. faecalis* isolates recovered from vultures exhibiting MDR phenotypes and harbored MGEs carrying ARGs and virulence-associated genes, is a cause for concern, since vultures may act as spreaders of these genes to the environment, domestic animals and humans.

Keywords: vultures; microbiota; *Enterococcus faecalis*; antimicrobial resistance; virulence genes; mobile genetic elements; genotyping

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1. Introduction

Antimicrobial resistance (AMR) is a complex problem that affects humans, animals and the environment. It is considered the greatest public and animal health threat of the 21st century [1] and requires a coordinated approach across different sectors to mitigate its impact [2]. Wildlife serves as a link between environmental, human and animal domains through direct and indirect contact, faecal contamination and shared environmental resources [3]. Although the role of wildlife has been highlighted with the application of the "One Health" concept to AMR, studies characterizing AMR in wild animals are not as exhaustive as those carried out with humans and livestock [4]. These studies show that antibiotic-resistant bacteria (ARBs) can be isolated from a multitude of wild animals across different geographical areas, including wild birds, mammals and reptiles [5–8]. Wildlife is often referred to as sentinels, reservoirs and bridging hosts, facilitating the persistence of AMR in their habitats [4,9]. In addition, because many wild animal species are highly mobile, they can play a crucial role in the transmission of ARBs over long distances [10]. Consequently, the role of wildlife in the dissemination of AMR might be underestimated [4,9], and further research in this area is needed to properly assess the public health implications of AMR in wild animals.

The Eurasian griffon vulture (*Gyps fulvus*) is the most widespread vulture across Europe, Asia and Africa (http://www.birdlife.org). These vultures are obligate scavengers, feeding primarily on carcasses of dead livestock left at supplementary feed stations but also on carcasses from other unmanaged sources, such as wild species and human waste [11,12]. Although vultures are wild animals that do not receive antibiotics, they can be exposed to active antibiotics ingested when feeding on livestock carcasses [13], or they can be contaminated or colonized by ARBs through environmental contamination resulting from human activities [14]. Previous studies have isolated different ARBs, such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp. and *Enterococcus* spp., from the faecal microbiota of vultures [14–16]. In addition, the diet of vultures has been identified as a frequent source of AMR genes (ARGs) in their gut microbiome [13]. Therefore, owing to their feeding habits, migratory behaviour, and capacity to adapt to different environments, these wild birds may serve as reservoirs of ARBs present in their microbiomes with the potential to spread resistance genes over long distances and into new areas [5].

Enterococci are among the indicator bacteria used to study the extent of AMR in populations owing to their ability to spread resistance and virulence genes [9], as demonstrated in the surveillance program implemented in the European Union [17]. These bacteria are common among the commensal microbiota of domesticated and wild animals and are also opportunistic pathogens associated with significant morbidity and mortality in humans and animals [18]. In a previous study, analysis of cloacal and pharyngeal samples of Eurasian griffon vultures identified *Enterococcus faecalis* as one of the most common bacterial species [19]. Strains of *E. faecalis* isolated from faecal samples of vultures have been found to carry clinically important resistance determinants and virulence traits [14,16]. Both ARGs and virulence genes can spread between bacterial species or genera via horizontal gene transfer (HGT) through different mobile genetic elements (MGEs), such as conjugative transposons or plasmids [20]. From the perspective of the "One Health" concept, the study of AMR is a field of growing interest in microbial ecology [21]. In addition, there is no information available on the presence of MGEs among E. faecalis in the vulture microbiota. Thus, the purpose of this study was (a) to determine the phenotypic resistance of cloacal and pharyngeal E. faecalis isolates from Eurasian griffon vultures to various antimicrobials used in animal health and human medicine and

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(b) to characterize ARGs, virulence genes and MGEs in the genome of ARBs to four critical antimicrobials: linezolid, chloramphenicol, ciprofloxacin and high-level resistance (HLR) to gentamicin.

2. Materials and Methods

2.1. *Enterococcus faecalis* Isolates and Susceptibility Testing

The study focused on 56 *E. faecalis* isolates recovered from cloacal (*n*=35) or pharyngeal (*n*=21) samples of 45 Eurasian griffon (*Gyps fulvus*) vultures [19]. The *E. faecalis* isolates were subjected to surveillance testing with the microdilution method [22] using a standardized panel of antimicrobials (Sensititre EU Surveillance *Enterococcus* EUVENC Antimicrobial Susceptibility Testing Plates). In brief, inocula of the isolates were prepared in Muller–Hinton broth, adjusted to a 0.5 McFarland standard, and further diluted 1/220 in sterile distilled water. They were then deposited into each well of the microdilution plates, which were subsequently incubated at 37°C for 24 h. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as control strains. The epidemiological cut-off (ECOFF) values (ECVs) used for the interpretation of the minimal inhibitory concentrations (MICs) of the isolates were in accordance with the guidelines of the European Committee for Antimicrobial Susceptibility Testing (https://www.eucast.org) and the European Decision 2020/1729 [23]. Based on the ECV, the isolates were classified into wild-type (WT, without phenotypically detectable resistance) and non-WT (NWT, with phenotypically detectable resistance) categories (Table 1). HLR to gentamicin was defined as strains presenting MICs > 500 µg/mL, in accordance with the recommendations of the Clinical and Laboratory Standards Institute [24]. Isolates with phenotypically detectable resistance to three or more antimicrobials were classified as multidrug resistant (MDR). To assess the contribution of putative active efflux, the MICs of gentamicin, erythromycin and ciprofloxacin were determined in the presence or absence of the inhibitor reserpine (final concentration, 20 µg/ml; Sigma–Aldrich). The experiments were repeated three times. An efflux mechanism was inferred to be present when the antibiotic MICs in the presence of reserpine were at least 4-fold lower than the corresponding MICs in the absence of this compound [25].

