Science of the Total Environment 825 (2022) 153831

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Oh, deer! How worried should we be about the diversity and abundance of the faecal resistome of red deer?

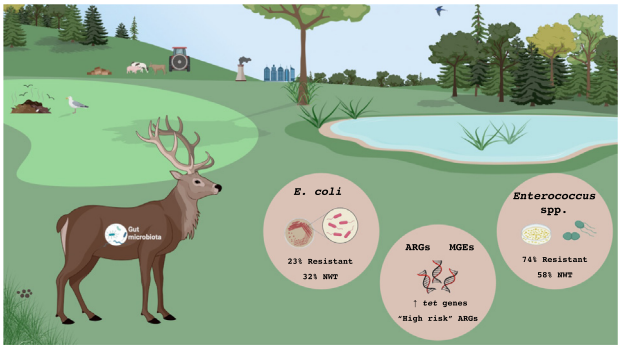
Diana Dias [a], Carlos Fonseca [a,b], Tânia Caetano [a], Sónia Mendo [a,*]

[a] CESAM and Department of Biology, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal
[b] ForestWISE - Collaborative Laboratory for Integrated Forest & Fire Management, Quinta de Prados, 5001-801 Vila Real, Portugal

HIGHLIGHTS

- Red deer resistome has "high-risk" ARGs that can pose a threat to human health.
- Tetracycline was the most abundant class of ARGs in red deer faeces.
- Higher diversity and abundance of AMR determinants in hunted animals (LousãM).
- Low resistant and multidrug-resistant bacteria were recovered from all locations.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 December 2021
Received in revised form 7 February 2022
Accepted 8 February 2022
Available online 11 February 2022

Editor: Rafael Mateo Soria

Keywords:
Antibiotic resistance genes (ARGs)
Mobile genetic elements (MGEs)
qPCR array
E. coli
Enterococcus spp.
Wildlife

ABSTRACT

The emergence of antimicrobial resistance (AMR) is a global threat to public health. Antimicrobials are used in animal production and human medicine, which contribute to the circulation of antibiotic resistance genes (ARGs) in the environment. Wildlife can be reservoirs of pathogens and resistant bacteria. Furthermore, anthropogenic pressure can influence their resistome. This work aimed to study the AMR of the faecal microbiome of red deer, one of the most important game species in Europe. To this end, a high-throughput qPCR approach was employed to screen a high number of ARGs and the antimicrobial susceptibility of indicator bacteria was determined. Several genes that confer resistance to different classes of antibiotics were identified, with the most abundant being tetracycline ARGs. Other genes were also present that are considered current and future threats to human health, and some of these were relatively abundant. Multidrug-resistant *E. coli* and *Enterococcus* spp. were isolated, although the overall level of antibiotic resistance was low. These results highlight the pressing need to know the origin and transmission of AMR in wildlife. Thus, and considering the One Health concept, studies such as this one shows the need for surveillance programs to prevent the spread of drug-resistant strains and ARGs.

1. Introduction

Antimicrobial resistance (AMR) is an emerging problem and one of the main health concerns of the 21st century, with the global overuse of antibiotics considered the major cause of this situation (WHO, 2014). AMR threatens modern medicine and the sustainability of effective public health response to the pressing threat of infectious and potentially fatal diseases (WHO, 2017). In Europe, the same antimicrobials or those belonging to the same classes are used in food-producing animals and in human health, and therefore the development of resistance to one specific antibiotic can lead to resistance to an entire related class (ECDC/EFSA/EMA, 2017; WHO, 2017). In addition, most European countries give animals a comparable or higher amount of antibiotics than those used in human medicine

* Corresponding author.
E-mail address: smendo@ua.pt (S. Mendo).

http://dx.doi.org/10.1016/j.scitotenv.2022.153831

(Vaz-Moreira et al., 2019), thus increasing their exposure to antibiotic selective pressure. The faeces and other waste from these animals are later used as manure (Muurinen et al., 2017). Even with the restrictions introduced in recent decades on the use of antibiotics, modern agriculture still deals with the problem of antibiotics and their metabolites in soils (Buta et al., 2021). Additionally, chemical substances such as pesticides, biocides, and metals contribute to AMR development (Sunde et al., 2018). Thus, antibiotic resistance determinants came to be considered environmental pollutants continuously disseminated by anthropogenic sources, such as animal faeces, wastewater treatment plants, hospitals, among others (Y. Zhang et al., 2021). Furthermore, there is a constant exchange of these determinants between the different compartments of the ecosystem, since drug-resistant bacteria are everywhere and circulate in different environments, contributing to the spread of AMR (WHO, 2017). Consequently, wildlife is exposed to antibiotics and the evolution of antibiotic resistance and, although they are not directly medicated, they have been considered important players in the dissemination of ARGs (Allen et al., 2010). Furthermore, it has been suggested that wildlife populations that inhabit close to humans are more likely to harbour clinically relevant AMR bacteria and ARGs than populations in remote areas (Arnold et al., 2016; Rwego et al., 2008; Sjölund et al., 2008).

Many wild animals are hunted for food, and game meat consumers consider it a natural and safe product, free of compounds such as antibiotics and hormones. In Europe, red deer (*Cervus elaphus*) is one of the most frequent big game species (Schulp et al., 2014).

Like those of other European countries, their populations in Portugal have been increasing and spreading, due to the reintroduction programs carried out during the 1990s, when this species disappeared from some places, mainly due to hunting pressure, habitat fragmentation and destruction (Valente et al., 2017; Vingada et al., 2010). As red deer often share the same habitat and resources with livestock, they can be excellent model species for investigating AMR. Previous studies have shown that it is a reservoir and a possible transmission vector of resistant bacteria as they can move quickly over large distances (Böhm et al., 2007; Dias et al., 2015; Rogers et al., 2018). Thus, following the One Health approach, AMR surveillance in game species is necessary and has been carried out mainly with human pathogenic bacteria and by evaluating the antimicrobial susceptibility of cultivable bacteria (Vittecoq et al., 2016). Yet, data on the occurrence and quantification of ARGs in wildlife (and across different environments) is scarce, and may help to better understand the acquisition, maintenance and dispersal of AMR (Arnold et al., 2016).

The major purpose of this study was to use culture-dependent and culture-independent approaches to assess AMR in red deer. We wanted to characterize its faecal resistome using a high-throughput qPCR array that targeted a variety of ARGs, mobile genetic elements (MGEs), and integrons. A standard technique was also used to determine the resistance profiles of two bacterial indicators (*E. coli* and *Enterococcus* spp.). To the best of our knowledge, this is the first study to investigate the diversity and abundance of ARGs in red deer populations that use a robust monitoring approach that allows the results to be compared across different sample types and countries, in addition to the phenotypic data.

2. Materials and methods

2.1. Study areas

101 red deer faecal samples were collected, between November 2017 and November 2019, from three different geographic locations (Fig. S 1): i) Montesinho Natural Park (MontesinhoNP; $n = 32$), ii) Lousã Mountain (LousãM; $n = 40$) and iii) Tapada Nacional de Mafra (TNMafra; $n = 29$). MontesinhoNP is located in the extreme northeast of Portugal, is bordered by Spain and has about 74.229 ha, integrating the Natura 2000 Network (site codes PTZPE0003 and PTCON0002). This is mostly a rural area, with tiny communities and low people and livestock densities. The Lombada National Hunting Zone is located in MontesinhoNP. LousãM is in the centre of Portugal. LousãM is also a member of the Natura 2000 Network due to its rich and diverse fauna and flora (site code PTCON0060, area with a total of 15,158 ha). In addition to its conservation value, LousãM has a variety of hunting places (national hunting areas, municipal hunting areas, and associative hunting areas), emphasizing its significance for regional and national hunting. This area has high human density and medium livestock density. TNMafra is an 819-hectare public gated area in central-west Portugal (Lisboa district). Animals in TNMafra live in semi-captivity; the main populations of game ungulates (deer, fallow deer, and wild boar) have no natural predators, and people provide food on a regular basis. TNMafra welcomes around 70,000 visitors/year, especially schools, elderly groups, families and tourists.

2.2. Sample collection and initial processing

The samples were collected and processed as described by Dias et al. (2022). The red deer samples from the LousãM were taken directly from the rectal area of hunted animals, and fresh faeces from the other two places were collected from their natural surroundings. The initial overnight culture from each faecal sample was used to isolate *E. coli* and *Enterococcus* spp.

