

## Antimicrobial resistance of *Enterococcus* species isolated from wild mammals in Aragón, Spain

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### Abstract

**Introduction:** Antimicrobial resistance is currently one of the major public health threats. In order to prevent its spread, the WHO, OIE and FAO have formed an alliance to promote the study of antibiotic resistance evolution in human, animal and environmental bacteria posing a public health threat; however, the studies performed in wild animals are scarce so far. The main objective of this study was to assess the antibiotic resistance of *Enterococcus* spp. isolated from wild mammals in Aragón, Spain. **Material and Methods:** Rectal samples were collected from 103 wild mammals – 70 hunt prey and 33 rescued animals. Isolates were identified by matrix-assisted laser desorption/ionisation–time of flight mass spectrometry and susceptibility tests to 10 antibiotics were also carried out. Statistical analysis was performed ( $P \leq 0.05$ ). **Results:** A total of 126 isolates of seven different *Enterococcus* species were recovered. Among them, *E. faecalis* (37.60%), *E. casseliflavus* (20.63%) and *E. faecium* (17.46%) were the most prevalent. The antibiotics quinupristin-dalfopristin and ciprofloxacin most frequently lost efficacy against the isolates. Multi-drug resistance was more prevalent in enterococci isolated from the rescued mammals. **Conclusion:** This study found resistance widely distributed among enterococci isolated from the studied mammals. This points to the need for additional study of its genetic determinants and investigation of the sources and measures to avoid contributory environmental contamination.

**Keywords:** wild mammals, *Enterococcus* spp., antibiotic resistance, epidemiology.

### Introduction

The discovery of antimicrobials has improved the quality of life of both humans and animals. Antimicrobials reduce mortality and morbidity by supporting recovery from surgical interventions and preventing diseases in immunocompromised patients, and they increase the lifespan of domestic animals or optimise animal production. However, inappropriate use of antibiotics exerts selective pressure on bacteria. The end result of this pressure is antimicrobial resistance (AMR) rising to levels which, for some infections previously easily treated, presently leave clinicians no treatment options (33).

Certain bacteria have developed a natural way to resist biomolecules produced by other microorganisms (33). Consequently, they contain a wide range of genes and genetic determinants of resistance naturally acquired that may be transmitted to other bacteria, including human and animal pathogens (12), leading to a decrease in or a complete loss of antibiotic efficacy.

Antimicrobial resistance is considered by the World Health Organization (WHO) “one of the top 10 global public health threats facing humanity” (34). The Tripartite Alliance between the WHO, the World Organisation for Animal Health (OIE), and the Food and Agriculture Organization of the United Nations (FAO) exists to address important health problems,

such as AMR, and to promote awareness, investigation and cooperation between countries and health professionals (13). One of its proposals is the monitoring of resistance in sentinel bacteria such as the vancomycin-resistant *Enterococcus* spp. (VRE), particularly *Enterococcus faecium* and *Enterococcus faecalis*.

The end of bacterial susceptibility to antimicrobials is a global anthropogenic threat affecting humans, animals and the environment, the last of these being regarded as an important vehicle of the transmission of AMR (18). Rectifying the lack of studies assessing the spread of AMR in wildlife, this study aimed to investigate the resistance to antibiotics of *Enterococcus* spp. isolated from wild mammals in the Autonomous Community of Aragón in Spain, and also to detect the possible sources of this resistance.

## Material and Methods

**Study samples.** Rectal samples from 103 wild mammals were collected in the Autonomous Community of Aragón in Spain between 2012 and 2015 (Table 1). Thirty-three of these samples were provided by the Centre of Wild Fauna Recovery of La Alfranca (CWFR-LA) (Zaragoza, Aragón, Spain) and came from rescued animals, and 70 were taken by veterinarians attending hunts in the Autonomous Community and came from hunt prey. The samples were collected by means of sterile swabs in Amies medium, in the first hours of the animal's arrival at the CWFR-LA or immediately after hunting.