Table 1. Minimal inhibitory concentrations for 12 antimicrobial agents of *E. faecalis* isolates from Eurasian griffon vultures.

Class	Antimicrobial	No. of isolates with MIC of (µg/ml)																	% non-WT isolates
		0.03	0.06	0.12	0.25	0.50	1	2	4	8	16	32	64	128	256	512	1024	≥2056	
β-Lactam	AMP					<2	38	16					>						0
Quinolone	CIP				<	3	35	9	1		2>	6							14.3
Macrolide	ERY						<10	8	5				2	>	31				58.9
Aminoglycoside	GEN									<31	11			1			1[a]>	12[a]	25.0
Liopeptide	DAP				<1	4	39	12				>							0
Tetracycline	TET						<10				1	1	5	37>	2				82.1
	TGC	<	16	32	8				>										0
Phenicol	CHL								<2	38	1	1	9	5>					25.0
Oxazolidinone	LZD					<	8	45	1		2		>						3.6
Streptogramin	SYN					<3	1		1	10	34	7	>						0
Glycopeptide	TEI					<56							>						0
	VAN						<29	20	7					>					0

|, lines indicate epidemiological cut-off values (ECOFF). The grey zone indicates the number of bacteria with decreased susceptibility above ECOFF (https://mic.eucast.org). <, minimum value of concentration used; >, maximum value of concentration used. [a]Number of HLR-Gn isolates. Abbreviations: non-WT, non-wild type; AMP, ampicillin; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; DAP, daptomycin; TET, tetracycline; TGC, tigecycline; CHL, chloramphenicol; LZD, linezolid; SYN, quinupristin/dalfopristin; TEI, teicoplanin; VAN, vancomycin.

2.2. Multilocus Sequence Typing (MLST)

The 56 isolates were characterized using primers and conditions for PCR amplification of seven housekeeping gene fragments (*gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt* and *yqiL*) included on the website of the *E. faecalis* MLST database (https://pubmlst.org/organisms/enterococcus-faecalis). The MLST alleles and resulting sequence types (STs) were assigned through the submission of the amplified sequences or respective allelic profiles to the *E. faecalis* MLST database. Genetic diversity (GD) was calculated as the ratio of the total number of STs to the total number of isolates [26]. The MLST profiles of *E. faecalis* available for download from the MLST website (https://pubmlst.org/organisms/enterococcus-faecalis) were used to generate a minimum-spanning tree with Phyloviz V2.0 and the goeBURST algorithm [27]. Clonal complexes (CCs) were defined as groups of isolates that differed in no more than two of the seven loci analysed, consisting of double-locus variants (DLVs) of a founder isolate.

2.3. Whole-Genome Sequencing (WGS)

A total of 19 *E. faecalis* isolates were selected for WGS based on phenotypically detectable resistance to four antimicrobials (linezolid, ciprofloxacin, chloramphenicol and/or HLR to gentamicin) (Table S1). The genomic DNA of 13 isolates was sent to STAB-VIDA (Caparica, Portugal) for WGS. Genomic DNA of the isolates was extracted using the MagMax core kit (Applied Biosystems) and the KingFisher Flex System automated extraction instrument (Thermo Fisher Scientific) according to the manufacturer's protocol. The concentration of genomic DNA was quantified and verified using the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific). The degree of genomic DNA degradation was evaluated through agarose gel electrophoresis. The sequencing libraries were prepared using the KAPA HyperPrep Library Preparation Kit (Roche) following the manufacturer's recommended protocol and sequenced using the Illumina NovaSeq platform with the TrueSeq Library Prep Kit (paired end 150 bp). Quality control of the raw data generated was performed using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). Trimming and de novo assembly were carried out with CLC Genomics Workbench v12.0.3 (Qiagen). All assemblies were performed with an automatic word size, a similarity fraction of 0.95, a length fraction of 0.95 and a minimum contig size of 500 bp. In addition, genomic DNA from six other isolates was sent to Secugen (Madrid, Spain) for WGS. Total DNA extraction and purification were performed with the MagAttract HMW DNA kit (Qiagen). Subsequently, the quality and concentration of DNA were assessed using NanoDrop (Thermo Fisher Scientific) and Qubit (Invitrogen; Thermo Fisher Scientific) devices. Genomic libraries were prepared in accordance with the 1D native barcoding genomic DNA protocol using SKQ-LSK114 and SQK-NBD114.96 kits (Oxford Nanopore Technologies). Sequencing was performed using MinION equipment on a FLO-MIN114 vR10.4.1 flow cell at a speed of 400 bp/s (5 kHz). Long-read assemblies were carried out with Flye v2.9.1 [28] using default parameters.

Raw Illumina and nanopore sequence data were deposited under project PRJNA1277192 (https://www.ebi.ac.uk/ena).

2.4. Bioinformatic Analysis of Whole-Genome Sequences

The assembled genomes were subjected to in silico screening for ARGs and virulence genes using the genomic tools ResFinder v4.0 and VirulenceFinder v2.0, respectively (http://www.genomicepidemiology.org), with the following cut-off values: a minimum of 90% coverage and 80% identity. ARGs were also screened against the Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/analyze/rgi) using the default criteria (perfect and strict hits only). LRE-Finder (http://www.genomicepidemiology.org) was used to detect 23S rRNA mutations and *optrA*, *cfr*, *cfr(B)*, and *poxtA* genes encoding linezolid resistance in enterococci from whole-genome sequences [29]. Mobile Element Finder was used to identify the MGEs in the genomes (http://www.genomicepidemiology.org), while plasmid replicon genes, incompatibility groups and associated contigs were determined using PlasmidFinder (http://www.genomicepidemiology.org) and MOBsuite v3.0.3 [30].