2.3. DNA extraction and pooling

DNA extraction from each faecal sample was performed as described in Dias et al. (2022). A pool containing an equal concentration of DNA from each sample ($n = 61$) was prepared and used for the high-throughput qPCR pre-screening analysis. The final high-throughput qPCR analysis was done with four DNA pools: i) pool 1: DNAs from MontesinhoNP ($n = 14$), ii) pool 2 ($n = 15$) and pool 3 (n = 15): DNAs from LousãM, and iii) pool 4: DNAs from TNMafra ($n = 17$). Each DNA pool had a final concentration of 20 ng/μl.

2.4. High-throughput qPCR

The high-throughput qPCR analysis was performed as described by Dias et al. (2022). Briefly, Resistomap (Helsinki, Finland) employed the ARG qPCR array 2.0 (Stedtfeld et al., 2018) to identify and quantify ARGs and MGEs determinants. A pre-screening was first carried out using a DNA pool representing the red deer samples as DNA template and using 384 primer sets. The results obtained were used to set up a personalized array of 57 primer sets and the 16S rRNA gene (Table S 1) that allowed the quantification of ARGs and MGEs using the pools of DNAs from MontesinhoNP (1 pool), LousãM (2 pools) and TNMafra (1 pool) prepared as above described.

2.5. Isolation and selection of E. coli and Enterococcus spp.

The isolation of *E. coli* and *Enterococcus* spp. was made following the protocol described in Dias et al. (2022). In short, the prepared dilutions from each overnight culture (Section 2.2) were platted on selective culture media, and the isolates were confirmed by colony-PCR. One *E. coli* and one *Enterococcus* spp. isolate from each sample were stored at −80 °C in 15% sterile glycerol and further used for antibiotic susceptibility testing (AST).

2.6. Antibiotic susceptibility testing

AST was performed using the disk diffusion susceptibility testing, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, using *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 as quality controls. The antibiotics tested and the interpretation of the inhibition zone diameters (IZDs) both with clinical breakpoints and to the epidemiological cut-off values (ECOFFs) were performed as in (Dias et al., 2022). *E. faecalis* is reported as intrinsically resistant to quinupristin-dalfopristin (QDA) (Singh et al., 2002; Zaheer et al., 2020), and therefore their IZDs were not considered for the AST results according to clinical breakpoints.

The epidemiological cut-offs for *E. coli* and *Enterococcus* spp. were calculated with the normalized resistance interpretation (NRI) method (Kronvall and Smith, 2016). This was performed because: i) EUCAST cut-offs were derived mainly from testing human bacterial isolates and ii) EUCAST does not provide data for some species/drug combinations. The NRI method was performed by testing bacteria isolated from the faeces of wild mammals collected by our group since 2017 (271 *E. coli* and 244 *Enterococcus* spp.) (Dias et al., 2022).

2.7. Data analysis

The NRI method was used with the permission of the patent holder, Bioscand AB, TÄBY, Sweden (European patent No 1383913, US Patent No. 7,465,559). The automatic and manual excel programmes were made available by courtesy of P. Smith, W. Finnegan, and G. Kronvall. Venn diagram was generated with the website tool http://bioinformatics.psb.ugent.be/webtools/Venn/.

3. Results

3.1. The overall analysis of ARGs and MGEs found in red deer

Following a pre-screening, an array to detect 57 gene assays (43 ARGs, 12 MGEs and 2 integrons) was chosen to investigate the diversity and abundance of ARGs and MGEs in the faeces of red deer from different geographic locations. In general, the ARGs identified confer resistance to aminoglycosides (28%), MLSBs (23%), β-lactams (19%), tetracyclines (19%), vancomycin (5%), quinolones (5%) and phenicols (2%) (Fig. 1A). The resistance mechanisms of these ARGs include antibiotic deactivation (47%), cellular protection (40%), efflux pumps (7%) and other/unknown (7%) (Fig. 1B). Genes from all MGE groups were detected and included 6 insertion sequences (IS), 3 transposases, 3 plasmid-associated genes and 2 integrases (Fig. 1C).

The World Health Organization (WHO) list of critically important antimicrobials (CIA) used in human medicine point out cephalosporins (3rd, 4th and 5th generation), glycopeptides, macrolides, ketolides, polymyxins and quinolones as highest priority antimicrobials (WHO, 2019). ARGs detected in this study may confer resistance to some of these antibiotics, especially (Fig. S 2): i) $bla_{SFO\text{-}1}$ and bla_{ACT} that encode a class A extended-spectrum β-lactamase (ESBL) and a cephalosporin-hydrolyzing class C β-lactamase, respectively, ii) several *erm* genes that promote the resistance to macrolides by mediating ribosome methylation, iii) *pikR2* that is associated with the ketolide biosynthetic gene cluster and encodes enzymes homologous to *erm* rRNA methyltransferases, iv) *ereA* and *mphA* which encode esterases involved in the inactivation of macrolides, and v) *qepA* and *qnrB4*, both plasmid-associated genes conferring resistance to fluoroquinolones. Genes conferring resistance to colistin, *mcr-1* and *mcr-2*, were not detected.

3.2. The overall abundance of ARGs and MGEs

In each of the samples, the ARGs were quantified relative to the abundance of the 16S rRNA gene. The average relative abundance was 9.85×10^{-5}, and ranged between ca. 10^{-6}–10^{-3} (Fig. S 3 and Fig. 2). In general, the most abundant ARGs confer resistance to tetracyclines (mean ca. 10^{-4}), followed by quinolones (mean ca. 10^{-4}), aminoglycosides (mean ca. 10^{-5}), β-lactams (mean ca. 10^{-5}), phenicols (mean ca. 10^{-5}), vancomycin (mean ca. 10^{-5}) and MLSBs (mean ca. 10^{-5}) (Fig. 2). The relative abundances of the MGEs varied between ca. 10^{-6}–10^{-3}, with a mean of 2.42×10^{-4} (Fig. S 3 and Fig. 2).

The three ARGs with higher relative abundance were *tetW* (1.6×10^{-3}), *tetQ* (8.54×10^{-4}) and *tetO* (7.49×10^{-4}), all of them coding

