

# Geographical variation in cloacal microflora and bacterial antibiotic resistance in a threatened avian scavenger in relation to diet and livestock farming practices

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## Summary

The impact on wildlife health of the increase in the use of antimicrobial agents with the intensification of livestock production remains unknown. The composition, richness and prevalence of cloacal microflora as well as bacterial resistance to antibiotics in nestlings and full-grown Egyptian vultures *Neophron percnopterus* were assessed in four areas of Spain in which the degree of farming intensification differs. Differences in diet composition, especially the role of stabled livestock carrion, appear to govern the similarities of bacterial flora composition among continental populations, while the insular vulture population (Fuerteventura, Canary Islands) showed differences attributed to isolation. Evidence of a positive relationship between the consumption of stabled livestock carrion and bacterial resistance to multiple antibiotics was found. Bacterial resistance was high for semisynthetic penicillins and enrofloxacin, especially in the area with the most intensive stabled livestock production. The pattern of antibiotic resistance was similar for the different bacterial species within each area. Bacterial resistance to antibiotics may be determined by resistance of bacteria present in the

livestock meat remains that constituted the food of this species, as indicated by the fact that resistance to each antibiotic was correlated in *Escherichia coli* isolated from swine carrion and Egyptian vulture nestlings. In addition, resistance in normal faecal bacteria (present in the microflora of both livestock and vultures) was higher than in *Staphylococcus epidermidis*, a species indicator of the transient flora acquired presumably through the consumption of wild rabbits. Potential negative effects of the use of antimicrobials in livestock farming included the direct ingestion of these drug residues and the effects of bacterial antibiotic resistance on the health of scavengers.

## Introduction

Emerging infectious disease of wildlife has gained a growing interest due to the increasing impact of human intrusions in wildlife habitat on human disease emergence and resurgence (Hubálek, 1994; Daszak *et al.*, 2000; Friend *et al.*, 2001; Bengis *et al.*, 2004). An increased interface between humans, domestic animals and free-ranging wildlife is a global dimension of these intrusions. However, little is known about the dynamics of this interface as a factor for antibiotic resistance as a component of disease emergence. Intensification of livestock production and management systems has been accompanied by an increase in the use of antimicrobial agents and other drugs to mitigate increasing disease in conditions of high livestock production, including high stocking densities and rapid growth (McEwen and Fedorka-Cray, 2002). Antimicrobial drugs are also intensively used as growth promoters and for prophylaxis in the absence of disease (Angulo *et al.*, 2004; Mølbak, 2004). These practices are associated with increased levels of bacterial resistance (Mateu and Martin, 2001; O'Brien, 2002), a factor the World Health Organization considers to be one of the major health problems in human and veterinary medicine (Angulo *et al.*, 2004; Mølbak, 2004). Thus, it has been recommended that the use of antibiotics and other drugs as growth promoters be avoided, especially those agents also used in humans (WHO, 1999). Several European

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countries have banned or reduced use of these antimicrobials in poultry and swine farm operations. The feared loss of competitive productivity has not occurred (Greko, 1999; Sorensen *et al.*, 2002), but a reduction in resistant bacteria in animals, food products and humans has (Pantosti *et al.*, 1999; Aarestrup *et al.*, 2001). Potential human health consequences from the intensive use of antibiotics in livestock include increases in the resistance or cross-resistance to these drugs by pathogenic and commensal bacteria resulting in increased human morbidity and mortality (Swartz, 2002; Travers and Barza, 2002; Mølbak, 2004). Similar effects may be affecting wildlife, especially around farm facilities, abattoirs and dumps of livestock refuse, where livestock and their residues come into contact with wildlife.

The emergence of bacterial resistance in wildlife and the spread of these strains within ecosystems have received little attention despite the importance of animals as sources for disease emergence in humans (Woolhouse, 2002). Antibiotic-resistant microbes due to livestock intensification supported by the use of antimicrobial agents and other drugs may also be affecting wildlife health. Avian scavengers, such as vultures, are prominent among wildlife directly associated with livestock intensification. Vultures greatly rely on food found at 'muladares', places where livestock carrion is intentionally discarded for elimination by these scavenger species, especially in Asia and the Mediterranean region (Donázar, 1992; Donázar *et al.*, 1997). Muladares and other refuse dumps have come to play a keystone role in the conservation of threatened avian scavengers in the Mediterranean because of the decline of their main wild prey (Donázar *et al.*, 1997). Also, recent changes in farming systems and health regulations as a result of the bovine spongiform encephalopathy crisis have made the carrion of unstabled livestock scarce or inaccessible (Donázar *et al.*, 1997; Tella, 2001), thereby reducing the potential availability of this historic food source for threatened avian scavengers in the Mediterranean region.

Spain is a main stronghold for threatened avian scavengers in Europe (Donázar *et al.*, 1997). The recently scarcity of carrion from unstabled, freely grazing livestock (hereafter unstabled livestock) may be resulting in the concentration of these scavengers at muladares. Stabled livestock reared under intensive conditions (hereafter stabled livestock), including confined animal feeding operations where antimicrobials are intensively used, are the primary carrion, especially fattening swine and poultry (authors' unpubl. data) deposited in these muladares. Although muladares provide a superabundant and predictable food supply for avian scavengers, there are health aspects to be considered. Specifically, the use of antimicrobial agents and other drugs may have direct negative effects on the health and survival of scavengers