2.5. Phylogenetic Analysis

Mapping and whole-genome and core-genome alignment of the 19 *E. faecalis* genomes were performed using Snippy v4.6.0 software. A maximum likelihood tree based on the concatenated alignment of high-quality single-nucleotide polymorphisms (SNPs) was constructed with FastTree v2.1.10 [31] using the GTR+CAT models of nucleotide evolution, and the phylogenetic tree was visualized with iTOL v6.9.1.

3. Results

3.1. Phenotypic Antimicrobial Resistance

The percentages of isolates showing the NWT phenotype to the 12 antimicrobials tested are presented in Table 1. No significant differences were found between enterococci from cloacal and pharyngeal swabs for any of the 12 antimicrobials (data not shown). Most (82.1%) of the enterococcal isolates exhibited NWT phenotypes to six antimicrobial agents (Table 2). The most prevalent NWT phenotype was to tetracycline (82.1%), followed by erythromycin (58.9%). The NWT rates for ciprofloxacin, gentamicin, chloramphenicol and linezolid ranged between 25.0% and 3.5%, whereas no NWT phenotypes were found for the remaining six antibiotics (vancomycin, teicoplanin, daptomycin, quinupristin/dalfopristin, tigecycline and ampicillin). HLR to gentamicin was detected in 13 isolates.

Table 2. Antimicrobial resistance profiles of *E. faecalis* isolates from Eurasian griffon vultures.

Antimicrobial resistance profile	**Nº isolates showing the antimicrobial resistance profile (%)**	**ST (CC)**[3]
WT isolates	10 (17.9)	ST4 (2; CC16); ST40 (1; CC16); ST441 (1; CC16); ST648 (4; CC16); ST1600 (1; CC860); ST1875 (1; CC863)
TET	**13 (23.2)** [1]	ST9 (1; CC16), ST40 (1; CC16); ST59 (1; CC16); ST82 (1; CC16); ST200 (1; CC16); ST256 (2; CC16); ST268 (1; CC16); ST631 (1; CC631); ST699 (1; CC1567); ST706 (2; CC376); ST860 (1; CC860)
ERY-TET	**14 (25.0)**[1]	ST7 (1; CC16); ST16 (1; CC16); ST287 (2; CC287); ST300 (8; CC863); ST1287 (1; 1861)
GEN-ERY-TET[2]	2 (3.6)	ST16 (2; CC16)
CHL-ERY-TET[2]	5 (8.9)	ST19 (1; CC16); ST21 (1; CC16); ST76 (1; CC1688); ST116 (1; CC16); ST1288 (1; CC863)
GEN-CIP-TET[2]	1 (1.8)	ST330 (1; CC16);
GEN-CIP-ERY-TET[2]	4 (7.1)	ST16 (2; CC16); ST82 (1; CC16); ST1768 (1; CC16)
GEN-CHL-ERY-TET [2]	4 (7.1)	ST4 (1; CC16); ST35 (2; CC57); ST1291 (1; Singleton)
GEN-CIP- CHL-ERY-TET[2]	1 (1.8)	ST480 (1; CC16)
GEN-CIP-CHL-LZD-ERY-TET[2]	2 (3.6)	ST116 (2; CC16)
Total NWT isolates	46 (82.1)	
Total MDR isolates	19 (33.9)	

[1]Most frequent profiles are in bolds.[2] Multidrug resistance phenotypes are underlined. [3]Number of isolates and CCs in brackets. Abbreviations: WT, wild type, NWT, non-wild type; MDR, multidrug resistant; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; TET, tetracycline; CHL, chloramphenicol; LZD, linezolid.

The antimicrobial resistance profiles are presented in Table 2. MDR was detected in more than 30% of the isolates (Table 2). MDR profiles were generally characterized by phenotypic resistance to gentamicin, tetracycline and erythromycin. The most common phenotypic resistance profile was erythromycin-tetracycline (ERY-TET, 25.0% of the isolates), followed by tetracycline (TET, 23.2% of the isolates).

3.2. Molecular Characterization of the Isolates

Overall, significant genetic heterogeneity (GD 0.55) was observed among the 56 *E. faecalis* isolates, which were classified into 31 STs after MLST analysis (Table 3). The most frequent genotypes were ST300, ST16 and ST648, represented by nine, five and four isolates, respectively. The other two STs included three isolates each (ST4 and ST116). Additionally, six STs were represented by two strains each (ST35, ST40, ST82, ST256, ST287 and ST706). The remaining STs were represented by single isolates.

Table 3. Multilocus sequence types of the 56 *E. faecalis* isolates from Eurasian griffon vultures and their relationship to data available on *E. faecalis* the MLST database.

ST	**CC (DVL)**[a]	**Nº vulture isolates**			**Nº isolates *of E. faecalis* in the MLST database**					
		MDR	**non-MDR**	**TOTAL**	**Animals**[b]	**Human**	**Foods**	**Environment**	**Unknown**	**TOTAL**
4	16	1	2	3	12	1			1	14
7	16		1	1	1					1
9	16		1	1	22		2			24
16	16	4	1	5	68	1			11	80
19	16	1		1	7	4	7		3	21
21	16	1		1	41	23	22		4	90
35	57	2		2		2				2
40	16		2	2	15	42	8	3	6	74
59	16		1	1	6	4			1	11
76	1688	1		1	1	1			3	5
82	16	1	1	2	13	1	25		2	41
116	16	3		3	9	8	1	1	1	20
200	16		1	1	1					1
256	16		2	2	6	1		3	3	13
268	16		1	1		1				1
287	287		2	2		4		1		5
300	863		9	9	4	1				5
330	16	1		1	3	3			3	9
441	16		1	1		1				1
480	16	1		1	11	4			1	16
631	631		1	1	10	1				11
648	16		4	4	4	1				5
699	1567		1	1	1					1
706	376		2	2	1					1
860	860		1	1			1			1
1287	1861		1	1	2			1		3
1288	863	1		1	5					5
1291	singleton	1		1			1			1
1600	860		1	1			1			1
1768	16	1		1			1			1
1875	863		1	1	1					1

[a] CC (DVL), clonal complexes using the double-locus variants (DLV) criterion. [b] Chicken, pets, livestock and wildlife animals. Abbreviations: MDR; multidrug resistant.