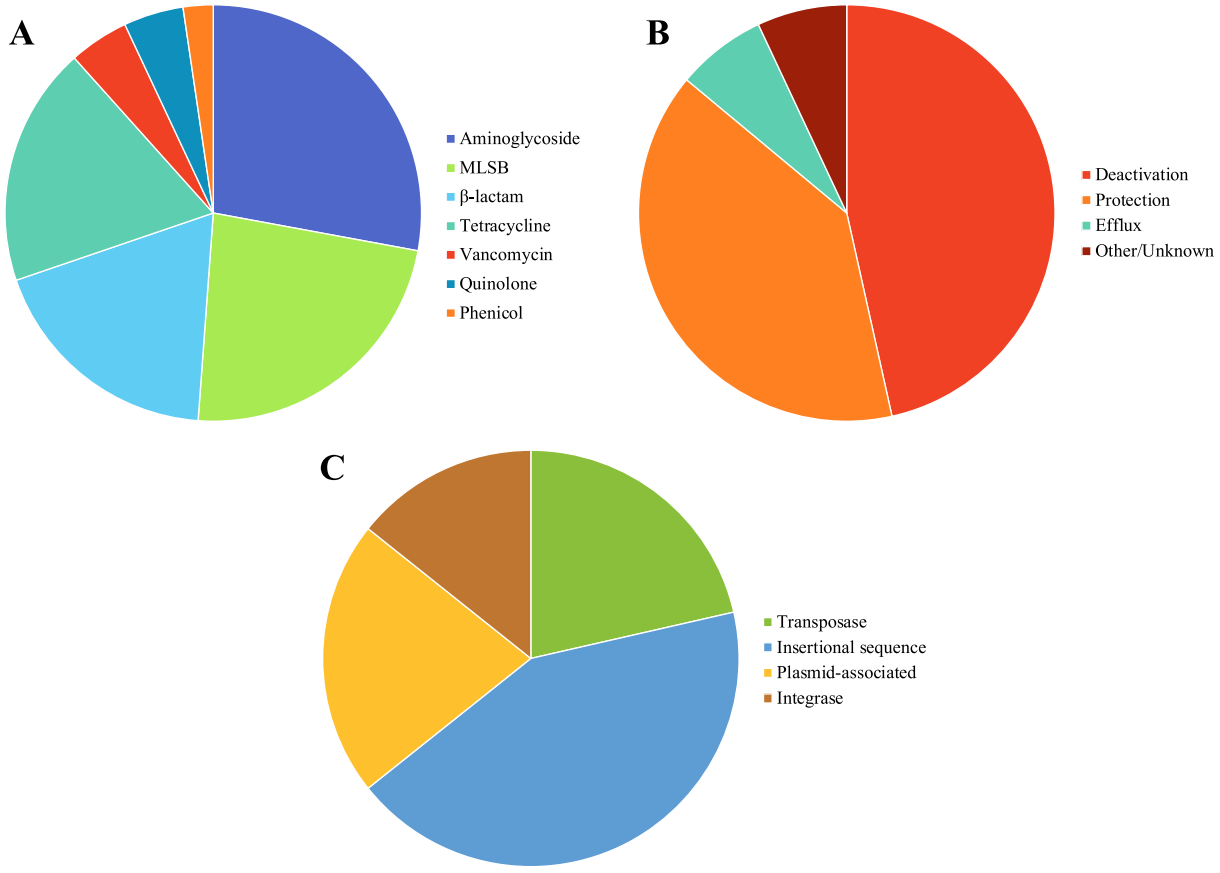


Fig. 1. Diversity of ARGs identified in red deer faecal samples classified according to the antibiotic class to which they confer resistance (A), their resistance mechanism (B) and types of MGEs (C).

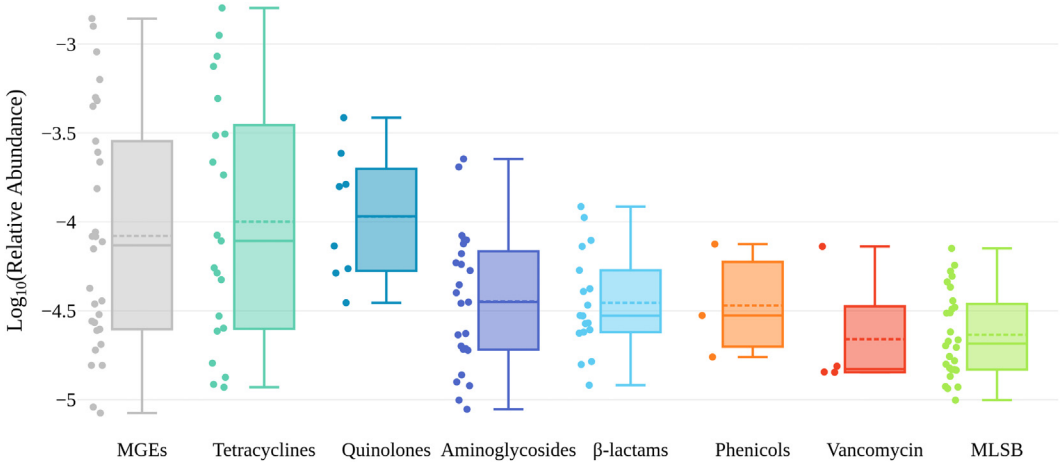


Fig. 2. Box plot showing the average of the relative gene abundances (log_{10} transformed values) for each ARG class and MGEs (including integrons) detected in red deer faecal samples. Error bars represent standard deviation.

for ribosomal protection proteins conferring resistance to tetracyclines (Fig. S 3). In addition to these, other abundant ARGs found were: i) *qepA*, a plasmid-mediated quinolone resistance determinant (3.85×10^{-4}), ii) *tet39*, coding for a tetracycline efflux pump (3.06×10^{-4}), iii) *aadA1*, which encodes an aminoglycoside nucleotidyltransferase that modifies streptomycin and spectinomycin (2.26×10^{-4}), iv) *qnrB4*, a plasmid-associated gene conferring resistance to quinolones (1.63×10^{-4}), and v) bla_{SFO} that encodes a low-occurrence extended-spectrum β-lactamase (1.22×10^{-4}).

The most abundant MGE encodes the *Tp*614 transposase (1.39×10^{-3}), followed by the insertion sequence *IS*613 (2.46×10^{-4}) (Fig. S 3). Focusing on integrons, class 1 and class 3 genes were identified and the first, which is considered an anthropogenic bioindicator, was more abundant (*intI1*: 1.26×10^{-3}; *intI3*: 6.32×10^{-4}) (Fig. S 3).

3.3. Diversity of ARGs and MGEs by location

Red deer inhabiting LousãM had a high diversity of ARGs and MGEs with 42 positive assays, followed by MontesinhoNP (36) and TNMafra (33) (Fig. 3A). Seventeen genes were detected in the three locations, and include ARGs for aminoglycosides (*aadA7*, *aac(3)-IIa/d* and *aph4-Ib*), tetracyclines (*tet44*, *tetQ* and *tetO*), β-lactams ($bla_{OXY\text{-}1}$ and bla_{SFO}), MLSBs (*ermE* and *ermF*) and quinolones (*qnrB4* and *qepA*), 3 MGEs (*Tp614*, *IS*1247 and *orf37-IS*26) and 2 integrases (*intI1* and *intI3*). A higher number of genes was shared between LousãM and MontesinhoNP ($n = 12$) than between LousãM and TNMafra ($n = 3$) or TNMafra and MontesinhoNP ($n = 5$) (Fig. 3A).

The 34 ARGs detected in red deer from LousãM confer resistance to MLSBs (26%), aminoglycosides (24%), β-lactams (18%), tetracyclines (18%), vancomycin (6%), quinolones (6%) and phenicols (3%). In MontesinhoNP samples, the 29 ARGs encode resistance to MLSBs (24%), aminoglycosides (24%), tetracyclines (21%), β-lactams (17%), quinolones (7%), vancomycin (3%) and phenicols (3%). The 22 ARGs identified in TNMafra are associated with aminoglycosides (27%), MLSBs (23%), tetracyclines (23%), β-lactams (18%) and quinolones (9%) resistance. Therefore, none of the tested ARGs of the phenicol and vancomycin groups was detected in TNMafra faecal samples (Fig. 3C).

3.4. The abundance of ARGs and MGEs by location

For the deer samples from LousãM, the most abundant ARGs detected confer resistance to tetracyclines (mean ca. 10^{-4}), followed by quinolones (mean ca. 10^{-4}), aminoglycosides (mean ca. 10^{-5}), phenicols (mean ca. 10^{-5}), β-lactams (mean ca. 10^{-5}), vancomycin (mean ca. 10^{-5}) and MLSBs (mean ca. 10^{-5}) (Fig. 3B). The most abundant ARGs in MontesinhoNP belong to quinolones (mean ca. 10^{-4}), followed by tetracyclines (mean ca. 10^{-5}), β-lactams (mean ca. 10^{-5}), aminoglycosides (mean ca. 10^{-5}), MLSBs (mean ca. 10^{-5}), phenicols (mean ca. 10^{-5}) and vancomycin (mean ca. 10^{-5}) classes (Fig. 3B). In TNMafra, the most abundant ARGs detected are involved in the resistance to tetracyclines (mean ca. 10^{-4}), followed by quinolones (mean ca. 10^{-5}), aminoglycosides (mean ca. 10^{-5}), β-lactams (mean ca. 10^{-5}) and MLSBs (mean ca. 10^{-5}) (Fig. 3B). The mean abundance of MGEs present in the three locations was ca. 10^{-4}.

Tetracycline was the most abundant ARG class in all the geographical areas studied, accounting for 76%, 62% and 24% of the sum of the ARGs total abundances in TNMafra, LousãM and MontesinhoNP, respectively (Fig. 3D). However, the total abundances were more evenly distributed in MontesinhoNP, where the percentages of ARGs for aminoglycosides (21%), quinolones (20%) and β-lactams (19%) were similar to those of tetracyclines (Fig. 3D).

3.5. Prevalence of resistance based on clinical breakpoints for E. coli

E. coli isolates were recovered from all faecal samples ($n = 101$) and further subjected to AST. According to the clinical breakpoints, 23% ($n = 23$) of them were resistant to at least one of the antibiotics tested. Resistance was detected for ampicillin (12%), amoxicillin/clavulanic acid (7%), tetracycline (6%), tobramycin (5%), cefoxitin (4%), streptomycin (3%), amikacin (2%) and co-trimoxazole (2%) (Fig. 4A). Therefore, the isolates were mostly resistant to β-lactams and aminoglycosides (Fig. 4A). A multidrug-resistant (MDR) phenotype was detected for two strains (2%), both of them isolated from samples collected in TNMafra (Table S 2).