if ingested as toxic residues or have indirect negative effects by aiding in the development of microbes with increased capacity to cause disease in these scavengers. Withdrawal times for antimicrobials are intended to prevent harmful drug residuals in meat that humans consume (McEwen and Fedorka-Cray, 2002). However, these waiting periods do not apply to carcasses and other waste disposal in muladares. Indirect effects on vulture health and survival derived from consuming carrion of intensively medicated livestock may include the acquisition of antimicrobial-resistant bacteria (McEwen and Fedorka-Cray, 2002) and the alteration of the normal protective flora by acquisition of a transient flora including pathogenic bacteria (Winsor *et al.*, 1981; Rodrigues *et al.*, 2003; Blanco *et al.*, 2006). Because of the importance of muladares as feeding areas for avian scavengers in Spain and the general lack of knowledge of health risks this may pose for these species, we investigated composition, richness and prevalence of cloacal microflora of nestlings and full-grown Egyptian vultures *Neophron percnopterus* in four areas in Spain in which farming practices differ, especially in the existence of stabled intensively medicated livestock versus unstabled livestock. Our focus was to evaluate whether or not different degrees of intensification in livestock management influence the availability and use of different food resources by avian scavengers and how resulting dietary differences affect cloacal microflora composition. Specifically, we assessed whether different degrees of farming intensification influenced the prevalence of bacterial resistance to 12 selected antibiotics commonly used in livestock farming and human medicine. Also, we tested the hypothesis that bacterial resistance within the microflora of the Egyptian vulture intestine may be acquired from resistant bacteria already present in carrion from treated stabled livestock used as a food source. We evaluated these hypotheses by: (i) assessing the relationships between the consumption of stabled livestock carrion and the degree of *in vitro* bacterial antibiotic resistance in each area; (ii) comparing antibiotic resistance in bacteria isolated from swine carrion and nestling Egyptian vultures; and (iii) comparing antibiotic resistance in normal faecal bacteria and that in *Staphylococcus epidermidis* isolated from vultures. This particular bacterial species was selected as a representative indicator of the transient flora acquired through the consumption of wild prey, specifically from the skin and intestinal flora of wild rabbits (Langan and O'Rourke, 2000). Rabbits are the main wild prey of Egyptian vultures. Resistance to antibiotics in this species presumably should be low (Livermore *et al.*, 2001). We predicted a positive relationship between the consumption of carrion from stabled livestock reared under intensive conditions, including the use of antibiotics, and the degree of bacterial resistance to antibiotics in each study area. We also predicted that if antibiotic

**Table 1.** Number of samples and prevalence (% of samples) of bacterial species isolated from the cloaca of Egyptian vultures in four Spanish areas.

	Ebro Valley		Segovia	Cádiz	Fuerteventura	
	Nestlings <i>n</i> = 11	Full-grown <i>n</i> = 9	Nestlings <i>n</i> = 8	Nestlings <i>n</i> = 12	Nestlings <i>n</i> = 17	Full-grown <i>n</i> = 11
Gram-negative bacteria						
<i>Salmonella</i> sp.	1 (9.1)	0 (0.0)	2 (25.0)	1 (8.3)	5 (29.4)	5 (45.5)
<i>Campylobacter jejuni</i>	1 (9.1)	1 (11.1)	0 (0.0)	1 (8.3)	0 (0.0)	5 (45.5)
<i>Clostridium</i> sp.	2 (18.2)	2 (22.2)	1 (12.5)	3 (25.0)	11 (64.7)	7 (63.6)
<i>Citrobacter</i> sp.	2 (18.2)	0 (0.0)	0 (0.0)	1 (8.3)	3 (17.6)	4 (36.4)
<i>Citrobacter freundii</i>	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	2 (11.8)	0 (0.0)
<i>Enterobacter cloacae</i>	3 (27.3)	2 (22.2)	4 (50.0)	2 (16.7)	3 (17.6)	5 (45.5)
<i>Enterobacter</i> sp.	3 (27.3)	5 (55.6)	2 (25.0)	1 (8.3)	0 (0.0)	0 (0.0)
<i>Escherichia coli</i>	9 (81.8)	8 (88.9)	5 (62.5)	8 (66.7)	12 (70.6)	10 (90.9)
<i>Hafnia alvei</i>	3 (27.3)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
<i>Klebsiella oxytoca</i>	1 (9.1)	1 (11.1)	2 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>Klebsiella</i> sp.	1 (9.1)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
<i>Morganella morganii</i>	0 (0.0)	0 (0.0)	1 (12.5)	1 (8.3)	0 (0.0)	0 (0.0)
<i>Proteus vulgaris</i>	8 (72.7)	6 (66.7)	3 (37.5)	6 (50.0)	0 (0.0)	0 (0.0)
<i>Proteus mirabilis</i>	3 (27.3)	1 (11.1)	2 (25.0)	1 (8.3)	0 (0.0)	0 (0.0)
<i>Proteus</i> sp.	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	12 (70.6)	5 (45.5)
<i>Providencia rettgeri</i>	2 (18.2)	1 (11.1)	1 (12.5)	1 (8.3)	3 (17.6)	0 (0.0)
<i>Providencia</i> sp.	1 (9.1)	1 (11.1)	0 (0.0)	1 (8.3)	0 (0.0)	5 (45.5)
<i>Pseudomonas</i> sp.	2 (18.2)	0 (0.0)	2 (25.0)	3 (25.0)	0 (0.0)	0 (0.0)
<i>Serratia marcencens</i>	2 (18.2)	2 (22.2)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)
<i>Serratia</i> sp.	1 (9.1)	1 (11.1)	0 (0.0)	0 (0.0)	4 (23.5)	7 (63.6)
<i>Yersinia</i> sp.	1 (9.1)	1 (11.1)	0 (0.0)	2 (16.7)	4 (23.5)	5 (45.5)
Gram-positive bacteria						
<i>Corynebacterium</i> sp.	3 (27.3)	2 (22.2)	4 (50.0)	2 (16.7)	3 (17.6)	3 (27.3)
<i>Enterococcus faecalis</i>	4 (36.4)	3 (33.3)	3 (37.5)	4 (33.3)	6 (35.3)	7 (63.6)
<i>Enterococcus faecium</i>	4 (36.4)	1 (11.1)	3 (37.5)	3 (25.0)	0 (0.0)	0 (0.0)
<i>Enterococcus durans</i>	2 (18.2)	0 (0.0)	1 (12.5)	1 (8.3)	0 (0.0)	0 (0.0)
<i>Staphylococcus epidermidis</i>	3 (27.3)	2 (22.2)	1 (12.5)	2 (16.7)	7 (23.5)	8 (72.7)
<i>Staphylococcus aureus</i>	0 (0.0)	1 (11.1)	0 (0.0)	1 (8.3)	1 (5.9)	3 (27.3)
<i>Staphylococcus simulans</i>	2 (18.2)	1 (11.1)	0 (0.0)	2 (16.7)	3 (17.6)	0 (0.0)

resistance is acquired by directly ingesting resistant bacteria through feeding on medicated livestock, we should find similar bacterial resistance in livestock carrion and vultures, and a lower susceptibility to antibiotics in bacterial species typical of the normal microflora than in *S. epidermidis*.