The 19 MDR isolates also exhibited significant genetic diversity (GD 0.68) and were classified into 13 STs, all of them previously documented in the *E. faecalis* MLST database from different sources (Table 3). Three STs (ST16, ST116 and ST35) were detected in 52.6% of the MDR isolates (Table 3). Except for ST4, ST16 and ST82, the remaining 10 STs included only MDR isolates.

The goeBURST analyses, using all STs available on the *E. faecalis* MLST database website, enabled the classification of the 31 STs identified in this study into 10 CCs and one singleton based on the DLV criterion (Tables 2 and 3). CC16 was the largest CC identified, comprising 18 STs, followed by CC863, which contained three STs. The isolates with the MDR phenotype included four CCs (CC16, CC57, CC863 and CC1688) (Table 3). CC16 included most STs containing NWT isolates to ciprofloxacin, linezolid and chloramphenicol and exhibiting HLR to gentamicin isolates (Table 4).

Table 4. Location of the antimicrobial resistance genes in the genome of 19 *E. faecalis* isolated from Eurasian griffon vultures.

Antimicrobial		**Nº Isolates**	**ST (Nº isolates)**	**Chromosome**		**MGEs**	
				QRDRs (Nº isolates)	**ARGs (Nº isolates)**	**REP (Nº isolates/ARG)**	**Tn (Nº isolates/ARG)**
Included in the commercial panel	HLR-Gn	13	ST4 (1); ST16 (4); ST82 (1); ST35 (2); ST116 (2); ST330 (1); ST480 (1); ST1768 (1)		*aac(6')-aph(2'')* (12)		
	CIP	8	ST16 (2); ST82 (1); ST116 (2); ST330 (1); ST480 (1); ST1768 (1)	gyrA_E87G/*parC*_S80I (4) *gyrA*_E87G/*parC*_S80I (3)			
	CIP (WT)	2	ST35 (2)	*parC*_S80I (2)			
	CHL	14	ST480 (1); ST116 (3); ST4 (1); ST16 (2); ST1291 (1); ST35 (2); ST1288 (1); ST76 (1); ST21 (1); ST19 (1)		*cat(pC221)* (1) *cat(pC223)* (2)	*rep7a (3/cat(pC221))* *repUS43 (3/cat(pC223))* *rpUS40 (2/fexA)*	
	LZD	2	ST116 (2)			*rpUS40 (2/optrA)*	
	TET	19	ST4 (1); ST16 (4); ST19 (1); ST21 (1); ST35 (2); ST76 (1); ST82 (1); ST116 (2); ST330 (1); ST480 (1); ST1288 (1); ST1291(1); ST1768 (1)		*tetM* (2) *tetL* (4)	*repUS43 (10/tetM)* *repUS43 (7/tetM&tetL)* *rep22 (2/tetL)*	*Tn6009 (10/ tetM)* *Tn6009 (2/tetM&tetL)* cn_43171_ISS1N (1/*tetM&teL*)
	ERY	19	ST4 (1); ST16 (4); ST19 (1); ST21 (1); ST35 (2); ST76 (1); ST82 (1); ST116 (2); ST330 (1); ST480 (1); ST1288 (1); ST1291(1); ST1768 (1)		*ermB* (16)	*rep7a (1/ ermB)*	cn_43171_ISS1N (1/*ermB*)
No included in the commercial panel	STR	-			*ant(6)-Ia* (10)	*rep7a (1/ant(6)-Ia)* *rep7a (4/str)*	
	KAN	-			*aph(3')-III* (12)	*rep7a (1/ aph(3')-III)*	
	TRM	-			*dfrD* (1) *dfrG* (16)		
	LIN	-			*lsaA* (19)		*Tn6260 (5/lnuG)*

	lsaE (9) *lnuA*(1) *lnuB* (10) *lnuG* (5)

Abbreviations: ST. sequence type; HLR-Gn, high level resistance to gentamicin AMR, Antimicrobial resistance; REP, replicon type; Tn, Transposon; ComTn, Composite Transposon; CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; CHL, chloramphenicol; LZD, linezolid; STR, streptomycin; KAN, Kanamycin; TRM, Trimethoprim; LIN, Lincomicin QRDRs, quinolone resistance determinant regions; MEG, mobile genetic elements; ARGs, antimicrobial resistance genes; REP, replicon; Tn, transposons.

Pairwise SNP differences among the 19 vulture *E. faecalis* with NWT phenotype to ciprofloxacin, linezolid and chloramphenicol and/or with HLR to gentamicin are shown in Table S2. The minimum and maximum SNP differences were 0 and 17168, respectively. The phylogenetic tree generated on the basis of whole-genome SNP (WG-SNP) revealed three small clades for the sequence types ST116 (3 isolates), ST16 (5 isolates) and ST35 (2 isolates), whereas the other STs were genetically diverse (Figure 1).