Considering each location, 28% of the *E. coli* population isolated from LousãM samples ($n = 11$) showed resistance to at least one antibiotic, and a higher percentage of resistance was found to β-lactams, followed by aminoglycosides and tetracycline (Fig. 4A). Regarding *E. coli* from MontesinhoNP, 8 isolates (25%) were resistant mainly to β-lactams, followed by aminoglycosides and tetracycline. The *E. coli* strains isolated from TNMafra exhibit resistance (14%; $n = 4$) to β-lactams, followed by aminoglycosides, tetracycline, and sulfonamides.

3.6. Prevalence of resistance based on ECOFFs for E. coli isolates

Considering the ECOFFs calculated with the NRI method (Table S 3), 32% ($n = 32$) of the *E. coli* strains were NWT to at least one of the antibiotics tested. An NWT phenotype was identified for ciprofloxacin (29%), tetracycline (6%), streptomycin (3%), ampicillin (2%) and co-trimoxazole (2%) (Fig. 4B). NWT phenotype was mainly detected for quinolone, followed by tetracycline, aminoglycoside, β-lactam and sulfonamide

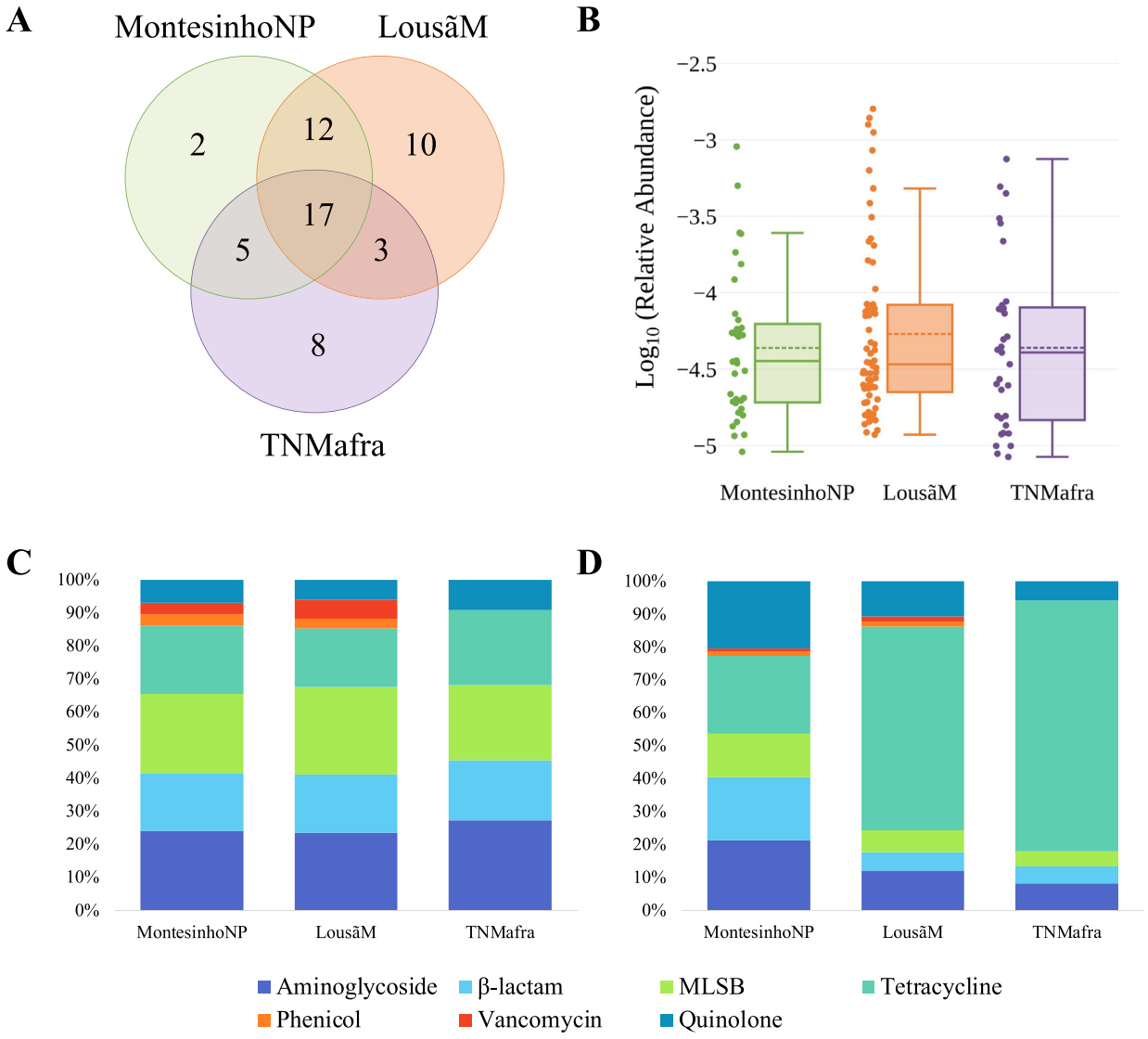


Fig. 3. AMR genetic determinants detected on red deer samples by location, including: a Venn diagram showing the number of shared genes (A), the relative abundance of genes (log_{10}; B), and the percentage of ARG's diversity (C) and abundance (D) according to the antibiotic class.

classes. Considering each location, 45% (n = 13), 38% (n = 12) and 18% (n = 7) of the strains with origin in TNMafra, MontesinhoNP and LousãM, respectively, had an NWT phenotype (Fig. 4B).

3.7. Prevalence of resistance based on clinical breakpoints for Enterococcus spp. isolates

Enterococcus spp. isolates were recovered from 75% of the faecal samples (n = 76) and belong to the following species: *E. faecium* (33%), *E. hirae* (30%), *E. faecalis* (17%), and other *Enterococcus* spp. (20%). According to the clinical breakpoints, AST results show that 74% (n = 56) were resistant to at least one of the antibiotics tested. Resistance was detected for quinupristin-dalfopristin (53%), tigecycline (24%), tetracycline (22%), erythromycin (7%), teicoplanin (5%), streptomycin (1%) and ciprofloxacin (1%) (Fig. 4C). Accordingly, the isolates were mainly resistant to the streptogramin, glycylcycline, tetracycline, macrolide, glycopeptide, aminoglycoside, and quinolone classes. A MDR phenotype was detected for 6 strains (8%), 3 isolated from hunted deer in LousãM, and 3 from MontesinhoNP (Table S 2).

Considering each location, 88% of the isolates from MontesinhoNP showed resistance to at least one antibiotic, and a higher percentage was found for streptogramin, followed by tetracycline, macrolide, glycylcycline and quinolone (Fig. 4C). 68% of the *Enterococcus* spp. from LousãM showed resistance, mainly to streptogramin, followed by tetracycline, glycylcycline, glycopeptide, macrolide and aminoglycoside groups (Fig. 4C). From TNMafra, 67% of the strains were resistant, mostly to streptogramin, followed by glycylcycline, tetracycline and glycopeptide classes (Fig. 4C).

3.8. Prevalence of resistance based on ECOFFs for Enterococcus spp. isolates

According to the ECOFFs calculated with the NRI methodology (Table S 4), 58% (n = 44) of the strains were considered NWT to at least one of the antibiotics tested. An NWT phenotype was identified for tetracycline (26%), gentamicin (22%), erythromycin (18%), tigecycline (15%), ampicillin (13%), quinupristin-dalfopristin (8%), streptomycin (5%) and teicoplanin (1%) (Fig. 4D).