## Results

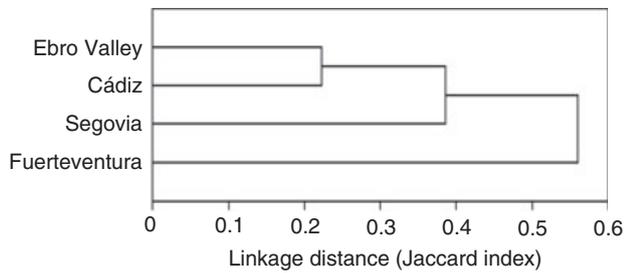
### Microflora composition

Cloacal microflora of Egyptian vultures were composed of cosmopolitan intestinal bacteria, bacteria commonly present in their food, and bacteria associated with sewage and sludge commonly occurring in sites for disposal of livestock carcasses and municipal solid waste (Table 1). Nestlings from the three areas in the Iberian Peninsula shared about half (48.1%) of the isolated species (13 of 27) (Table 1). Microflora composition of nestlings from Fuerteventura (Canary Islands) somewhat differed from that of nestlings from other areas, as indicated by the fact that only seven of 28 (25.0%) bacterial species found there were also found in the three areas of the Iberian Peninsula

(Table 1). Microflora composition of full-grown and nestling birds was similar in both Ebro Valley and Fuerteventura populations. They shared 18 of 25 (72.0%) and 12 of 17 (70.6%) bacterial species respectively (Table 1). Full-grown individuals from both areas shared half (50.0%) of the bacterial species (11 of 22). According to the cluster analysis for present versus absent species (Jaccard's similarity index), a higher similarity in microflora composition existed between Ebro Valley and Cádiz (dissimilarity value = 0.223) while flora from Fuerteventura showed the lowest similarity with the other areas (Fig. 1).

### Geographical variations in bacterial prevalence and richness

Bacterial prevalence was similar among the Iberian populations and differed from that of Fuerteventura, as indicated by the fact that prevalence was intercorrelated significantly in nestlings from the three Iberian areas (Spearman rank correlation, all  $P < 0.001$ ,  $n = 27$ ). Prevalence was also significantly correlated between full-grown and nestling birds from the Ebro Valley ( $r_s = 0.562$ ,  $P < 0.003$ ,  $n = 25$ ) and Fuerteventura ( $r_s = 0.599$ ,



**Fig. 1.** Dendrograms of dissimilarity (Jaccard coefficients) between study areas in Spain based on cluster analysis of presence–absence of bacterial species in Egyptian vultures, using the UPGMA algorithm.

$P = 0.011$ ,  $n = 17$ ), and between nestlings from Segovia and Cadiz and full-grown birds from Ebro Valley (Spearman rank correlation, both  $P < 0.029$ ,  $n = 27$ ). Bacterial prevalence from Fuerteventura was, however, not significantly correlated with that of the mainland Iberian areas, both for full-grown and nestling birds (Spearman rank correlation, all  $P > 0.097$ ,  $n = 28$ ). No significant differences in prevalence of any bacterial species were found between nestlings and full-grown individuals in Ebro Valley and Fuerteventura, nor between nestlings in each area after applying the Bonferroni correction ( $G$ - and Fisher's exact tests, Table 1).

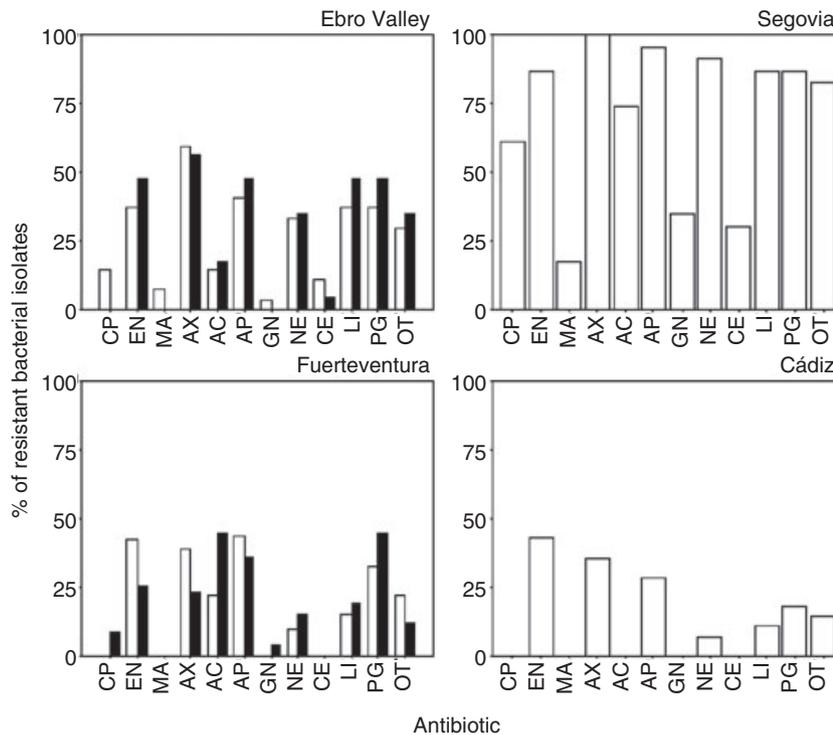
Overall, 18, 13, 16 and 10 bacterial species or genera of unidentified species were isolated in cloacal samples of nestlings from Ebro Valley, Segovia, Cádiz and Fuerteventura respectively (Table 1). In full-grown individuals, 19 and 14 bacterial species or genera were isolated from Ebro

Valley and Fuerteventura. The number of bacterial species isolated per sample did not differ between areas for nestlings (Kruskal–Wallis test,  $H = 5.590$ , d.f. = 3,  $P = 0.133$ , pooled mean =  $4.71 \pm 1.77$ ,  $n = 48$ , range = 1–8). Bacterial richness per sample did not differ between full-grown birds ( $4.67 \pm 1.58$ ,  $n = 9$ ) and nestlings ( $5.73 \pm 1.73$ ,  $n = 11$ ) from Ebro Valley (Mann–Whitney  $U$ -test,  $z = 1.48$ ,  $P = 0.139$ ), but it was higher in full-grown birds ( $7.18 \pm 2.56$ ,  $n = 11$ ) than in nestlings ( $4.47 \pm 0.70$ ,  $n = 17$ ) from Fuerteventura ( $z = 2.600$ ,  $P = 0.009$ ). Bacterial richness per sample was also higher in full-grown individuals from Fuerteventura than from Ebro Valley ( $z = 2.201$ ,  $P = 0.028$ ).