The epidemiological data compiled were the order and species, source of sampling, animal age (infant (<1 year), young (from 1 to 2 years) or adult (>2 years)), sex, main diet (apart from the general consideration, the main diet is the one most frequently ingested by the mammal: carnivorous, herbivorous, omnivorous, piscivorous, or insectivorous), and scavenging (if habitual on carrion or not). The year and season of sampling and the geographical location of the mammal's hunting or rescue were also recorded.

**Isolation and identification of enterococci.** Samples were seeded in Slanetz and Bartley Agar (CM0377; Oxoid, Madrid, Spain) with and without 4 µg/mL of vancomycin. Selected colonies were subcultured in Columbia blood agar base (sheep Blood Agar Base PB0115; Oxoid) in order to be identified by proteomic profiling using a Biotyper 3 matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry biotyper (Bruker, Billerica, MA, USA), following the manufacturer's instructions.

**Antibiotic susceptibility test.** Antibiotic resistance was evaluated using the Kirby–Bauer disc diffusion (DD) test, following the instructions of the Clinical & Laboratory Standards Institute (CLSI) (8). The antibiotics vancomycin (VAN, 30 µg), teicoplanin (TEI, 30 µg), ampicillin (AMP, 10 µg), streptomycin

(S, 300 µg), gentamicin (GEN, 120 µg), chloramphenicol (CL, 30 µg), tetracycline (TE, 30 µg), erythromycin (ERL, 15 µg), quinupristin-dalfopristin (QD, 15 µg), and ciprofloxacin (CIP, 5 µg) were studied. Vancomycin-resistant isolates detected by the DD test were also subjected to a minimum inhibitory concentration (MIC) M.I.C. Evaluator test (Oxoid) because it is considered the reference test to detect resistance to the VAN phenotype. Enterococci with VAN MIC values of 8 µg/mL, which indicated intermediate susceptibility, were further analysed to detect *vanA* and *vanB* genes in the Department of Food and Agriculture, Area of Biochemistry and Molecular Biology at the University of La Rioja, Spain. Gene detection and amplification were carried out by PCR, with the primers and conditions presented in Table 2.

The DD test reading was based on the criteria set by the CLSI. In the case of the M.I.C. Evaluator test, the manufacturer provides the range of concentrations of antibiotics to distinguish resistant, intermediate and susceptible bacteria, also based on CLSI criteria. Multidrug-resistant (MDR) isolates were defined as those not susceptible to at least one agent in three or more antimicrobial categories (22).

**Statistical analysis.** The distribution of frequency was calculated for the main epidemiological factors, *Enterococcus* spp. isolation, and the detected antibiotic resistance. Statistical analysis was performed with Epi Info 7.1.5.2 software (<https://www.cdc.gov/epiinfo>). The chi square value ( $\chi^2$ ) was estimated for qualitative variables to detect the existence of an epidemiological association ( $P \leq 0.05$ ). Occasionally Fisher's exact test was applied.

## Results

One hundred and twenty-six enterococci isolates were recovered, 64 from hunted mammals and 62 from rescued mammals. The enterococci were collected from 14 species as listed in Table 1. No enterococci were retrieved from Iberian ibex and weasel samples, while all hedgehog samples carried these bacteria.

The frequency of the isolates was similar among orders. The Lagomorpha and Carnivora provided 34 enterococci isolates each (26.98% of the total enterococci retrieved), Artiodactyla yielded 32 (25.40%), and Erinaceomorpha gave 24 (19.05%). The single representative of the Chiroptera carried two enterococci (1.59%). Considering the main diet, herbivores provided 50 isolates, (39.68% of the total isolates), insectivores 26 isolates (20.63%), omnivores 22 isolates (17.46%), carnivores 20 isolates (15.87%), and piscivores 8 isolates (6.35%) (Table 1).

Seven different *Enterococcus* spp. were identified in this study, *E. faecalis* predominating (37.60% of the total of enterococci identified) and *E. casseliflavus* (20.63%) and *E. faecium* (17.46%) constituting large proportions as shown in Table 3.