Considering each location, in MontesinhoNP, an NWT profile was detected for 79% (n = 19) of the strains, and to the antibiotics erythromycin, gentamicin, tetracycline, ampicillin, tigecycline, quinupristin-dalfopristin and teicoplanin (Fig. 4D). For LousãM, an NWT phenotype was identified in 55% (n = 17) of the isolates to tetracycline, gentamicin, erythromycin, tigecycline, ampicillin, quinupristin-dalfopristin and streptomycin (Fig. 4D). The *Enterococcus* spp. isolated from TNMafra samples exhibited this phenotype in 38% (n = 8) of the strains, to tetracycline, ampicillin,

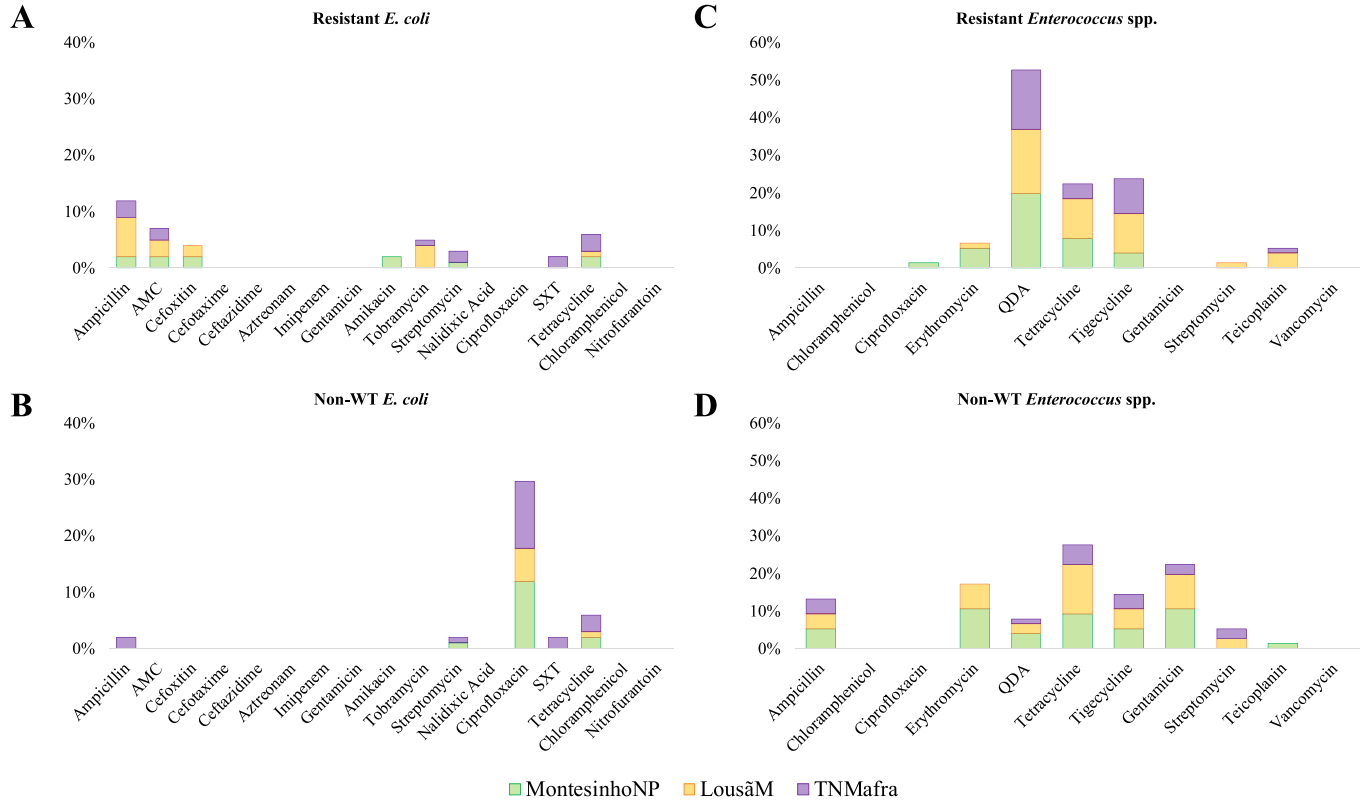


Fig. 4. Antibacterial susceptibility of *E. coli* and *Enterococcus* spp. isolates identified in this study and interpreted according to clinical breakpoints (resistant strains; A and C) and ECOFFs (non-wildtype strains; B and D). AMC corresponds to amoxicillin/clavulanic acid, SXT to trimethoprim-sulfamethoxazole and QDA to quinupristin-dalfopristin.

tigecycline, gentamicin, streptomycin and quinupristin-dalfopristin (Fig. 4D).

4. Discussion

AMR bacteria have been frequently reported in peridomestic wildlife, although the described resistance rates vary between different species and countries (Arnold et al., 2016). It is usually assumed that (acquired) resistance in wildlife results from the contact with antibacterial compounds through environmental contamination due to anthropogenic activities (Vittecoq et al., 2016). Human populations are expanding, and wild animals are forced to forage on resources contaminated by human 'pollution' (Arnold et al., 2016). Cervids, for instance, have been observed to transit and feed on grass and crop fields that were fertilized with manure (Rogers et al., 2018), which is considered a hotspot matrix for the spread of metals, antimicrobials and ARGs (Lima et al., 2020). Freshwaters, frequently used as a source of drinking water for wildlife, are especially liable to antibiotic contamination from agricultural runoffs, sewage, and other sources (Cacace et al., 2019; Nnadozie and Odume, 2019; Zhou et al., 2018; Zhu et al., 2017). Red deer is worldwide distributed, occupying different habitats, including natural environments, farms and urban areas. It is one of the main food game species, and its meat is consumed in all European countries (Schulp et al., 2014; Vingada et al., 2010).

So far, studies on AMR in cervids have focused on cultivable bacteria, characterizing the antimicrobial susceptibility of indicator strains (as *E. coli* and *Enterococcus* spp.) or pathogenic strains (Dias et al., 2015; Plaza-Rodríguez et al., 2021; Turchi et al., 2019). Some studies have investigated the occurrence of few ARGs in ESBL positive strains (Alonso et al., 2016; Križman et al., 2017; Mateus-Vargas et al., 2017). As such, research on deer resistome is sparse and limited in the number of ARGs analysed (Rogers et al., 2018). This study took advantage of a high-throughput ARGs and MGEs quantification method to document the diversity and abundance of AMR-associated genetic determinants using total DNA from faeces of red deer as a template. In total, 41 different ARGs (corresponding to 43 ARG assays) that confer resistance to 7 antibiotic groups and 14 MGEs were identified. This result is lower than the reported by studies applying the same approach but that were conducted with different matrices, such as soils receiving swine and dairy manure (77 ARGs from 8 groups and 12 MGEs) (Chen et al., 2019) and swine faeces (146 ARGs from 9 groups and 10 MGEs) (Zhao et al., 2018).

Regarding the relative abundances of ARGs, they were within the range found in soil ecosystems (10^{-6} to 10^{-4}) (McCann et al., 2019) or livestock wastes in more than 90 countries (varied from 10^{-3} to 10^{-1}) (He et al., 2020). A high abundance of tetracycline ARGs was observed, followed by quinolone, aminoglycoside, and β-lactam groups. These correspond to the main classes of antibiotic used intensively in animal husbandry worldwide (He et al., 2020). Likewise, the most frequent and abundant ARGs classes identified in samples associated with livestock farming confer resistance to tetracyclines, MLSBs, aminoglycosides and β-lactams (Chen et al., 2019; Muurinen et al., 2017; Qian et al., 2018; Wu et al., 2020; Zhao et al., 2018). In south China, an investigation in farmed sika deer reported tetracycline ARGs as the most abundant resistance determinants (Huang et al., 2016). Furthermore, in Poland, a metagenomic shotgun sequencing study performed in wild and domestic animals gut showed a dominance of tetracycline resistance ARGs, in which the *tetQ* was the most abundant gene in almost all tested animals (Skarżyńska et al., 2020). In the USA, the increased prevalence of *tetQ* and *ermB* ARGs in faecal samples of wild deer in proximity to manure and biosolids applications seems to support that proximity to anthropogenic sources impacts the AMR profiles of wildlife gut bacteria (Rogers et al., 2018). Our results are in agreement with this study, as *tetQ* was identified in all the geographical areas studied, but it was more abundant in locations close to anthropogenic activities (LousãM and TNMafra; Fig. S 3), and *ermB* was found only in TNMafra samples. Based on cumulative abundances of ARGs, resistance to tetracycline was higher

in all the studied areas, but it is more prevalent in LousãM and TNMafra. In contrast, in MontesinhoNP (the environment with lower anthropogenic activity), is similar to the resistance to aminoglycosides, quinolones and β-lactams. However, if the averages are analysed, MontesinhoNP was the only location where ARGs for quinolones were more abundant than the ARGs for tetracyclines. The genes exclusively found in MontesinhoNP were few, and LousãM have more ARGs in common with this area than with TNMafra. In addition, only two ARG types (vancomycin and phenicols) were not detected in TNMafra samples. These results suggest that ARGs diversity might be influenced by factors such as the ecosystem location and resources, and not mainly by anthropogenic activities. Although MontesinhoNP and LousãM are differently impacted by human population, both are open areas, contrary to the gated TNMafra, in which deer live in semi-captivity and are frequently fed by humans. In TNMafra, deer do not interact with livestock, agriculture, and the contact with other wildlife is also restricted to the species inhabiting that area.