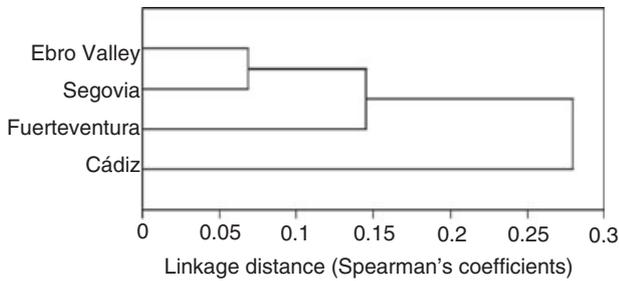
*Salmonella* sp. was isolated together with other bacterial species in nestlings from all areas, and in full-grown birds from Fuerteventura (Table 1). Serotype *enterica* was found in Ebro Valley ( $n = 1$ ) and Cádiz ( $n = 1$ ), while *typhimurium* ( $n = 1$ ) and *gallinarum* ( $n = 1$ ) were found in Segovia. Serotype *enteritidis* ( $n = 4$ ), *typhimurium* ( $n = 3$ ), *gallinarum* ( $n = 1$ ), as well as an unidentified serotype ( $n = 1$ ) were isolated from Fuerteventura.

#### Geographical variations in antibiotic susceptibility

We found bacterial resistance to all tested antibiotics in Segovia and Ebro Valley, while bacterial isolates did not present resistance to several antibiotics in Fuerteventura and especially in Cádiz (Fig. 2). Overall, the pattern of resistance to the tested antibiotics showed a similar profile between areas (Fig. 2). Thus, the highest bacte-



**Fig. 2.** Profile of bacterial resistance to 12 antibiotics in nestling (white bars) and full-grown (black bars) Egyptian vultures in four Spanish areas. Antibiotics: ciprofloxacin (CP), gentamicin (GN), enrofloxacin (EN), marbofloxacin (MA), amoxicillin (AX), amoxicillin-clavulanic (AC), ampicillin (AP), cephalixin (CE), lincomycin (LI), neomycin (NE), oxitetracycline (OT), penicillin G procaine (PG).



**Fig. 3.** Dendrograms of dissimilarity (Spearman coefficients) between study areas in Spain based on cluster analysis of the percentage of isolates resistant to each antibiotic in Egyptian vultures, using the UPGMA algorithm.

rial resistance was found for  $\beta$ -lactams (amoxicillin, ampicillin) and enrofloxacin in all areas. Lincomycin, neomycin, oxitetracycline and penicillin G procaine showed variable resistance ranging from about 75–80% of resistant isolates in Segovia to about 10–20% in Cádiz (Fig. 2). Nestlings and full-grown individuals did not differ in the percentage of resistant bacterial isolates to each antibiotic, both in Ebro Valley and Fuerteventura (Wilcoxon matched-pair test, both  $P > 0.637$ ). Regarding between-area differences for nestlings, we found the highest values of bacterial resistance to each antibiotic in Segovia and the lowest values in Cádiz (Fig. 2); the proportion of resistant isolates to each antibiotic was significantly higher in Segovia than in the other areas (Wilcoxon matched-pair test, all  $P = 0.002$ ). It was also higher in Ebro Valley than in Cádiz and Fuerteventura (both  $P < 0.028$ ), and higher in Fuerteventura than in Cádiz ( $P = 0.017$ ). Full-grown individuals did not differ in the proportion of bacterial resistance to antibiotics between Ebro Valley and Fuerteventura (Wilcoxon matched-pair test,  $P = 0.110$ ).

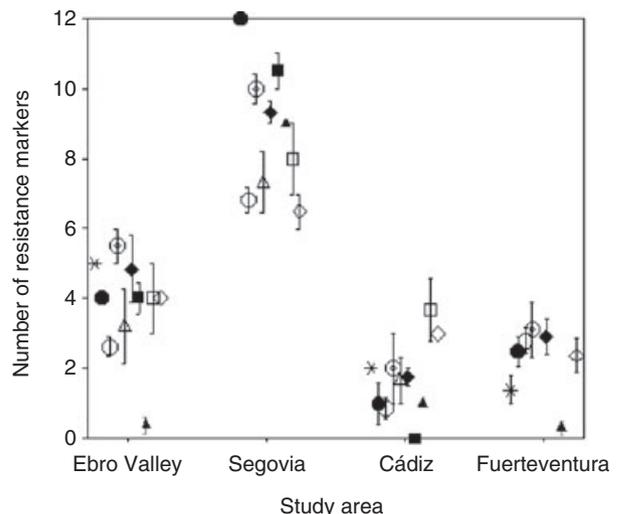
The proportion of resistant isolates (pooling all bacterial isolates) to each antibiotic were intercorrelated, both in nestlings from the four areas and in full-grown individuals from Ebro Valley and Fuerteventura (Spearman rank correlation, all  $P < 0.029$ ,  $n = 12$ ). According to the cluster analysis for quantitative similarities between areas in antibiotic susceptibility (Spearman dissimilarity values), a higher affinity existed between Ebro Valley and Segovia (dissimilarity value = 0.069, Fig. 3). Fuerteventura showed intermediate values and Cádiz showed the lowest similarity with the other areas (Fig. 3).

#### Multiple resistance according to area and bacterial species

Bacterial strains isolated from nestlings and full-grown individuals did not differ in the number of antibiotics to which they were resistant (pooling all bacterial species), both in Ebro Valley (Mann–Whitney  $U$ -test,  $z = 0.513$ ,

$P = 0.608$ ,  $n = 27, 23$ ) and in Fuerteventura ( $z = 0.381$ ,  $P = 0.703$ ,  $n = 40, 47$ ). These differences remained non-significant for particular bacterial species with a sufficient sample size in each area (all  $P > 0.370$ ,  $n = 6$  tests for particular bacterial species). On this basis, we pooled data on multiple resistance from nestlings and full-grown individuals for subsequent analyses.