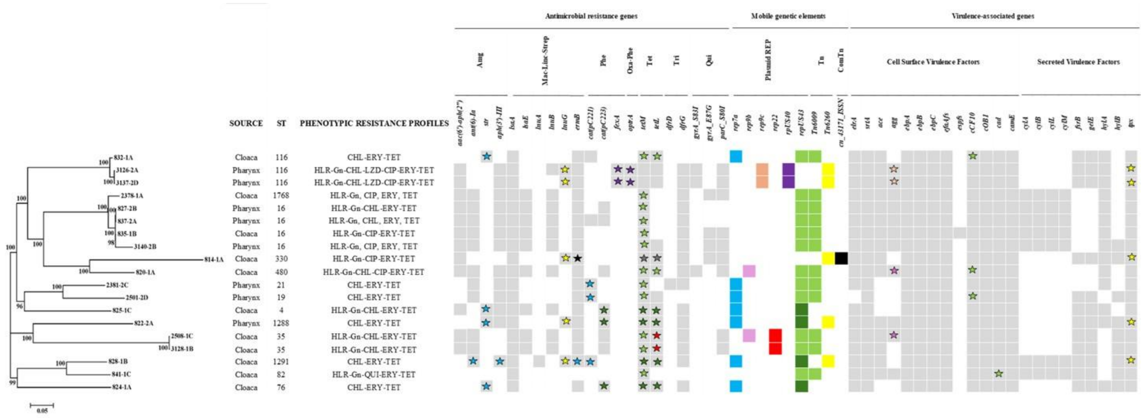

Figure 1. Phylogenetic tree generated from whole-genome SNP (WG-SNP) analysis of 19 non-wide type vulture enterococci with resistance to ciprofloxacin, linezolid and chloramphenicol and/or high-level resistance to gentamicin. The figure shows the in silico identification of virulence-associated genes, antimicrobial resistance genes and mobile genetic elements (MGEs). The colour of the symbols and leaves link ARGs and virulence genes to the corresponding MGE. Except for isolates 822-2A, 824-1A, 825-1C, 828-1B and 835-1B, *repUS43* and *Tn*6009 were found co-located with tetracycline resistance genes. Abbreviations: Amg, Aminoglycoside; Mac-Linc-Strep, Macrolide-Lincosamide-Streptogramin; Phe, Phenicols; Oxa-Phe, Oxazolidinone-Phenicols; Tet, Tetracyclines; Tri; Trimethoprim; Qui, Quinolones; REP, replicon type; Tn, Transposon; ComTn, Composite Transposon.

3.3. Antimicrobial Resistance Genes and Mobile Gene Elements (Plasmids, Transposons, and Integrative and Conjugative Elements)

Table 4 shows the presence of ARGs detected in the genomes of the 19 isolates exhibiting an NWT phenotype to linezolid, ciprofloxacin and chloramphenicol and/or with HLR to gentamicin. Twelve NWT isolates to chloramphenicol carried *cat* genes encoding resistance to this antibiotic, *cat(pC223)* (58.3% isolates) and *cat(pC221)* (41.7%), whereas the two isolates showing phenotypic resistance to chloramphenicol and linezolid (3126-2A and 3137-2D) were found to contain genes encoding resistance to phenicols (*fexA*) and phenicols/oxazolidinones (*optrA*). The *aac(6')-Ie-aph(2")-Ia* gene conferring resistance to aminoglycosides was detected in 12 isolates with the HLR phenotype to gentamicin. Seven isolates with an NWT phenotype to ciprofloxacin carried chromosomal point mutations in the quinolone resistance-determining regions (QRDRs) of the DNA gyrase subunit A (*gyrA*) and topoisomerase IV subunit A (*parC*) genes. Three amino acid mutations were detected in the *gyrA* gene, two at codon 83 (Ser to Tyr [*n* = 1] and Ser to Ile [*n* = 2]) and another at codon 87 (Glu to Gly [*n* =4]), whereas a single amino acid mutation was found at *parC* codon 80 (Ser to Ile [*n* = 7]). This amino acid change at codon 80 in *the parC* gene was also detected in two WT isolates.

Genes conferring resistance to other antimicrobials included in the commercial panel used for phenotypic resistance screening were also identified. The *tetM* gene and *tetM*/*tetL* genes were found in 31.5% and 68.4% of the isolates with an NWT phenotype to tetracycline, respectively (Table 4). The *ermB* gene was identified in 94.7% of the isolates with the NWT phenotype to erythromycin.

In addition, genes conferring resistance to other antimicrobials not included in the commercial panel used for phenotypic resistance screening were also identified (Table S1). Thus, the *ant(6)-Ia* and *str* genes, which confer resistance to streptomycin, were detected in 11 and four isolates, respectively, whereas the *aph(3')-III* gene, which confers resistance to kanamycin, was detected in 13 isolates. The presence of the *dfrG* gene, which confers resistance to trimethoprim, was identified in the genomes of 16 isolates. The *lnu (lnuA, LnuB* and *lnuG)* genes and *lsa (lsaA* and *lsaE)* genes conferring resistance to lincomycin were also frequently detected.

One isolate (841-1C) with an NWT phenotype to ciprofloxacin and erythromycin and with HLR to gentamicin but without resistance genes or chromosomal point mutations in the *gyrA* and *par*C genes was further analysed using an antibiotic/reserpine (A/R) MIC test. The isolate had lower MICs for these antibiotics after exposure to reserpine than the corresponding MICs in the absence of this compound (MICs of 16 µg/ml vs 1024 µg/ml, 0.25 µg/ml vs 16 µg/ml and <1 µg/ml vs >128 µg/ml for gentamicin, ciprofloxacin and erythromycin, respectively). The *efrA* and *erfB* genes, which encode efflux pumps for different antimicrobials, were detected in the genome of this isolate (data not shown).