Thus, in addition to identifying and quantifying AMR determinants, it is also necessary to assess the danger they may represent (Pärnänen et al., 2019). Recently, A.N. Zhang et al. (2021) proposed a classification that separates ARGs into four ranks (I to IV), depending on their risk, from the highest to the lowest. Rank I ARGs include "current threats" and Rank II "future threats". 11 of these ARGs were identified in red deer faecal samples (Fig. 5). Two Rank I ARGs, *qnrB* and *aac(3)-II* were identified on the three locations. Furthermore, *tetW*, *tetO* and *aadA*, categorized on Rank II were found in relatively high abundances (10^{-4}–10^{-3}). Additionally, we also identified some ARGs in the red deer resistome that were proposed by (Berendonk et al., 2015) as indicators of resistance in the environment, that include: i) *ermB*, associated with macrolide resistance, which was only present in TNMafra and that is a Rank I and Rank II gene, ii) *ermF*, also associated with macrolide resistance, iii) *aph* ARGs encoding aminoglycoside phosphotransferases and iv) the clinical *intI1* gene, considered as a good proxy for pollution because it is associated with genes that confer resistance to antibiotics, disinfectants and metals, and that is believed to have evolved under the selective pressure of human activity (Gillings et al., 2015).

Culture-dependent approaches are still commonly used to access the bacterial AMR profile of many animals and environments (Guitor et al., 2019). Therefore, herein, we have also accessed the prevalence of antibiotic resistance by testing the susceptibility of *E. coli* and *Enterococcus* spp. bacteria isolated from deer faeces. *E. coli* strains were recovered from 100% of the samples, which corresponds to a higher percentage than that reported for red deer populations of other European countries (Spain and Poland, 59% and 78%, respectively) (Alonso et al., 2016; Wasyl et al., 2018). Considering the clinical breakpoints, 23% of the *E. coli* isolates showed resistance to at least one antibiotic, a higher percentage than that reported for red deer faeces in Spain (7%) and game meat in Germany (9%) (Alonso et al., 2016; Mateus-Vargas et al., 2017). In our study, *E. coli* were mainly resistant to β-lactams and aminoglycosides, whereas in the above referred two studies, the isolates were primarily resistant to tetracyclines (Spain; Alonso et al., 2016) and β-lactams (Germany; Mateus-Vargas et al., 2017). A MDR phenotype was detected only in two *E. coli* isolates (2%) similar to that observed in farmed red deer in Spain, where only one *E. coli* with this phenotype was detected (Alonso et al., 2016). Regarding *Enterococcus* spp., the second indicator species, it was isolated from 75% of the samples, which corresponds to a lower rate than that reported in farmed red deer samples from New Zealand (100%; Pattis et al., 2017). According to the clinical criteria, a high number of resistant strains was obtained (74%). This was mostly due to QDA resistance (53%), usually detected in *Enterococcus* spp. from food animals, but uncommon in clinical isolates (Hershberger et al., 2004; Jones et al., 1998). A high level of resistance to QDA (83%) was also detected in enterococci isolated from roe deer meat in Spain (Guerrero-Ramos et al., 2016). The enterococci resistance determined in our study was also lower than that reported in Spanish roe deer meat: tetracycline (22% vs 57%), erythromycin (7% vs 86%), teicoplanin (5% vs 14%), ciprofloxacin (1% vs 91%), with streptomycin being an exception (1% vs 0%). Six *Enterococcus* spp. strains were MDR, which is a lower rate than that identified in wild game meat, where all the strains were MDR (Guerrero-Ramos et al., 2016).

The type of cut-offs chosen to investigate the prevalence of AMR in a given bacterial population significantly impact the reported resistance rates (Silva et al., 2020). This is why it can be more appropriate to use ECOFFs to interpret resistance in environmental studies. Our results showed that, according to these cut-offs, 32% of the *E. coli* were considered NWT and mainly to quinolones class, which is higher than that observed in red deer from Poland (3.4% NWT) (Wasyl et al., 2018). Regarding *Enterococcus* spp., an NWT phenotype was detected in 58% of the isolates, primarily for aminoglycoside and tetracycline classes.

In general, we found a higher diversity and abundance of ARGs in samples from LousãM, and higher rates of resistance were also detected for both bacterial indicators recovered from this location. All the samples analysed from LousãM were collected from hunted animals, whose meat was for human consumption. This highlights the risk of dissemination through direct contact with the people who handle the carcasses or also through the foodchain.

The results presented here emphasizes that using culture-independent and dependent approaches to investigating AMR can be beneficial as they

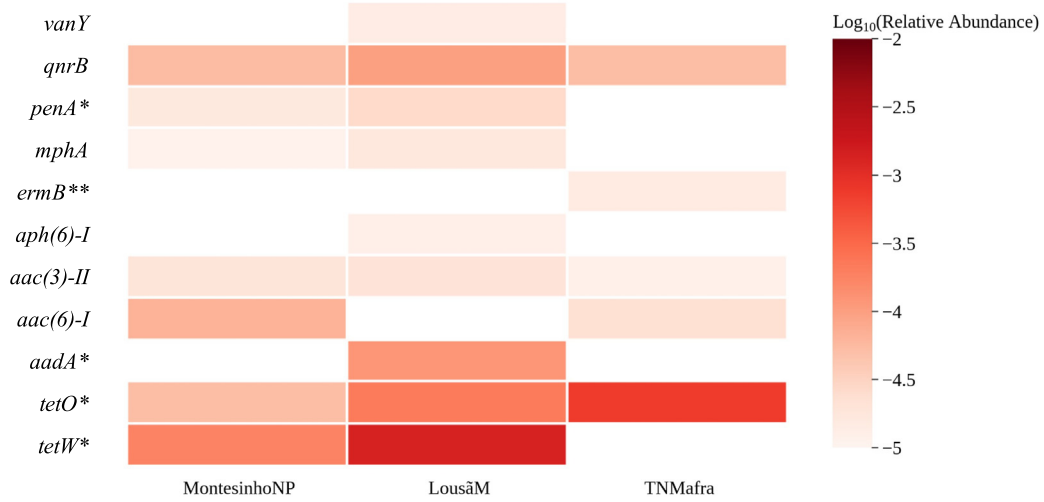


Fig. 5. Heatmap of the relative abundance of high-risk ARGs (Rank I and Rank II) detected in this study. ARGs belonging to Rank II are marked with an *, and ARGs of both ranks are marked with **.

complement each other. For instance, qPCR did not detect any trimethoprim or sulfonamide ARG in the samples, although a phenotype of resistance to co-trimoxazole (2%) was identified in the 2 multidrug-resistant *E. coli* isolates from TNMafra. However, comparisons are difficult to make, and standardized methodologies for both approaches must be implemented worldwide. The qPCR array applied here is excellent for standardizing results based on metagenomics, although upstream methodologies are still diverse.

5. Conclusions

To the best of our knowledge, this is the first study aimed at identifying a high number of ARGs using a metagenomic approach with red deer, one of the main game species in Europe. ARGs conferring resistance to 7 different groups were identified in the red deer faecal microbiome, and tetracycline genes were the ARGs with the highest relative abundance. ARGs considered as current and future threats to human health (Rank I and Rank II, respectively) were detected, some of them at high relative abundance, as for example, the plasmid-associated *qnrB* conferring resistance to quinolones. Regarding indicator bacteria, a high prevalence of antibiotic resistance was observed mainly for *Enterococcus* spp., but only for a few antibiotics. A low MDR rate was observed, but some MDR strains were isolated from hunted animals, that were intended for human consumption, what may represent a public health problem.