The resistance pattern was similar for the different bacterial species within each area (Kruskal–Wallis test,  $P > 0.05$  in all cases, Fig. 4), but it clearly differed between areas, with the highest multiple resistance in Segovia and the highest susceptibility to different antibiotics in Cádiz (Fig. 4). Bacterial species or genera with sufficient sample sizes differed between areas in the number of antibiotics to which they were resistant (Kruskal–Wallis test,  $H = 21.97$ , d.f. = 3,  $P < 0.0001$ ,  $n = 51$ ;  $H = 11.64$ , d.f. = 3,  $P = 0.009$ ,  $n = 27$ ;  $H = 16.55$ , d.f. = 3,  $P = 0.001$ ,  $n = 26$  for *Enterococcus faecium*, *Escherichia coli* and *Enterobacter*, including *Enterobacter cloacae* and *Enterobacter* sp., respectively, Fig. 4). These differences were due to the higher number of multiresistant strains in Segovia ( $8.47 \pm 0.38$ , range = 6–12) than in the remaining areas (Mann–Whitney test, all  $P < 0.026$ ,  $n = 9$  comparisons involving the species with a sufficient sample size). These between-area differences remained significant when all isolates from all bacterial species were pooled (Kruskal–Wallis test,  $H = 72.05$ , d.f. = 3,  $P < 0.0001$ ,  $n = 188$ ). The number of resistance markers was also higher in Ebro Valley ( $3.32 \pm 0.27$ , range = 0–9) than in Cádiz ( $1.57 \pm 0.23$ , range = 0–5) and Fuerteven-



**Fig. 4.** Mean SE number of antibiotics to which each bacterial species is resistant. Each symbol represents a bacterial species: *Salmonella* sp. (white diamond), *C. jejuni* (asterisk), *E. cloacae* (filled circle), *Enterobacter* sp. (black square), *Clostridium* sp. (black circle), *Pseudomonas* sp. (white square), *E. coli* (white circle), *E. faecalis* (black diamond), *E. faecium* (white triangle), *S. epidermidis* (black triangle).

**Table 2.** Percentage of each type of prey among food remains found in Egyptian vulture nests from Spain.

Prey type	Study area			
	Ebro Valley (n = 2202)	Segovia (n = 211)	Cádiz (n = 667)	Fuerteventura (n = 632)
Wild prey	49.2	27.0	84.0	64.9
Unstabled livestock carrion	25.0	20.9	9.4	27.1
Stabled livestock carrion (total)	25.8	52.1	6.6	8.0
Swine	17.2	43.6	3.0	1.7
Poultry	8.3	6.1	3.6	6.3
Domestic rabbit	0.3	2.4	0.0	0.0

tura ( $2.32 \pm 0.19$ , range = 0–7) (Mann–Whitney test, both  $P < 0.003$ ), but it did not differ between Cádiz and Fuerteventura ( $z = 1.815$ ,  $P = 0.070$ ).

In both areas where sufficient sample size allowed statistical comparisons, isolates of *S. epidermidis* showed resistance to fewer different antibiotics ( $0.40 \pm 0.20$ ,  $n = 5$ , range = 0–1 and  $0.33 \pm 0.19$ ,  $n = 12$ , range = 0–2 in Ebro Valley and Fuerteventura respectively) than other tested bacteria (pooled mean:  $3.64 \pm 0.26$ ,  $n = 45$ , range = 1–9 and  $2.64 \pm 0.20$ ,  $n = 75$ , range = 0–7 in Ebro Valley and Fuerteventura, Mann–Whitney test, both  $P < 0.0001$  respectively) (Fig. 4).

#### Diet and antibiotic resistance

The proportion of stabled livestock carrion in the diet of Egyptian vultures was greatest in Segovia and lowest in Cádiz, while the proportion of unstabled livestock carrion was lowest in Cádiz and similar in the remaining areas (Table 2). Pig remains dominated stabled livestock carrion, both in Segovia and Ebro Valley, but not in Fuerteventura where poultry remains were more frequent than pig carrion. Both pig and poultry remains found in Cádiz were likely acquired from feeding on carrion from familiar non-intensive farm operations but were included as stabled livestock in our evaluations in order to be conservative in the analyses. Remains from domestic rabbits were only found in low numbers in Segovia and Ebro Valley (Table 2). Thus, stabled livestock carrion increased in the diet of Egyptian vultures from Cádiz to Fuerteventura to Ebro Valley and Segovia. Considering this transition, a significant positive relationship was found between the number of antibiotics to which each bacterial isolate (pooling all bacterial species) was resistant and the consumption of intensive livestock carrion (Kendall tau = 0.466,  $P < 0.001$ ,  $n = 188$ , Fig. 5).

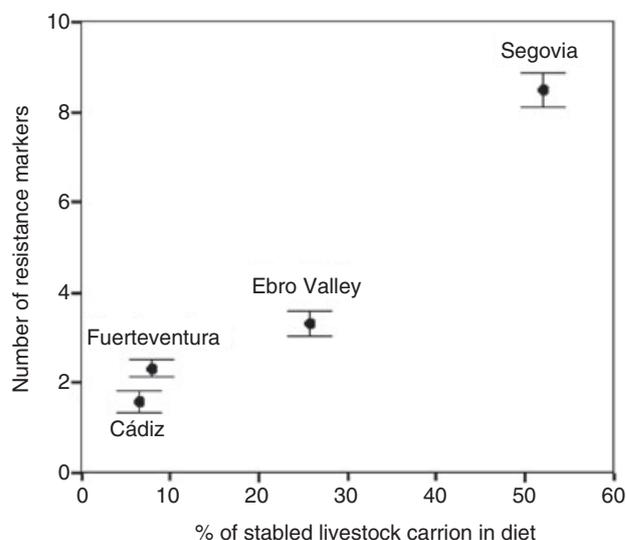
#### Antibiotic resistance in swine carrion

The total proportion of antibiotic-resistant isolates from swine carrion varied between 25.0% and 95.83% for gentamicin and amoxicillin in *E. coli* ( $n = 24$ ), and between 31.3% and 100% for gentamicin and ampicillin in