Four plasmid replicon types harbouring ARGs were detected in the 19 isolates (Table 4; Table S1, Figure 1). The most prevalent was *repUS43,* which was detected in 16 isolates (89.5%). This replicon was found to be chromosomally integrated and co-located on the same contig with *tetM* (10 genomes) or *tetM/temL* (3 genomes; 820-1A, 828-1B and 832-1A) genes in 13 *E. faecalis* isolates exhibiting an NWT to tetracycline. In addition, *cat* genes, together with *tetM/temL,* were bound to *repUS43* in three genomes (822-2A, 824-1A and 825-1C). The *tetL* gene was also associated with the *rep22*-type plasmid, whereas the *str, aph(3')-III*, *ant(6)-Ia*, *ermB* and *cat* genes were associated with *rep7a* (Table 4; Table S1, Figure 1). The *repUS40* replicon was associated with the *optrA* and *fexA* genes in both isolates exhibiting NWT phenotypes to linezolid and chloramphenicol.

In addition, other MGEs, such as transposons (Tns) and composite transposons (ComTn), were also associated with ARGs. *Tn*6009 was detected in 57.9% of the isolates. This MGE was associated with *repUS43* and tetracycline resistance genes. The *lnuG* gene was found to be embedded in *Tn6260* in five genomes, whereas ComTn, *cn_43171_ISS1N,* was linked to tetracycline (*tetM, tetL*) and macrolide (*ermB*) resistance genes in one genome.

3.4. Virulence Factors

A total of 22 virulence factors were found among the 19 genomes (Figure 1). All isolates harboured genes encoding sex pheromone-associated proteins (*cad, cCF10, camE, cOB1*), protection against oxidative stress (*tpx*), cell wall adhesion (*efaAfs*), biofilm-associated pili (*ebpA, ebpB, ebpC*) and the cell wall anchor surface protein sortase A (*srtA*). Genes associated with the cytolysin toxin (*cylA, cylB, cylL* and *cylM*) were identified in seven isolates (12.5%). Other virulence genes identified in most of the 19 isolates were *elrA* (Rgg-like regulator gene associated with macrophage persistence; 94.7% of the isolates), *agg* (aggregation substance; 57.9% of the isolates), *frsB* (quorum-sensing regulator; 63.2% of the isolates), *gelE* (gelatinase toxin; 63.2% of the isolates), *ace* (collagen adhesion precursor; 78.9% of the isolates), *hylA* and *hylB* (hyaluronidase genes; 73.7% and 47.4% of the isolates, respectively). The *espfs* gene (enterococcal surface protein) was identified in 5.3% of the isolates. Genes associated with the cytolysin toxin (*cylA*, *cylB*, *cylL* and *cylM*) were found only in the ST16, ST82, ST1291 and ST1768 isolates.

Some virulence genes were associated with MGEs (Figure 1). Thus, the *cCF10* and *cad* genes bound to *repUS43* in three and one isolates, respectively. In addition, the *agg* gene bound to replicons *rep9b* and *rep9c*, in two isolates each, and the *tpx* gene to transposon *Tn6260*, in five isolates.

4. Discussion

The impact of wild animals as reservoirs of AMR and ARGs, that can later be disseminated among different hosts, and the environment, has received particular attention from the "One Health" perspective in recent years [3]. Thus, the present study investigated the prevalence of AMR in a collection of 56 *E. faecalis* isolates from the pharynx and cloaca of Eurasian griffon vultures. These birds were chosen because of their wide geographic distribution and their scavenging habits, and because they live in close proximity to humans. In addition, enterococci are widely considered key microbiota indicators for tracing the spread and evolution of MDR bacteria in environments and wildlife [9]; furthermore, *E. faecalis* is a common inhabitant of the cloacal and pharyngeal microbiota of Eurasian griffon vultures [19].

Different studies have shown high rates of AMR and MDR in enterococci from various wild bird species, including vultures [14,32,33]. In this study, a high percentage of *E. faecalis* isolates (82.1%) exhibited NWT phenotypes to six antimicrobials, indicating the widespread presence of AMR in isolates of *E. faecalis* in this vulture population. The highest resistance rates were detected for erythromycin and tetracycline (Table 1), and one-third of the isolates were phenotypically MDR (Table 2). Accordingly, the most common antimicrobial resistance profile observed was resistance to erythromycin and tetracycline (Table 2), which is in line with previous studies on *E. faecalis* collected from different animal and human sources [8,34]. Of special concern is the detection of isolates with an NWT phenotype to linezolid, chloramphenicol and ciprofloxacin and with HLR to gentamicin (Table 1), antimicrobials considered clinically important in human medicine [21]. Furthermore, over half (63%; 12 out 19 isolates) of the MRD isolates exhibited an NWT phenotype to at least two of these antimicrobials (Table 2). *E. faecalis* strains with a resistant phenotype to any of these four antimicrobial agents have been reported in various wild bird species, including vultures [6,16,32].

Human activities and food-animal production can contribute to the development and spread of AMR in the environment, which has a negative impact on wildlife. Thus, vultures living in close contact with anthropogenically impacted areas or high densities of livestock may be colonized by ARB strains, which are likely selected by antimicrobial agents used in humans and domestic animals [35]. In this way, the rates of *E. faecalis* with an NWT phenotype to antimicrobials authorized only for human use [21], such as linezolid, could be linked to the exposure of these vultures to antimicrobials via contaminated water [36]. The feeding habits of Eurasian griffon vultures can also contribute to the acquisition of ARBs. These animals feed almost exclusively on carrion, primarily mammals from intensive farming that have died from disease or accidents and are discarded at predictable sites that represent an abundant food source [37]. Consequently, the concentration of vultures at these feeding sites may facilitate their exposure to active antimicrobial residues that are present in the carcasses of medicated livestock [13]. Erythromycin and tetracycline are widely used in veterinary medicine in Spain (https://www.aemps.gob.es/), and the direct feeding of livestock carcasses treated with these antimicrobials could explain the high rates of *E. faecalis* isolates with the NWT phenotype to both antimicrobials (Table 2). Once ARBs are introduced into wildlife, they can persist for extended periods even in the complete absence of selection pressure from antimicrobial agents [38]. These bacteria can also spread to other species and environments, which could explain the relatively high rates of *E. faecalis* isolates with an NWT phenotype to chloramphenicol (Table 1), despite the ban on its use in humans, pets and non-food-producing animals in the mid-1990s in the EU [https://www.aemps.gob.es/, 39].