When comparing the prevalence of *E. faecalis* by factors, a higher frequency was observed in hunted mammals than in rescued ones, young than in adult mammals, females, herbivores, and those eating no carrion. The factor-predicated differences in the prevalence of *E. faecium* were that it was more frequent in rescued animals than hunted ones, adults than in young, males, carnivores, and carrion eaters. The

percentage of *E. faecalis* isolates was higher as host age decreased but the opposite was true of the percentage of *E. faecium* isolates and the difference between the prevalences of *E. faecalis* and *E. faecium* in the young age category was significant ( $P = 0.0009$ ) (Table 4). Ten *Enterococcus casseliflavus* were isolated from rescued mammals samples (38.46%; 10/26), and 16 from hunted ones (61.54%; 16/26).

**Table 1.** Species of wild mammals included in the study classified by origin, main diet and scavenging habit together with number of *Enterococcus* spp. isolated

Mammal order	Species	Scientific name	Origin	Main diet	Scavenging habit**	Individuals (n)	Isolates (n)
Artiodactyla	Iberian ibex	<i>Capra pyrenaica</i>	CWFR-LA	Herbivorous	No	1	0
	Mouflon	<i>Ovis orientalis</i>	Hunting	Herbivorous	No	4	4
	Red deer	<i>Cervus elaphus</i>	Hunting	Herbivorous	No	9	10
	Roe deer	<i>Capreolus capreolus</i>	CWFR-LA	Herbivorous	No	1	2
	Wild boar	<i>Sus scrofa</i>	Hunting	Omnivorous	No	17	16
Total						32	32
Carnivora	American mink	<i>Neovison vison</i>	CWFR-LA	Carnivorous	Yes	6	11
	Badger	<i>Meles meles</i>	CWFR-LA	Omnivorous	Yes	3	6
	Beech marten	<i>Martes foina</i>	CWFR-LA	Carnivorous	Yes	2	4
	Common genet	<i>Genetta genetta</i>	CWFR-LA	Carnivorous	Yes	1	2
	Common otter*	<i>Lutra lutra</i>	CWFR-LA	Piscivorous	Yes	3	8
	Red fox	<i>Vulpes vulpes</i>	CWFR-LA	Carnivorous	Yes	3	3
	Weasel	<i>Mustela nivalis</i>	CWFR-LA	Carnivorous	Yes	1	0
Total						19	34
Chiroptera	European free-tailed bat	<i>Tadarida teniotis</i>	CWFR-LA	Insectivorous	No	1	2
Total						1	2
Erinaceomorpha	Hedgehog	<i>Erinaceus europaeus</i>	CWFR-LA	Insectivorous	No	11	24
Total						11	24
Lagomorpha	Wild rabbit	<i>Oryctolagus cuniculus</i>	Hunting	Herbivorous	No	38	33
	Granada hare	<i>Lepus granatensis</i>	Hunting	Herbivorous	No	2	1
Total						40	34
TOTAL	16					103	126

\* – one enterococcus isolated from a common otter was missing after identification; \*\* – occasional carrion eaters were excluded; CWFR – Centre of Wild Fauna Recovery of La Alfranca (Aragón, Spain)

**Table 2.** Primers and conditions for detecting *vanA* and *vanB* genes by PCR

Primers (5'→3')	Amplification	Reference (length of the amplicon)
<i>vanA</i> F: ATGGCAAGTCAGGTGAAGATGG R: TCCACCTCGCCAACAACCTAACG	96°C2 min, 1 cycle	Woodford <i>et al.</i> (32) (399 bp)
	94°C30 s	
	50°C30 s, 35 cycles	
	72°C1 min	
<i>vanB</i> F: CAAAGCTCCGCAGCTTGCATG R: TGCATCCAAGCACCCGATATAC	72°C10 min, 1 cycle	Dahl <i>et al.</i> (10) (484 bp)
	94°C3 min, 1 cycle	
	94°C30 s	
	58°C2 min, 40 cycles	
	72°C2 min	
	72°C6 min, 1 cycle	

**Table 3.** Frequency of *Enterococcus* spp. isolated from wild mammals in this study and their resistance to quinupristin-dalfopristin