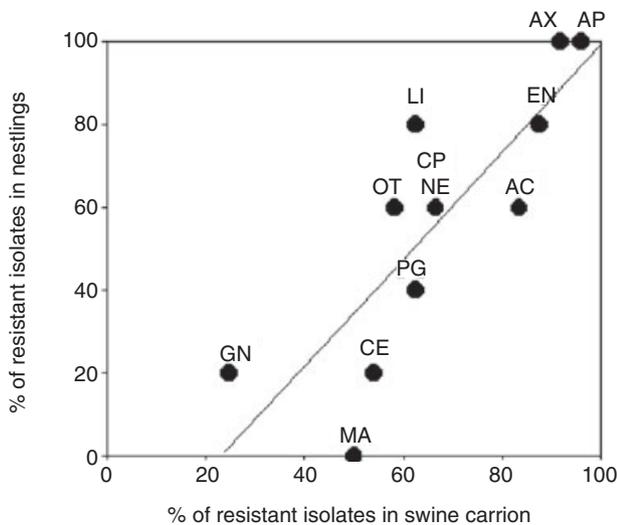
*Streptococcus suis* ( $n = 16$ ). These values were similar between the two tested bacteria as indicated by the highly significant correlation between the average proportion of resistant isolates to each antibiotic (Spearman rank correlation index,  $r_s = 0.910$ ,  $P < 0.0001$ ,  $n = 12$  antibiotics). In addition, the relationship between the proportion of resistant isolates to each antibiotic in *E. coli* from swine carrion and Egyptian vulture nestlings was highly significant ( $r_s = 0.867$ ,  $P < 0.0001$ ,  $n = 12$ , Fig. 6).

#### Discussion

Differences in diet composition, especially of stabled livestock carrion, seemed to influence the affinities of bacterial flora composition among continental populations. Microflora composition from continental populations of Egyptian vultures was clearly different from the Fuerteventura island population. The higher number of unidentified bacterial species found in the Fuerteventura population may include more undescribed taxa. There was a greater affinity in flora



**Fig. 5.** Relationship between the mean ( $\pm$ SE) number of antibiotics to which each bacterial isolate (pooling all bacterial species) was resistant and the consumption of stabled livestock carrion in each study area.



**Fig. 6.** Relationship between resistance to each antibiotic in *E. coli* from swine carrion and nestling Egyptian vultures. Least square regression line is shown for representation of tendency.

composition between Cádiz and Ebro Valley where Egyptian vultures consumed a high proportion of wild prey and a low to intermediate proportion of livestock. The lower affinity of microflora composition between Segovia and the remaining continental areas suggests that a diet based on stabled livestock carrion may somewhat influence flora composition of Egyptian vultures. However, these affinities were not clearly associated with differences in richness of bacterial species, number of bacterial species per sample or bacterial species prevalence between Segovia and the other continental areas. This suggests that a high proportion of stabled livestock carrion in the diet does not have clear implications in terms of flora richness or prevalence of bacterial species, although the small sample size in Segovia may have precluded detection of some species present in the population. Potential alteration of the normal flora as a consequence of feeding on livestock carrion may include interactions between the native host flora and transient flora acquired as a consequence of particular diets (Bangert *et al.*, 1988; Blanco *et al.*, 2006). Nevertheless, Egyptian vultures consumed a proportion of livestock carrion in all the study areas that may be sufficient to acquire most bacteria associated with their food and places where that food was obtained. If so, opportunistic pathogenic and commensal bacteria competing with native and protective normal microflora (Schneitz, 2005) may form part of the normal flora of Egyptian vultures and other avian scavengers (Winsor *et al.*, 1981; Bangert *et al.*, 1988; Rodrigues *et al.*, 2003; Blanco *et al.*, 2006).

Insufficient information about the cloacal microflora of avian scavengers exists to assess whether the prevalence of pathogenic bacteria found in this study may be influencing vulture population health and the conservation

status of this endangered species. However, potentially pathogenic bacteria, including *Salmonella* sp., *Campylobacter jejuni* and *Clostridium* sp. showed a relatively high prevalence when compared with other wild non-passerines (Bangert *et al.*, 1988; Fallacara *et al.*, 2001; Waldenström *et al.*, 2002; Reche *et al.*, 2003; Refsum *et al.*, 2003). Further investigation is warranted because these bacterial species have potential negative effects for wildlife and human health (Friend and Franson, 1999). The prevalence of *Salmonella* sp. was greatest in the area with the highest consumption of stabled livestock (see also Blanco *et al.*, 2006) and in the more isolated and endangered insular population respectively.

Antibiotic resistance among the bacteria isolated is another important consideration associated with the biological significance of the intestinal microflora of Egyptian vultures. Intensive agriculture systems often include the heavy use of multiple antimicrobial agents and other drugs. This approach may increase bacterial resistance to those agents in livestock (McEwen and Fedorka-Cray, 2002; Swartz, 2002) and in scavengers consuming their carrion, although the latter has not been previously documented. Our findings document bacterial resistance to multiple antibiotics in Egyptian vultures, both in nestlings and full-grown individuals, consuming stabled livestock carrion. The prevalence profile of antibiotic resistance showed a higher affinity between Ebro Valley and Segovia, the areas in which Egyptian vultures consumed the highest proportion of carrion from stabled livestock, especially fattening pigs and poultry. Fuerteventura showed intermediate values and Cádiz showed the lowest affinity, probably because of the high consumption of wild prey instead of livestock carrion by those vultures. The antibiotic resistance rate among bacterial isolates found in this study was very high compared with the available data from wild birds (Tsubokura *et al.*, 1995; DeBoer *et al.*, 2001; Livermore *et al.*, 2001; Nascimento *et al.*, 2003; Dobbin *et al.*, 2005), especially in Segovia where the proportion of stabled livestock in the diet was highest. High consumption by vultures of stabled livestock carrion was associated with increasing antimicrobial resistance in animal pathogens (e.g. *Clostridium*), human pathogens with animal reservoirs (*Salmonella* sp., *C. jejuni*) and commensal bacteria (e.g. *E. coli*, *Enterococcus faecalis*, *E. faecium*) present in Egyptian vulture microflora. This generalized resistance suggests that the selective pressure exerted by the use of antimicrobial agents was high, although variable depending on the degree of farming intensification in each study area. The isolation of bacterial strains multiresistant to 5–12 antibiotics (all tested antibiotics) in Segovia suggests the intensive use of these antimicrobial agents in food animals in this area.

Bacterial resistance was high for semisynthetic penicillins and enrofloxacin in all areas, while resistance to lin-

comycin, neomycin, oxytetracycline and penicillin G procaine ranged from high in Segovia to intermediate in the remaining areas. Precise data on current use of each particular antibiotic are difficult to obtain, but these and other antibiotics are commonly used in disease treatment, prophylaxis and growth enhancement in pigs and poultry in Segovia (authors' unpubl. data). The increasing number of fattening pig and poultry farms in Ebro Valley is illustrated by the greater amount of carrion consumed by Egyptian vultures from these species than from wild prey and unstabled livestock in recent years (Donázar, 1993; this study). This apparent shift in diet may be contributing to the intermediate to high bacterial resistance to antibiotics recorded in Ebro Valley and suggests its potential increase in the future, as has already occurred in pigs exploitation in this area (Agustín *et al.*, 2005).