Red deer's habitat often overlaps that of livestock and other wild species and, in addition to that, they usually feed on agricultural lands. Thus, long-term monitoring of wildlife species (and also livestock, water sources, and soils) is urgent and is an essential tool for national and global AMR surveillance efforts from a One Health perspective.

CRediT authorship contribution statement

D. Dias: Investigation, Data curation, Formal analysis, Writing - original draft.

C. Fonseca: Resources, Funding acquisition, Writing - review & editing.

T. Caetano: Conceptualization, Resources, Supervision, Writing - review & editing.

S. Mendo: Resources, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Diana Dias was supported by the Fundação para a Ciência e a Tecnologia (FCT) grant (SFRH/BD/118618/2016). Tânia Caetano was funded by national funds (OE), through FCT – Fundação para a Ciência e a Tecnologia, I.P., in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July (CEECIND/01463/2017). Thanks are due to FCT/MCTES for the financial support to CESAM (UIDP/50017/2020 + UIDB/50017/2020), through national funds. We thank Doctor Rita T. Torres, Ana Figueiredo and Dário Hipólito from the Wildlife Research Unit (CESAM and DBio, UA), and to Tapada Nacional de Mafra for their contribution to the collection of samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153831.

References

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8, 251–259. https://doi.org/10.1038/nrmicro2312.

Alonso, C.A., González-Barrio, D., Tenorio, C., Ruiz-Fons, F., Torres, C., 2016. Antimicrobial resistance in faecal Escherichia coli isolates from farmed red deer and wild small mammals. Detection of a multiresistant E. coli producing extended-spectrum beta-lactamase. Comp. Immunol. Microbiol. Infect. Dis. 45, 34–39. https://doi.org/10.1016/j.cimid.2016.02.003.

Arnold, K.E., Williams, N.J., Bennett, M., 2016. 'Disperse abroad in the land': the role of wildlife in the dissemination of antimicrobial resistance. Biol. Lett. 12, 20160137. https://doi.org/10.1098/rsbl.2016.0137.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439.

Böhm, M., White, P.C.L., Chambers, J., Smith, L., Hutchings, M.R., 2007. Wild deer as a source of infection for livestock and humans in the UK. Vet. J. 174, 260–276. https://doi.org/10.1016/j.tvjl.2006.11.003.

Buta, M., Korzeniewska, E., Harnisz, M., Hubeny, J., Zieliński, W., Rolbiecki, D., Bajkacz, S., Felis, E., Kokoszka, K., 2021. Microbial and chemical pollutants on the manure-crops pathway in the perspective of "One health" holistic approach. Sci. Total Environ. 785, 147411. https://doi.org/10.1016/j.scitotenv.2021.147411.

Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.-N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: a pan-European survey of urban settings. Water Res. 162, 320–330. https://doi.org/10.1016/j.watres.2019.06.039.

Chen, Z., Zhang, W., Yang, L., Stedtfeld, R.D., Peng, A., Gu, C., Boyd, S.A., Li, H., 2019. Antibiotic resistance genes and bacterial communities in cornfield and pasture soils receiving swine and dairy manures. Environ. Pollut. 248, 947–957. https://doi.org/10.1016/j.envpol.2019.02.093.

Dias, D., Torres, R.T., Kronvall, G., Fonseca, C., Mendo, S., Caetano, T., 2015. Assessment of antibiotic resistance of Escherichia coli isolates and screening of Salmonella spp. in wild ungulates from Portugal. Res. Microbiol. 166, 584–593. https://doi.org/10.1016/j.resmic.2015.03.006.

Dias, D., Fonseca, C., Mendo, S., Caetano, T., 2022. A closer look on the variety and abundance of the faecal resistome of wild boar. Environ. Pollut. 292, 118406. https://doi.org/10.1016/j.envpol.2021.118406.

ECDC/EFSA/EMA, 2017. ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. EFSA J. 15. https://doi.org/10.2903/j.efsa.2017.4872.

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.-G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226.

Guerrero-Ramos, E., Cordero, J., Molina-González, D., Poeta, P., Igrejas, G., Alonso-Calleja, C., Capita, R., 2016. Antimicrobial resistance and virulence genes in enterococci from wild game meat in Spain. Food Microbiol. 53, 156–164. https://doi.org/10.1016/j.fm.2015.09.007.

Guitor, A.K., Raphenya, A.R., Klunk, J., Kuch, M., Alcock, B., Surette, M.G., McArthur, A.G., Poinar, H.N., Wright, G.D., 2019. Capturing the resistome: a targeted capture method to reveal antibiotic resistance determinants in metagenomes. Antimicrob. Agents Chemother. 64. https://doi.org/10.1128/AAC.01324-19.

He, Y., Yuan, Q., Mathieu, J., Stadler, L., Senehi, N., Sun, R., Alvarez, P.J.J., 2020. Antibiotic resistance genes from livestock waste: occurrence, dissemination, and treatment. npj CleanWater 3, 4. https://doi.org/10.1038/s41545-020-0051-0.

Hershberger, E., Donabedian, S., Konstantinou, K., Zervos, M.J., 2004. Quinupristin-dalfopristin resistance in Gram-positive bacteria: mechanism of resistance and epidemiology. Clin. Infect. Dis. 38, 92–98. https://doi.org/10.1086/380125.

Huang, F., An, X.-L., Chen, Q., Ren, H., Su, J., 2016. Distribution characteristics of antibiotic resistance genes in sika deer farm. Huanjing Kexue/Environ. Sci. 37, 4402–4409. https://doi.org/10.13227/j.hjkx.201605196.

Jones, R.N., Ballow, C.H., Biedenbach, D.J., Deinhart, J.A., Schentag, J.J., 1998. Antimicrobial activity of quinupristin-dalfopristin (RP 59500, Synercid®) tested against over 28,000 recent clinical isolates from 200 medical centers in the United States and Canada. Diagn. Microbiol. Infect. Dis. 31, 437–451. https://doi.org/10.1016/S0732-8893(98)80002-3.

Križman, M., Kirbiš, A., Jamnikar-Ciglenečki, U., 2017. Antimicrobial-resistant bacteria in wild game in Slovenia. IOP Conf. Ser. Earth Environ. Sci. 85, 012083. https://doi.org/10.1088/1755-1315/85/1/012083.

Kronvall, G., Smith, P., 2016. Normalized resistance interpretation, the NRI method. APMIS 124, 1023–1030. https://doi.org/10.1111/apm.12624.

Lima, T., Domingues, S., Da Silva, G.J., 2020. Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events. Vet. Sci. 7, 110. https://doi.org/10.3390/vetsci7030110.

Mateus-Vargas, R.H., Atanassova, V., Reich, F., Klein, G., 2017. Antimicrobial susceptibility and genetic characterization of Escherichia coli recovered from frozen game meat. Food Microbiol. 63, 164–169. https://doi.org/10.1016/j.fm.2016.11.013.

McCann, C.M., Christgen, B., Roberts, J.A., Su, J.-Q., Arnold, K.E., Gray, N.D., Zhu, Y.-G., Graham, D.W., 2019. Understanding drivers of antibiotic resistance genes in High Arctic soil ecosystems. Environ. Int. 125, 497–504. https://doi.org/10.1016/j.envint.2019.01.034.

Muurinen, J., Stedtfeld, R., Karkman, A., Pärnänen, K., Tiedje, J., Virta, M., 2017. Influence of manure application on the environmental resistome under Finnish agricultural practice with restricted antibiotic use. Environ. Sci. Technol. 51, 5989–5999. https://doi.org/10.1021/acs.est.7b00551.

Nnadozie, C.F., Odume, O.N., 2019. Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. Environ. Pollut. 254, 113067. https://doi.org/10.1016/j.envpol.2019.113067.

Pärnänen, K.M.M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V.Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5, eaau9124. https://doi.org/10.1126/sciadv.aau9124.

Pattis, I., Moriarty, E., Billington, C., Gilpin, B., Hodson, R., Ward, N., 2017. Concentrations of Campylobacter spp., Escherichia coli, Enterococci, and Yersinia spp. in the feces of farmed red deer in New Zealand. J. Environ. Qual. 46, 819–827. https://doi.org/10.2134/jeq2017.01.0002.