<i>Enterococcus</i> spp.	Isolates (n)	Isolates (%)	Resistance to QD n (%)	
<i>Enterococcus faecalis</i>	47	37.60	38 (80.85)	
<i>Enterococcus casseliflavus</i>	26	20.63	8 (30.77)	
<i>Enterococcus faecium</i>	22	17.46	12 (54.55)	
<i>Enterococcus hirae</i>	13 (-1)	9.60	9 (75.00)	
<i>Enterococcus gallinarum</i>	9	7.14	5 (55.56)	
<i>Enterococcus mundtii</i>	8	6.35	6 (75.00)	
<i>Enterococcus avium</i>	1	0.79	1 (100.00)	
TOTAL	125*	100	79 (63.20)	
Species Order	Isolates (n)	Isolates (%)	Resistance to QD n (%)	P value for associations
Artiodactyla	32	25.60	11 (34.38)	Lag. vs Art. F 0.0000
Carnivora	33	26.40	20 (60.61)	Carn. vs Art. 0.0195
Chiroptera	2	1.6	2 (100.00)	
Erinaceomorpha	24	19.2	16 (66.67)	Erin. vs Art. 0.0100
Lagomorpha	34	27.2	30 (88.24)	
Age				P value for associations
Adult	70	56.00	37 (52.86)	Young vs Adult 0.0052
Young	50	40.00	38 (76.00)	
Infant	5	4.00	4 (80.0)	Not applicable
TOTAL	125*	100	79 (63.20)	

Art. – Artiodactyla; Car. – Carnivora; Erin. – Erinaceomorpha; Lag. – Lagomorpha; QD – quinupristin-dalfopristin; \* – A total of 126 isolates were retrieved. However, one of the enterococci identified as *E. hirae* was lost and could not be analysed for antibiotic resistance. The isolate percentages are based on the total of 125

**Table 4.** Results of the statistical analysis of *E. faecalis* and *E. faecium* isolation related to source of sampling, mammal age and sex, main diet and scavenging habit in the studied mammals

Factor	Variable (n)	<i>E. faecalis</i> n (%)	<i>E. faecium</i> n (%)	P value*
Source of sampling	CWFR-LA (36)	19 (52.78)	17 (47.22)	0.0025
	Hunting (33)	28 (84.85)	5 (15.15)	
Age	Adult (32)	15 (46.88)	17 (53.13)	0.0009
	Young (32)	27 (84.38)	5 (15.63)	
	Infant (5)	5 (100.00)	0	Not applicable
Sex**	Female (29)	25 (86.21)	4 (13.79)	F 0.0078
	Male (39)	22 (56.41)	17 (43.59)	
Main diet	Carnivorous (15)	8 (53.33)	7 (46.67)	0.0152
	Herbivorous (33)	28 (84.85)	5 (15.15)	
Scavenging habit	No (48)	36 (75.00)	12 (25.00)	0.0383
	Yes (21)	11 (52.38)	10 (47.62)	

CWFR – Centre of Wild Fauna Recovery of La Alfranca (Spain); F – Fisher's exact test; \* –  $\chi^2$  or Fisher's test where appropriate; statistical significance at  $P < 0.05$ ; \*\* – one animal did not have its sex determined

The highest frequency of resistance to QD was identified for *E. faecalis* (Table 3), and the difference to that of *E. faecium* was significant ( $P = 0.0153$ ). The Lagomorpha order species were the main carriers of bacteria with resistance to QD (88.24%; 30/34)

followed by the Erinaceomorpha (66.67%; 16/24) and Carnivora (60.61%; 20/33) species, and the lowest carriage was detected in Artiodactyla (34.38%; 11/32). Young mammals carried a higher percentage of resistant isolates (76.00; 38/50) than adults (52.86;

37/70) and four of the five isolates from infant mammals were resistant to QD. No influence of sex or scavenging habit on hosting QD-resistant *Enterococcus* spp. was observed.