The pattern of antibiotic resistance found in our study was similar for the different bacterial species within each area, but it clearly differed between areas. The greatest amount of multiple resistance was found in samples from Segovia and the greatest susceptibility to different antibiotics in Cádiz. The presence of resistant bacterial strains in Egyptian vulture microflora may be explained by two alternative hypotheses that are not mutually exclusive. First, the acquisition of resistant bacteria may be due to the direct ingestion of resistant strains already present in livestock carrion fed upon by this species. Second, antibiotic resistance may be acquired through transference of mobile genetic elements from bacteria to bacteria, in or across bacterial species and genera (Henriques Normark and Normark, 2002; McEwen and Fedorka-Cray, 2002; Mølbak, 2004). The similarity between bacterial species in their antibiotic resistance within areas may be due to their common presence in livestock and Egyptian vulture microflora. This is supported by the similar resistance to antibiotics in *E. coli* and *S. suis* from swine carrion and, especially, by the highly significant relationships between antibiotic resistance in *E. coli* isolated from Egyptian vulture nestlings and swine meat on which vultures fed upon in Segovia. Further support for this hypothesis lies in the finding that antibiotic resistance in normal faecal bacteria (present both in livestock and Egyptian vulture microflora) was higher than in *S. epidermidis*.

The need for greater understanding of the potential hazards for wildlife that feed on livestock carrion has been emphasized by events on the Indian subcontinent and in North America. Residues of antimicrobial agents and other drugs may also be released together with the meat remains or entire corpses of treated animals, with the subsequent risk of ingestion and associated problems in scavenging birds. The recent precipitous decline of vultures on the Indian subcontinent has been associated with residues of diclofenac, a common veterinary drug present

in the tissues of livestock carcasses fed upon by these birds (Shultz *et al.*, 2004). In the USA and Canada, bald eagles *Haliaeetus leucocephalus* have died from the residues of euthanasia solutions in livestock carcasses upon which they fed (Langelier, 1993; Thomas, 1999). The ingestion of antibiotic residuals by wildlife scavengers needs to be assessed by directly searching for them in livestock carrion and wildlife that feed on this material. Antimicrobial drugs have been suggested to cause a transient decrease in host resistance upon exposure to pathogens (Barza, 2002; Barza and Travers, 2002). Understanding of the indirect effects of bacterial flora resistance on health and survival of avian hosts will require focused experiments. One possibility suggested for human pathogens is that antimicrobial-resistant bacteria may be more virulent than antimicrobial-sensitive bacteria because of a possible co-selection of virulence traits in resistant agents (Barza and Travers, 2002). Therefore, the hypothesis that antibiotic resistance may confer bacterial resistance against the host's immune system necessitates further examination for wildlife conservation purposes, especially for species potentially affected by pathogenic bacteria (Friend and Franson, 1999; Blanco *et al.*, 2006).

It is likely that muladares will increase in importance as feeding areas for avian scavengers in Spain and other regions as feeding strategies for these species become further restricted by changes in landscape and other factors associated with human population growth. Therefore, given the precarious population status for some of these species, there is an elevated need to better understand potential health risks for avian scavengers so that muladares can be managed in a manner that minimizes health risks and benefits the conservation of avian scavenger species. Also, the mobility of these avian species requires that their role in the elimination of livestock wastes at muladares is not accompanied by increased potential for the dissemination of pathogens and antibiotic-resistant microbes of significance for human and livestock health. A first step in achieving this goal is to consider the ban or reduction of antimicrobial agents and other drugs for livestock growth promotion and prophylaxis as recommended by the World Health Organization.

## Experimental procedures

### *Study species*

The Egyptian vulture is a small vulture inhabiting open landscapes in arid and rugged regions in the Mediterranean, Africa and Asia. Mainland populations in Europe are migratory, with wintering areas in Africa, while island populations are sedentary. This species has suffered a sharp decline since the end of the nineteenth century in most regions of

the Mediterranean mainly attributed to illegal persecution through poisoning, habitat alteration and reduction of food supplies (Donázar *et al.*, 1997). The Egyptian vulture populations in our investigation have all experienced a recent sharp decline (Donázar *et al.*, 2002; Benitez *et al.*, 2003; Donázar, 2004; Martínez *et al.*, 2004) as has occurred throughout most of the range for this species in Spain (Donázar, 2004). The primary food sources for this species are carcasses of small and medium-sized wild animals, livestock carcasses, and a relatively high proportion of faeces from wild ungulates and livestock (Negro *et al.*, 2002). Thus, Egyptian vultures exploit the feeding opportunities provided by livestock operations (Donázar *et al.*, 1997). These feeding strategies make them potential reservoirs and long-distance disseminators of pathogens that may be acquired from such carrion.

#### Study areas and diet

The study was conducted in four areas representative of the habitat used by Egyptian vultures in Spain. Three of the areas were located in the Iberian Peninsula (the middle segment of Ebro Valley, Segovia and Cádiz) and the remaining one in the Canary Islands (Fuerteventura). The middle segment of Ebro Valley (hereafter Ebro Valley) is an arid zone in Northern Spain (provinces of Zaragoza, Navarra and Huesca) where Egyptian vultures form a relatively abundant population (around 100 breeding pairs). These vultures feed mostly on wild prey, unstabled and stabled livestock carrion and domestic refuse dumped in municipal dumps and muladares (Donázar, 1993; authors' unpubl. data). The study area in Segovia province (central Spain) holds a population of about 25 pairs mostly feeding on carrion from intensive livestock, especially swine, as well as on wild prey, mostly birds (authors' unpubl. data). Cádiz Province (southern Spain) holds a population of 23 breeding pairs mostly feeding on wild prey, especially wild rabbit, and unstabled livestock, especially goats (Benitez *et al.*, 2003). Fuerteventura is the nearest island to the African continent (100 km) within the Canary Islands. This island represents the main stronghold for the species in this archipelago, comprising a breeding population of 23–24 pairs and a total population estimated at around 130 individuals (Donázar *et al.*, 2002). Vultures in Fuerteventura primarily feed on the carcasses of wild prey, mainly wild rabbits and feral pigeons (*Columba livia*) and carcasses of domestic and feral goats (Medina, 1999).