Plaza-Rodríguez, C., Alt, K., Grobbel, M., Hammerl, J.A., Irrgang, A., Szabo, I., Stingl, K., Schuh, E., Wiehle, L., Pfefferkorn, B., Naumann, S., Kaesbohrer, A., Tenhagen, B.-A., 2021. Wildlife as sentinels of antimicrobial resistance in Germany? Front. Vet. Sci. 7. https://doi.org/10.3389/fvets.2020.627821.

Qian, X., Gu, J., Sun, W., Wang, X.-J., Su, J.-Q., Stedfeld, R., 2018. Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. J. Hazard. Mater. 344, 716–722. https://doi.org/10.1016/j.jhazmat.2017.11.020.

Rogers, S.W., Shaffer, C.E., Langen, T.A., Jahne, M., Welsh, R., 2018. Antibiotic-resistant genes and pathogens shed by wild deer correlate with land application of residuals. EcoHealth 15, 409–425. https://doi.org/10.1007/s10393-018-1316-7.

Rwego, I.B., Isabirye-Basuta, G., Gillespie, T.R., Goldberg, T.L., 2008. Gastrointestinal bacterial transmission among humans, mountain gorillas, and livestock in Bwindi Impenetrable National Park,Uganda. Conserv. Biol. 22, 1600–1607. https://doi.org/10.1111/j.1523-1739.2008.01018.x.

Schulp, C.J.E., Thuiller, W., Verburg, P.H., 2014. Wild food in Europe: a synthesis of knowledge and data of terrestrial wild food as an ecosystem service. Ecol. Econ. 105, 292–305. https://doi.org/10.1016/j.ecolecon.2014.06.018.

Silva, N., Phythian, C.J., Currie, C., Tassi, R., Ballingall, K.T., Magro, G., McNeilly, T.N., Zadoks, R.N., 2020. Antimicrobial resistance in ovine bacteria: a sheep in wolf's clothing? PLoS One 15, e0238708. https://doi.org/10.1371/journal.pone.0238708.

Singh, K.V., Weinstock, G.M., Murray, B.E., 2002. An Enterococcus faecalis ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. Antimicrob. Agents Chemother. 46, 1845–1850. https://doi.org/10.1128/AAC.46.6.1845-1850.2002.

Sjölund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G., Olsen, B., 2008. Dissemination of multidrug-resistant bacteria into the Arctic. Emerg. Infect. Dis. 14, 70–72. https://doi.org/10.3201/eid1401.070704.

Skarżyńska, M., Leekitcharoenphon, P., Hendriksen, R.S., Aarestrup, F.M., Wasyl, D., 2020. A metagenomic glimpse into the gut of wild and domestic animals: quantification of antimicrobial resistance and more. PLoS One 15, e0242987. https://doi.org/10.1371/journal.pone.0242987.

Stedtfeld, R.D., Guo, X., Stedtfeld, T.M., Sheng, H., Williams, M.R., Hauschild, K., Gunturu, S., Tift, L., Wang, F., Howe, A., Chai, B., Yin, D., Cole, J.R., Tiedje, J.M., Hashsham, S.A., 2018. Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. FEMS Microbiol. Ecol. 94, 1–8. https://doi.org/10.1093/femsec/fiy130.

Sunde, M., Urdahl, A.M., Norström, M., Madslien, K., Danielsen, A.V., Barstad, A.S., Welde, H., Slettemeås, J.S., Neves, C.G.das, 2018. Antibiotic resistance in terrestrial wild mammal species in Norway - roe deer and wild reindeer as indicators species.

Turchi, B., Dec, M., Bertelloni, F., Winiarczyk, S., Gnat, S., Bresciani, F., Viviani, F., Cerri, D., Fratini, F., 2019. Antibiotic susceptibility and virulence factors in Escherichia coli from sympatric wildlife of the Apuan Alps Regional Park (Tuscany, Italy). Microb. Drug Resist. 25, 772–780. https://doi.org/10.1089/mdr.2018.0191.

Valente, A., Valente, J., Fonseca, C., Torres, R., 2017. The success of species reintroductions: a case study of red deer in Portugal two decades after reintroduction. Int. J. Biodivers. Sci. Ecosyst. Serv. Manag. 13, 134–138. https://doi.org/10.1080/21513732.2016.1277265.

Vaz-Moreira, I., Ferreira, C., Nunes, O.C., Manaia, C.M., 2019. Sources of antibiotic resistance. Antibiotic Drug Resistance. Wiley, pp. 211–238 https://doi.org/10.1002/9781119282549.ch10.

Vingada, J., Fonseca, C., Cancela, J., Ferreira, J., Eira, C., 2010. Ungulates and their management in Portugal. European Ungulates And Their Management in the 21st Century. Cambridge University Press, Cambridge.

Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., Arnal, A., Aberkane, S., Jean-Pierre, H., Gauthier-Clerc, M., Thomas, F., Renaud, F., 2016. Antimicrobial resistance in wildlife. J. Appl. Ecol. 53, 519–529. https://doi.org/10.1111/1365-2664.12596.

Wasyl, D., Zając, M., Lalak, A., Skarżyńska, M., Samcik, I., Kwit, R., Jabłoński, A., Bocian, Ł., Woźniakowski, G., Hoszowski, A., Szulowski, K., 2018. Antimicrobial resistance in Escherichia coli isolated from wild animals in Poland. Microb. Drug Resist. 24, 807–815. https://doi.org/10.1089/mdr.2017.0148.

WHO, 2014. Antimicrobial Resistance: Global Report on Surveillance France.

WHO, 2017. Global Action Plan on Antimicrobial Resistance. World Health Organization.

WHO, 2019. Critically Important Antimicrobials for Human Medicine, 6th Revision Geneva.

Wu, N., Xie, S., Zeng, M., Xu, X., Li, Y., Liu, X., Wang, X., 2020. Impacts of pile temperature on antibiotic resistance, metal resistance and microbial community during swine manure composting. Sci. Total Environ. 744, 140920. https://doi.org/10.1016/j.scitotenv.2020.140920.

Zaheer, R., Cook, S.R., Barbieri, R., Goji, N., Cameron, A., Petkau, A., Polo, R.O., Tymensen, L., Stamm, C., Song, J., Hannon, S., Jones, T., Church, D., Booker, C.W., Amoako, K., Van Domselaar, G., Read, R.R., McAllister, T.A., 2020. Surveillance of Enterococcus spp. reveals distinct species and antimicrobial resistance diversity across a one-health continuum. Sci. Rep. 10, 3937. https://doi.org/10.1038/s41598-020-61002-5.

Zhang, A.-N., Gaston, J.M., Dai, C.L., Zhao, S., Poyet, M., Groussin, M., Yin, X., Li, L.-G., van Loosdrecht, M.C.M., Topp, E., Gillings, M.R., Hanage, W.P., Tiedje, J.M., Moniz, K., Alm, E.J., Zhang, T., 2021. An omics-based framework for assessing the health risk of antimicrobial resistance genes. Nat. Commun. 12, 4765. https://doi.org/10.1038/s41467-021-25096-3.

Zhang, Y., Zheng, Y., Zhu, Z., Chen, Y., Dong, H., 2021. Dispersion of antibiotic resistance genes (ARGs) from stored swine manure biogas digestate to the atmosphere. Sci. Total Environ. 761, 144108. https://doi.org/10.1016/j.scitotenv.2020.144108.

Zhao, Y., Su, J.-Q., An, X.-L., Huang, F.-Y., Rensing, C., Brandt, K.K., Zhu, Y.-G., 2018. Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. Sci. Total Environ. 621, 1224–1232. https://doi.org/10.1016/j.scitotenv.2017.10.106.

Zhou, Z.-C., Feng, W.-Q., Han, Y., Zheng, J., Chen, T., Wei, Y.-Y., Gillings, M., Zhu, Y.-G., Chen, H., 2018. Prevalence and transmission of antibiotic resistance and microbiota between humans and water environments. Environ. Int. 121, 1155–1161. https://doi.org/10.1016/j.envint.2018.10.032.

Zhu, Y.-G., Zhao, Y., Li, B., Huang, C.-L., Zhang, S.-Y., Yu, S., Chen, Y.-S., Zhang, T., Gillings, M.R., Su, J.-Q., 2017. Continental-scale pollution of estuaries with antibiotic resistance genes. Nat. Microbiol. 2, 16270. https://doi.org/10.1038/nmicrobiol.2016.270.