According to the DD test, the percentage of VAN-resistant isolates was high (66%). A total of 52 enterococci were randomly selected and re-evaluated with the M.I.C. Evaluator test to corroborate this finding, including *E. faecalis* (n = 38), *E. faecium* (n = 4), *Enterococcus hirae* (n = 4), *E. casseliflavus* (n = 3), *Enterococcus gallinarum* (n = 2), and *Enterococcus mundtii* (n = 2). When comparing the DD and MIC tests, no correlation was identified (P = 0.009). Six enterococci isolates corresponding to *E. casseliflavus*, *E. gallinarum* and *E. faecalis* showed a VAN MIC of 8 µg/mL, indicative of intermediate susceptibility, but none of the selected enterococci carried the *vanA* or *vanB* genes.

As seen in Table 5, the highest frequency of enterococci resistance to antibiotics was for QD (63.20%) and the lowest for AMP (7.20%), with frequencies of some concern also emerging for CIP, TE, ERI and S.

Regarding the mammal species (Table 5), the lowest frequency of bacteria resistant to CIP was found in wild boar (18.75%; 3/16); however, the scarcity of resistant isolates from these mammals detracts from the reliability of the results. Wild rabbits carried enterococci with the highest percentage of resistance to CIP (66.67%; 22/33), and the difference to

the percentage with resistance among isolates from hedgehogs (37.50%; 9/24) was significant (P = 0.0172). As regards resistance to TE, its frequency in wild rabbit isolates (12.12%; 4/33) was significantly lower than that in hedgehog (41.67%; 10/24), and red and roe deer isolates (41.67%; 5/12). The number of *Enterococcus* spp. showing resistance to any of the tested antibiotics in beech martens, common otters, badgers, American mink, and the single European free-tailed bat was too low for any statistical analysis to be performed.

Regarding the source of samples (Table 6), it was observed that the rescued mammals carried enterococci with higher levels of resistance, except to AMP, CL, CIP and GEN, for which the results were not significant. The highest frequencies of resistant isolates in rescued mammals were observed for TE (55.74%), ERI (34.43%) and S (29.51%).

There was an association between order and resistance to CIP (P = 0.0002) and TE (P = 0.0000), *Enterococcus* spp. from samples from the Carnivora order showing the highest frequency of resistance (72.73% to CIP and 66.67% to TE) (Table 6). The enterococci isolated from mammals belonging to the Lagomorpha also presented a high percentage of resistance to CIP (64.71%). In the case of resistance to TE, the lowest frequency of resistant isolates came from samples from the Lagomorpha (11.76%) and Artiodactyla (15.63%).

**Table 5.** Frequency of *Enterococcus* spp. isolates resistant to the studied antibiotics by mammal species

Species	<i>Enterococcus</i> spp. isolates (n)	<i>Enterococcus</i> spp. (%)	Antibiotic tested							
			AMP	CL	CIP	ERI	GEN	QD	S	TE
American mink	11	8.80	3		9	4	2	6	3	8
Badger	6	4.80	1		3	2	1	4	3	4
Beech marten	4	3.20	2	2	3	2		1	2	3
Common genet	2	1.60			2			2		2
Common otter	7	5.60		2	5	4		5	3	3
European free-tailed bat	2	1.60			1	1		2		
Granada hare	1	0.80						1		
Hedgehog	24	19.20		2	9	6	2	16	4	10
Mouflon	4	3.20	1	1					1	
Red deer	10	8.00		1	4	4		2		3
Roe deer	2	1.60	1	1	2	1		2	2	2
Red fox	3	2.40		1	2	1	1	2	1	2
Wild boar	16	12.80			3	1		7		
Wild rabbit	33	26.40	1	2	22	6	5	29	6	4
TOTAL	N	125	9	12	65	32	11	79	25	41
	%	100	7.20	9.60	52.00	25.60	8.80	63.20	20.00	32.80

AMP – ampicillin; CL – chloramphenicol; CIP – ciprofloxacin; ERI – erythromycin; GEN – gentamicin; QD – quinupristin-dalfopristin; S – streptomycin; TE – tetracycline. No enterococci resistant to any of the studied antibiotics were recovered from the Iberian ibex or weasel