The degree of intensification of livestock management and its influence on the diet of Egyptian vultures was assessed by comparing the proportion of stabled livestock (pigs, poultry and domestic rabbits) versus wild prey and unstabled livestock (sheep, goats) among food remains found at nests during the study periods in each area. Methods to collect, identify and quantify prey remains were standardized by the authors prior to data collection. Prey remains were identified by macroscopic comparisons with skeletal and skin reference collections, and quantified assuming the smallest possible number of individuals (Martí, 1987). We excluded livestock faeces used as food by Egyptian vultures because of the difficulty in quantification in relation to other food remains.

#### Cloacal microflora

We sampled bacterial microflora from the cloaca of nestling Egyptian vultures in the nest in July 2004. Nests were accessed by climbing when nestlings were about 45- to 55-day-old. Full-grown, non-breeding subadults and adults were sampled after capturing them with canyon nets baited with livestock carrion close to communal roosting and foraging sites at Ebro Valley and Fuerteventura during June–July 2004 and January 2005 respectively. Cloacal microflora were sampled with sterile microbiological swabs that were subsequently inserted into tubes containing Amies transport medium. Samples were transported on ice to the laboratory within 4–5 days after collection and processed within 1–2 h following their arrival at the laboratory. Overall, we sampled nestlings in Segovia ( $n=8$ ), Cádiz ( $n=12$ ), Ebro Valley ( $n=11$ ) and Fuerteventura ( $n=17$ ). In addition, full-grown individuals were sampled in Ebro Valley ( $n=9$ ) and Fuerteventura ( $n=11$ ).

The prevalence of each bacteria species (or genera in the case of unidentified species) between areas and between collection periods within each area was compared by means of Fisher's exact and *G*-tests. When multiple comparisons were performed on the prevalence of the different bacterial species or genera between areas and ages, the sequential Bonferroni correction was used to adjust the significance level to control Type I error rates. To assess differences in the bacterial communities among areas, we calculated similarity of species composition with the Jaccard index (Magurran, 1988) for each pair of areas using EstimateS Version 7.5 (<http://viceroy.eeb.uconn.edu/estimates>). For classification (clustering), the UPGMA (unweighted pair-group method using arithmetic averages) was used considering Jaccard's dissimilarity index to identify possible affinities in flora composition among areas.

#### Microflora culture and identification

For the enterobacteriae, samples were cultured on 5% sheep blood agar, chocolate agar and McConkey agar, to avoid *Proteus* sp. overgrowth. Plates were incubated at 37°C, either in normal atmospheric, microaerophilic (10% CO<sub>2</sub>) or anaerobic conditions, respectively, for 24 h. All suspect colonies were then subcultured on appropriate medium and identified as species by multi-substrate identification (API 20 E; Bio Merieux). Isolates of *Campylobacter* were identified at species level by flagellin gene A polymerase chain reaction/restriction fragment length polymorphism and pulsed-field gel electrophoresis.

For *Salmonella*, samples were cultured using two selective media: SM ID agar (BioMerieux) and xylose-lysine-desoxycholate (XLD) agar (Difco). A selective enrichment procedure was performed on cloacal samples. Pre-enrichment was carried out in peptone broth (Difco), and then 1 ml was cultured onto selenite brilliant green broth (Difco) and subcultured on solid media SM ID agar and XLD agar. Plates were incubated at 37°C, either in normal atmospheric and microaerophilic (10% CO<sub>2</sub>) conditions, respectively, for 24 h, except for the peptone broth, which was incubated at 37°C for 16 h. Confirmation of the identification of *Salmonella* species was performed using standard methods (Holt *et al.*,

1994). *Salmonella* serological typing was performed at the Laboratorio Central Veterinario (Algete, Madrid, Spain).

#### Antibiotic susceptibility testing

Antibiotic susceptibility and resistance testing for all the isolates were performed by disc diffusion assay. We selected *Salmonella* sp., *C. jejuni*, *E. cloacae*, *Enterobacter* sp., *Clostridium* sp., *Pseudomonas* sp., *E. coli*, *E. faecalis* and *E. faecium* because of their potential pathogenic effects and high prevalence (especially *E. coli*, *E. faecalis*, *E. faecium*) allowing us to assess antibiotic susceptibility in a greater number of individuals. In addition, susceptibility testing was also performed for *S. epidermidis*, as a potential indicator of flora acquired through the consumption of wild rabbits. Inoculates of the microorganisms were prepared from 24 h cultures. Bacterial suspensions in normal saline were adjusted to the McFarland n° 1 turbidity standard. In all cases, blood agar was used, except for *Salmonella* isolates that were cultured in SM ID agar.

We looked for susceptibility to 12 antibiotics chosen because of their use in humans and farm animals, including quinolones (ciprofloxacin, enrofloxacin, marbofloxacin),  $\beta$ -lactams (amoxicillin, amoxicillin-clavulanic, ampicillin), aminoglycosides (gentamicin, neomycin) and others (cephalexin, lincomycin, penicillin G procaine, oxitetracycline). Each bacterial isolate was classified as either susceptible, intermediate or resistant, depending on the growth inhibition diameter although subsequently intermediate isolates were considered as susceptible to be conservative and in order to simplify the analyses as only five of 2256 isolates were intermediate. Disc contents and growth inhibition zones were in accordance with criteria set by the National Committee for Clinical Laboratory Standards for bacteria isolated from animals.

All antibiotic resistance tests were performed for all bacterial isolates and pooled to compare the profile of antibiotic resistance between areas. Quantitative affinities between areas in antibiotic susceptibility were identified by computing Spearman correlation rank coefficients using the percentage of isolates resistant to each antibiotic between areas. UPGMA clustering was used considering dissimilarity values from Spearman rank correlation coefficients. Relationships between the number of antibiotics to which each bacterial isolate (pooling all bacterial species) was resistant and the consumption of stabled and unstabled livestock carrion was analysed with two-tailed Kendall rank order correlation because of the high frequency of tied data (Zar, 1984).

Resistance to the same antibiotics was tested on cultures of *E. coli* and *S. suis* isolated from samples collected with sterile swabs from liver, muscle, kidney, mouth and anus of swine carrion ( $n=27$  cultures from seven pigs) found in several muladares located in the Segovia province.

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