MLST analysis identified 31 STs with high genetic heterogeneity (GD 0.55) among the 56 *E. faecalis* isolates. The genetic diversity was similar between non-MRD and MRD *E. faecalis* isolates (GD 0.56 and GD 0.68, respectively). Despite the similar genetic heterogeneity of the MRD and non-MRD isolates, most STs identified in MRD isolates (10 out of 13) were detected exclusively in these isolates.

Similarly, 19 of the 21 STs identified in the non-MRS isolates were detected in only these isolates (Table 3). Moreover, ST16 and ST116 accounted for almost 40% of the MRD isolates, whereas only one non-MRD isolate belonged to either of these two genotypes. These results suggest a different genetic background for the *E. faecalis* populations of MRD and non-MRD isolates. Additionally, SNP differences among the 19 *E. faecalis* isolates from vultures with an NWT to ciprofloxacin, linezolid and chloramphenicol and/or with HLR to gentamicin also indicated high genetic heterogeneity. Except for isolates 3126-2A and 3137-2D, both belonging to ST116, all other 17 isolates exhibited SNP differences greater than 100 SNP, which is above the proposed cut-off value for considering isolates as clonal [40]. Thus, the genetic diversity observed by MLST suggests that the high rates of antimicrobial resistance detected in the *E. faecalis* isolates are more likely a consequence of exposure to multiple strains rather than the clonal spread of resistant isolates. All the STs identified have been detected previously in animals, humans, foods and the environment according to the information available on the *E. faecalis* MLST database website (https://pubmlst.org/organisms/enterococcus-faecalis). These data suggest that the detection of NWT phenotypes to different antimicrobial agents in the *E. faecalis* vulture isolates is likely a consequence of anthropogenic ARB contamination of the ecosystems inhabited by vultures [41].

Given the importance of understanding AMR mechanisms, the genomes of a subset of 19 selected isolates with an NWT phenotype to the clinically important antimicrobials linezolid, chloramphenicol and ciprofloxacin and with HLR to gentamicin were sequenced, and an in-depth analysis of their genetic mechanisms of resistance was performed. Several ARGs encoding resistance to chloramphenicol and gentamicin were identified on the chromosomes of the *E. faecalis* isolates (Table 4). Notably, the *aac(6')-Ie-aph(2")-Ia* gene was detected in most isolates (92.3%) with an HLR phenotype to gentamicin, which is consistent with [42] that described this resistance gene as the most frequently modifying enzyme underlying HLR to aminoglycosides in enterococci. The NWT phenotype to chloramphenicol was associated with the *cat(pC221)* and *cat(pC223)* genes (Table 4), which are *cat*A variants that have been detected in a wide variety of bacteria [39]. Both isolates with an NWT phenotype to chloramphenicol and linezolid carried the *optrA* and *fexA* genes in their genomes (Table 4), which are the main mechanisms of resistance of *Enterococcus* to oxazolidinone and phenicol antimicrobials [43]. Seven of the eight isolates with an NWT phenotype to ciprofloxacin and MICs of ≥16 µg/ml exhibited amino acid changes in the GyrA (S83I or S83Y, and E87G) and ParC (S80I) proteins, which have been previously reported in ciprofloxacin-resistant *E. faecalis* isolates [44,45].

ARGs to other antimicrobials included in the commercial panel that exhibited NWT phenotypes were also identified on the chromosome of the *E. faecalis* isolates. Thus, the NWT phenotype to erythromycin and tetracycline was associated with the detection of the *emrB* (94.7% isolates) and *tetM*/*tetL* genes, in accordance with previous studies, which indicated that these genes are the most prevalent among erythromycin- and tetracycline-resistant enterococci [6,32]. We also identified other genes associated with resistance to antimicrobials not included in the commercial panel, such as the *aph(3')-III gene,* which confers resistance to kanamycin and amikacin, and the *ant(6)-Ia* and *str* genes, which are associated with resistance to streptomycin [46,47]. However, phenotypic resistance to these antimicrobials was not tested in this study. The *efrA*, *erfB* and *lsa* genes, encoding efflux pumps for different antimicrobials in *E. faecalis* isolates [48], were detected in the genome of one isolate with an NWT phenotype to ciprofloxacin and erythromycin and with HLR to gentamicin but without resistance genes or chromosomal point mutations in the *gyrA* and *par*C genes. The decrease in the MIC values for these antimicrobials by six- to seven-fold in the presence of reserpine versus in the absence of this compound suggests that antibiotic efflux pumps are involved in resistance to these antimicrobials [49]. Although the efflux pump system has not been involved in erythromycin resistance in enterococci, it has been reported in other gram-positive bacteria, such as streptococci [25].