**Table 6.** Antibiotic resistance of *Enterococcus* spp. isolates in relation to sample source and order

Factor	Antibiotic	Factor category (n)	Antibiotic resistance n (%)	P value*
Source of samples	ERI	CWFR-LA (61)	21 (34.43)	0.0149
		Hunting (64)	11 (17.19)	
	S	CWFR-LA (61)	18 (29.51)	0.0024
		Hunting (64)	6 (9.38)	
	TE	CWFR-LA (61)	34 (55.74)	0.0000
		Hunting (64)	7 (10.94)	
Order	CIP	Artiodactyla (32)	9 (28.13)	0.0018
		Carnivora (33)	24 (72.73)	
		Erinaceomorpha (24)	9 (37.50)	
		Lagomorpha (34)	22 (64.71)	
		Chiroptera (2)	1 (50.00)	
	TE	Artiodactyla (32)	5 (15.63)	0.0000
		Carnivora (33)	22 (66.67)	
		Erinaceomorpha (24)	10 (41.67)	
		Lagomorpha (34)	4 (11.76)	
		Chiroptera (2)	0	

CIP – ciprofloxacin; ERI – erythromycin; S – streptomycin; TE – tetracycline; \* –  $\chi^2$ , statistical significance at  $P < 0.05$ . Only statistically significant associations are included

**Table 7.** Antibiotic resistance of *Enterococcus* spp. isolates in relation to sex, main diet and scavenging habit

Factor	Antibiotic	Factor category (n)	Antibiotic resistance n (%)	P value*
Sex	CIP	Female (48)	31 (64.58)	0.0096
		Male (75)	32 (42.67)	
	GEN	Female (48)	8 (16.67)	F 0.0200
		Male (75)	3 (4.00)	
Main diet	AMP	Carnivorous (20)	5 (25.00)	F 0.0376
		Herbivorous (50)	3 (6.00)	
	CIP	Carnivorous (20)	16 (80.00)	Carn. vs Omn: F 0.0008
		Herbivorous (50)	28 (56.00)	
		Insectivorous (26)	10 (38.46)	
		Omnivorous (22)	6 (27.27)	
		Piscivorous (7)	5 (71.43)	
	TE	Carnivorous (20)	15 (75.00)	Carn. vs Herb. 0.0000 Carn. vs Ins. 0.0084 Carn. vs Omn. F 0.0003 Ins. vs Herb. 0.0313
		Herbivorous (50)	9 (18.00)	
		Insectivorous (26)	10 (38.46)	
		Omnivorous (22)	4 (18.18)	
	Scavenging habit	AMP	No (92)	3 (3.26)
Yes (33)			6 (18.18)	
CL		No (92)	6 (6.52)	0.0368
		Yes (33)	6 (18.18)	
CIP		No (92)	41 (44.57)	0.0029
		Yes (33)	24 (72.73)	
ERI		No (92)	19 (20.65)	0.0215
		Yes (33)	13 (39.39)	
S		No (92)	12 (13.04)	0.0032
		Yes (33)	12 (36.36)	
TE		No (92)	19 (20.65)	0.0000
		Yes (33)	22 (66.67)	

AMP – ampicillin; CIP – ciprofloxacin; CL – chloramphenicol; ERI – erythromycin; GEN – gentamicin; S – streptomycin; TE – tetracycline; F – Fisher's exact test; Carn. – carnivorous; Herb. – herbivorous; Omn. – omnivorous; \* –  $\chi^2$  or Fisher's test where appropriate; statistical significance at  $P < 0.05$ . Only statistically significant associations are included

Female mammals (Table 7) showed the highest percentage of isolates resistant to CIP (64.58%; 31/48) ( $P = 0.0228$ ). Concerning the main diet, no statistical significance was identified for resistance to CL or GEN; none of the 22 isolates achieved from omnivorous mammals was resistant to CL. Overall, the carnivorous and piscivorous animals yielded the highest percentages of isolates resistant to most of the studied antibiotics and the herbivorous and omnivorous species isolates showed the lowest percentages of resistance. The frequency of resistant isolates to CIP obtained from the carnivores was significantly higher than that of the omnivores, and a similar disparity was observed for resistance to TE. In this case, there was also significance to the differences in frequency of resistance between enterococci isolated from herbivores (18.00%) and those isolated from insectivores (38.46%). A scavenging habit was associated with a higher percentage of resistant isolates to CIP (72.73%), TE (66.67%), ERI (39.39%), S (36.36%), CL (18.18%) and AMP (18.18%) compared to those eating no carrion ( $P \leq 0.005$ ).

In this study, a total of 27 isolates were classified as MDR (21.60%; 27/125). The higher percentage of MDR isolates was found in isolates from rescued mammals (32.26%; 20/62), and greatly exceeded the low proportion obtained from isolates from hunted mammals (11.11%; 7/63). The Carnivora order carried more MDR enterococci (39.39%; 13/33) than the Artiodactyla (6.25%; 2/32). Carrion eaters also gave a higher percentage of MDR isolates (39.39%; 13/33), than animals which did not scavenge for it (15.22%; 14/92). All these differences were statistically significant ( $P \leq 0.005$ ). Regarding the animal species, it was not possible to perform any statistical analysis because of the low number of isolates obtained from the majority of them. It is of note that none of the 16 isolates achieved from wild boar was MDR, while 1 out of the 2 isolates from the European free-tailed bat and 2 out of the 4 isolates from beech martens were.

## Discussion

Enterococci are found as part of the gastrointestinal microbiome in humans and animals (mammals, birds, fish, reptiles and insects) (25), in nosocomial infections (31), and in soil, plants, water and sewage (5). We identified enterococci from all samples from hedgehogs, which could be related to their predominantly insect diet (15), suggesting that the environment is involved. Wild animals should be studied as an important component of the environment in order to assess the expansion of AMR, since they are not directly treated with antibiotics. Research on wild fauna also gives a picture of the magnitude of this healthcare problem (9, 16, 24).

*Enterococcus faecalis* and *E. faecium* were two of the bacterial species most frequently isolated in this

study. The former commonly causes human infections, the latter shows a higher percentage of intrinsic resistance to antibiotics (20), and both are found in mammals. As also seen by other authors (6, 16), *E. faecalis* was the species more frequently isolated in our study. It was mainly recovered from hunted mammals (*E. faecium* being isolated more from rescued individuals, contrastingly), predominating in females and healthy young individuals, probably being part of the intestinal flora. The main diet of the animals may explain the frequency of isolation of *E. faecalis*, as it was mostly isolated from the herbivores. In contrast, *E. faecium* was most frequently carried by carnivores. Both enterococci were also commonly found in scavengers. Environmental contamination with antibiotics is likely to favour the location of *E. faecium* in the intestine of wild animals that are not directly exposed to these drugs (3, 14, 24). Interestingly, we found that omnivorous and adult mammals and wild boar carried a higher percentage of enterococci other than *E. faecalis* and *E. faecium*.

One of the main hindrances to treatment of enterococci infections is resistance to widely used antibiotics (17), and it is of note that resistant enterococci have the ability to easily exchange AMR genes with other enterococci and Gram-positive bacteria species (5). The highest level of resistance detected in this study was to QD, this level being higher than those found by other authors (29). Quinupristin-dalfopristin is a combination of two synthetic streptogramins developed to treat VRE and MDR *E. faecium* human infections (29). In animal production, virginiamycin, another streptogramin combination, was used as a growth promoter for 30 years, which led to resistance developing in *E. faecium* in chicken and pigs (1), but this practice was banned in Europe in 1999 (30). It is known that this resistance may be transferred from humans to animals and *vice versa*, and may reach wild animals through food and water (1, 26). The same transfer would seem to have occurred to endow *E. faecium* with the resistance identified in it to chloramphenicol. Although this frequency can be regarded as low, this antibiotic was banned in livestock production for more than 30 years, and restricted to human use. Therefore, near-zero levels of resistance were to be expected, but other authors also found CL-resistant *E. faecium* (24). Resistance to CL was associated with the scavenging habit, and slightly predominated in the area of the Pyrenees, which suggests its relationship with sheep or cattle herding or horse grazing. Remarkably, soil is considered the main source of resistant genes, including in areas where there are no human activities (2), and genes of chloramphenicol resistance may persist in it after use of the antibiotic has stopped (28).