Enterococci readily acquire ARGs through MGEs such as transposons or plasmids, which play a key role in the acquisition and dissemination of ARGs via HGT [50]. In addition to chromosomal

ARGs, MGEs, including plasmid replicons and transposons, were identified among the *E. faecalis* vulture isolates, suggesting the potential of these isolates to acquire and transfer AMR. Notably, resistance genes to chloramphenicol, linezolid, tetracycline, erythromycin, streptomycin and kanamycin more frequently co-existed in different replicons and transposons (Figure 1; Table S1). Chloramphenicol resistance genes were detected in *rep7a*, *repUS43* and *rpUS40* replicons. Tetracycline resistance genes (*tetM, tetL*) were detected in transposons cn_43171_ISS1N and *Tn6009* and in the plasmid replicons *repUS43* and *rep22*, whereas a macrolide resistance gene (*ermB*) was also detected in cn_43171_ISS1N and *rep7a* (Table 4). The association between the *tetM* and *tetL* genes and the *repUS43* replicon, as well as with *Tn6009*, has been previously reported [45,51]. *Tn6260* was associated with the *lnuG* gene (Table 4), which is involved in resistance to lincosamide, in agreement with previous studies [52,53]. The detection of ARGs for different antimicrobials in the same MGE has also been reported [51]. In line with the observations of these authors, we found that different MGEs carried the same ARG and that different ARGs were carried by the same MGE, which could increase their dissemination potential. Thus, genes conferring resistance to different classes of antimicrobial agents were found in six isolates co-located with a plasmid replicon on the same contig (31.6%; Table S1): three isolates carried the *tetM, tetL* and *cat(pC223)* genes located in tandem close to the plasmid replicon *repUS43;* two isolates carried the *fexA and optrA* genes on the *repUS40* replicon; and one isolate carried the *ant(6)-Ia, aph(3')-III, ermB* and *cat(pC221)* genes on the same contig as the *rep7a* replicon. Other resistance genes located on MGEs not reported previously in *E. faecalis* were also detected in this study. Notably, *rep22* carried the *tetL* gene, which has been reported in other Gram-positive bacteria, such as *Macrococcus caseolyticus* [54], while the *optrA* and *fexA* genes located on the *repUS40* plasmid replicon have been reported in *Enterococcus hirae* and *Enterococcus casseliflavus* [53,55]. In this study, ComTra with ISS1N on both sides was linked to the *tetM, tetL* and *ermB* genes in one isolate. This MGE has been reported in *Listeria* [56] but not in *Enterococcus* or in association with resistance genes. Thus, it is the first description of these three MGEs (*rep22*, *repUS40* and ComTra) carrying antimicrobial resistance genes in *E. faecalis.* The acquisition of ARGs carried by MGEs can lead to the establishment of multidrug resistance [57]. In this context, the detection of different MGEs in the 19 sequenced isolates could explain why one-third of the 56 isolates were MDR (Table S1; Figure 1). According to the available data (https://www.ncbi.nlm.nih.gov), all replicons and transposons identified in the present study have been reported previously in *E. faecalis* and other enterococcal species isolated from humans, foods and livestock. Hence, the detection of *E. faecalis* isolates in vultures with NWT phenotypes to several antimicrobials could result from the exchange of ARBs and/or ARGs from these sources to vultures. This finding also supports the MLST data, suggesting that AMR in these vultures is likely a consequence of anthropogenic pollution.

Most enterococci are not virulent and are considered relatively harmless, with little potential for human infection. However, they have also been identified as nosocomial opportunistic pathogens with increased resistance to antimicrobial-approved agents [58]. The 19 isolates investigated in this study harboured 22 different virulence-related genes (Figure 1), which could facilitate colonization and cause infections [59]. In particular, several isolates carried genes (*cylA, cylB, cylL* and *cylM*) associated with cytolysin production, which is related to increased severity of infection in humans [59]. These genes are more prevalent among ST16 isolates [42], the second most frequently detected genotype in our study. In addition, vulture isolates harboured genes encoding sex pheromone-associated proteins, protection against oxidative stress, cell wall adhesion, biofilm formation and macrophage persistence (Figure 1), which are also involved in enterococcal pathogenicity [59]. Some virulence genes were found on the same MGEs, such as the replicon *repUS43* and the transposon *Tn6260,* which simultaneously carried ARGs. The co-presence of ARGs and virulence-related genes on the same MGE could facilitate the ability of *E. faecalis* vulture isolates to survive under antibiotic pressure and increase their pathogenic potential [58]. Consequently, they could represent a risk factor for domestic animals and humans.

5. Conclusions

Overall, the results of this study show that a significant proportion of *E. faecalis* strains recovered from cloacal or pharyngeal samples of vultures were phenotypically MDR and harboured MGEs (plasmid replicons, transposons and composite transposons) that carried AMR and virulence-associated genes. These findings are cause for concern, since vultures may act as spreaders of virulence and antimicrobial resistance genes to the environment and even to other hosts.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Phenotypic, genomic antimicrobial resistance and mobile genetic elements associated with antimicrobial resistance genes in 19 E. faecalis isolates investigated in this study; Table S2: Pairwise SNP differences between E. faecalis isolates with a non-wide type to ciprofloxacin, linezolid, chloramphenicol and/or high-level resistance to gentamycin.

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Abbreviations

The following abbreviations are used in this manuscript:

AMR	Antimicrobial resistance
ARBs	Antibiotic-resistant bacteria
ARGs	AMR genes
HGT	Horizontal gene transfer
MGEs	Mobile genetic elements
HLR	High-level resistance
ECOFF	Epidemiological cut-off
ECVs	Epidemiological cut-off F values
MICs	Minimal inhibitory concentrations
WT	Wild-type
NWT	non-WT
MDR	Multidrug resistant
MLST	Multilocus sequence typing
STs	Sequence types
GD	Genetic diversity
CCs	Clonal complexes
DLVs	Double-locus variants
WGS	Whole-genome sequencing
SNPs	Single-nucleotide polymorphisms
TET	Tetracycline
ERY-TET	Erythromycin- tetracycline
A/R	Antibiotic/reserpine

Tns	Transposons
ComTn	Composite transposons

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