Resistance to CIP was also high in isolated enterococci, showing a similar prevalence to that observed in isolates from domestic mammals (19, 24). The mammal species which provided the isolates

demonstrating the greatest CIP-resistance in this study were American mink, common otters and beech martens. A high prevalence of resistance to CIP was observed in carnivore, piscivore and female mammal isolates, suggesting the presence of interacting factors. Fluoroquinolones such as ciprofloxacin are frequently used together with  $\beta$ -lactams or vancomycin to treat human infections caused by *Enterococcus* spp. (4), which could ultimately favour the development of resistance to fluoroquinolones and other drugs.

Resistance to TE, ERI and S was also high in this study. Mammal feeding habits might contribute to this resistance and resistance to other antibiotics, implying a variety of sources for its acquisition and the importance of the agricultural environment (20, 24). The highest frequency of resistance to TE was detected in *E. faecium* isolates from carnivores (especially in badgers), isolates resistant to ERI and S were remarkably strongly associated with piscivores (common otters), and resistance to the three antibiotics was linked to the scavenging habit. Aminoglycosides (except for spectinomycin) have been classified into group C in animals; they only have to be used when the antibiotics of group D (prudent use) fail or are not available (26). The percentage of resistance to streptomycin detected in the studied mammals' isolates was higher than expected, but lower than that detected by Nowakiewicz *et al.* (24) in four carnivorous species in Poland. Our study found it distributed between carnivorous, piscivorous, herbivorous, and insectivorous mammals and predominating in geographical areas where human population, livestock production farms and rivers are abundant (7, 23, 27, 28).

In general, resistance to ampicillin is frequent in *Enterococcus* species. However, the resistance to AMP observed in this study was low and was usually in *E. faecium*. The scarce resistance in these findings contrasts with the more abundant resistance to this antibiotic found in human isolates. It is important to highlight that *E. faecalis* is usually susceptible to this  $\beta$ -lactam, and the isolates obtained from humans in hospitals were also found to be so, only 1.6% resisting AMP (14, 21). The high level of antibiotic resistance detected in the isolates of this bacterium in our study may explain the high level of MDR isolates – a level which, while lower than that observed in Lublin, Poland (24), is also higher than that reported by other authors in Tuscany (11). That is worrying, because wild mammals also contribute to maintaining and disseminating bacteria and mobile genetic elements in the environment (18, 24).

In this study, the DD test gave false positives for resistance to VAN and TEI, indicating its low reliability. As other authors found, the M.I.C. Evaluator test is the most suitable technique to detect resistance to vancomycin, but results need to be confirmed by molecular techniques (especially to identify *vanA* and

*vanB* genes, and particularly for *E. casseliflavus* and *E. gallinarum*, the *vanC* gene) (8).

The main limitation of this study is the number of species included in the final analysis: because samples needed to originate from wild mammals, this criterion imposed conditions on obtaining samples and made it difficult. Further studies concomitantly testing human, animal and environmental sources (rivers, waste water, soil and plants) are required in order to assess the extent of the dissemination of bacterial resistance and AMR determinants.

In conclusion, resistance to antibiotics with sanitary implications was detected in a high percentage of enterococci isolated from wild mammals in the Autonomous Community of Aragón, Spain. The results of this study suggest that animal medication, where administered in animal husbandry, agriculture and livestock production; human medication; and both, where residues of therapeutic antimicrobials may contaminate rivers, soil and vegetation, are pathways for resistance genes to reach bacteria in wild mammals. This implies that efforts to control AMR might tackle this problem perceiving it from a wider perspective, extending to particular study and monitoring of the environment in order to avoid the dissemination of AMR, as the global health concept proposes.

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