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Wild Bonelli's eagles (*Aquila fasciata*) as carrier of antimicrobial resistant *Salmonella* and *Campylobacter* in Eastern Spain

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ABSTRACT

Wild birds have repeatedly been found to be involved in the dissemination of enteric bacterial pathogens in the environment. The aim of this study was to determine the occurrence of *Salmonella* and *Campylobacter* as well as the antimicrobial resistance in wild Bonelli's eagles nestlings in Eastern Spain. In addition, we compared the efficiency of two sampling methods (fresh faecal samples from nest and cloacal swabs from nestlings) for detection of both bacteria. A total of 28 nests with 45 nestlings were analysed. In the nest, *Salmonella* occurrence was 61 ± 9.2%, while *Campylobacter* occurrence was 11 ± 5.8% ($p < 0.05$). In the nestlings, *Salmonella* occurrence was 36 ± 7.1%, while *Campylobacter* occurrence was 11 ± 4.7% ($p < 0.05$). Eight *Salmonella* serovars were identified, and the most frequently isolated were *S.* Enteritidis, *S.* Typhimurium, *S.* Houston, and *S.* Cerro. Only one *Campylobacter* species was identified (*C. jejuni*). Regarding antimicrobial resistance, the *Salmonella* strains isolated were found to be most frequently resistant to ampicillin and to tigecycline; however, the sole *Campylobacter* strain recovered was multidrug resistant. In conclusion, this study demonstrated that wild Bonelli's eagles nestlings are greater carriers of *Salmonella* than of *Campylobacter*. Both *Salmonella* and *Campylobacter* isolates exhibited antimicrobial resistance. In addition, faecal samples from nests were most reliable for *Salmonella* detection, while cloacal swab from nestlings were most reliable for *Campylobacter* detection.

1. Introduction

Wild birds have been highlighted as carriers of several microorganisms and involved in their dissemination in the environment [1]. A large number of *Salmonella* spp. have been isolated from wild birds, sometimes in birds with signs, but quite often in birds without signs of disease [2]. Hence, the occurrence of *Salmonella* and *Campylobacter* in wild bird reservoirs has been well documented [1,3–9]. Thus, *Salmonella enterica* serotypes Enteritidis, Typhimurium, monophasic Typhimurium 1,4,[5],12:i:-, Newport, Derby and Arizonae among others have been recorded in psittacines, passerines, charadriiformes, pigeons, and raptors [1,6,10–14]. In adittion, *Campylobacter jejuni*, *lari* and *coli* have been recorded in ducks, finches, seabirds, passerines and raptors [11,13,15,16]. Both genera of bacteria could be asymptomatic in wild birds [17,18], but for *Salmonella* when there is immunosuppression, clinical signs can vary from gastrointestinal and nonspecific signs [3] to septicaemia, embryonic and neonatal death [19]. Outbreaks can affect large proportions of populations [20,21], that could have potential implications for conservation. Also note that several authors have indicated that less obvious infections with host adapted strains seem to have consequences on the birds' reproductive success [22–24]. Moreover, *Salmonella* and *Campylobacter* are zoonotic pathogens, with special importance in public health due to the severity of symptoms and the large host range they can affect [25–27]. Due to their migratory patters, wild birds are could be an important source of direct or indirect contamination of raw plant food material or livestock farms [28,29].

The Bonelli's eagle (*Aquila fasciata*) is widespread a raptor, with a range extending from the Iberian Peninsula, representing 65% of Europe population. Bonelli's eagles are large birds of prey that feed on small mammals, birds and reptiles. This species have a marked decline in number since the early 1980, and is included in Annex I of the Birds Directive (79/409/CEE), considered "vulnerable" in Spain (Royal

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The Bonelli's eagle is considered a top avian predator in the food-chain of Mediterranean ecosystems [30–32]. This species feeds mostly on European wild rabbit (*Oryctolagus cuniculus*) and red-legged partridge (*Alectoris rufa*) playing a major dietary role [33]. However in the last decades they have suffered considerable privation of these preys species, due to game hunting and infectious diseases [33]. This condition has forced Bonelli's eagles to feed on other species like pigeons, which could carry multiresistant microorganisms, and this could lead to treatment failures in wildlife rescue centres [31].

Till now, only one study has assessed the occurrence of *Salmonella* in Bonelli's eagles [34], but to our best knowledge the occurrence of *Campylobacter* spp. has not been evaluated in this species. In this context, the aims of this study were (i) to determine the occurrence of *Salmonella* spp. and *Campylobacter* spp. in wild Bonelli's eagles nestlings in Eastern Spain, (ii) to determine the best sample type for detection of *Salmonella* spp. and *Campylobacter* spp. and (iii) to analyse the occurrence of antimicrobial resistance.

2. Materials and methods

All animals were handled according to Directive 2010/63/EU EEC for animal experiments. The Department of Infrastructure, Planning and Environment of the Valencian Regional Government granted permission to take samples, in order to improve conservation projects for endangered raptors.

2.1. Study species and study area

Sample collection was carried out during the breeding season in all Bonelli´s nests registered in the Valencian Region (Eastern Spain), concomitantly with the ringing programme implemented by the Regional Ministry (Fig. 1). The sampling period was from March to May of 2015 and 2016. All animals tested for this study were wild-bred nestlings of Bonelli's eagles, tested in their corresponding nest (during this study each nest was tested only once). The age of each nestling was determined by its feather development and by the lay and incubation records, and the sex was determined by DNA analysis (Spanish Animal Health Reference Laboratory, Ministry of Agriculture and Rural Affairs, Algete, Madrid) [6,35].

2.2. Collection of faecal samples

To take the samples it was necessary to descend the cliff to reach the nest (Fig. 2). If present, a pooled faecal dropping (5-10gr) was taken from the nest. In addition, two cloacal samples were collected from each nestling (Fig. 2), one for *Salmonella* spp. and another for *Campylobacter* spp. detection, using sterile cotton swabs (Cary-Blair sterile transport swabs, DELTALAB, Barcelona Spain). The swab was inserted approximately 1 cm into the cloaca to obtain the sample, and then kept in Cary-Blair transport medium. All samples were transported on ice and processed at the laboratory within 24 h after collection.

2.3. Salmonella isolation and identification

The detection procedure was performed according to European official method ISO 6579:2002 [36]. First, the samples were pre-enriched in buffered peptone water 2.5% (BPW, Scharlau, Barcelona, Spain), in 1:10 vol/vol proportion, and incubated at 37 ± 1 °C for 18 ± 2 h. The pre-enriched samples were then transferred onto a semi-solid agar medium, Rappaport Vasiliadis (MSRV, Difco, Valencia, Spain), and incubated at 41.5 ± 1 °C for 24–48 hours. For the positive plates, the colonies obtained were inoculated onto two specific agar plates for *Salmonella* spp. detection: Xylose-Lysine-Deoxycholate (XLD, Liofilchem, Valencia, Spain) and a selective chromogenic medium for detection of C8-esterase activity (ASAP, bioMerieux, Marcy l'Étoile, France). These agar plates were incubated at 37 ± 1 °C for 24–48 hours. After incubation, suspected colonies were collected and inoculated into a pre-dried nutrient agar plate, then incubated at 37 ± 1 °C for 24 h. Finally, biochemical test was performed to confirm *Salmonella* spp. (API-20, bioMerieux, Marcy l'Étoile, France). *Salmonella* strains isolated were serotyped at the Centre of Poultry Quality and Food Nutrition of the Valencia Region (CECAV), using the Kauffman-White-Le Minor technique [37].

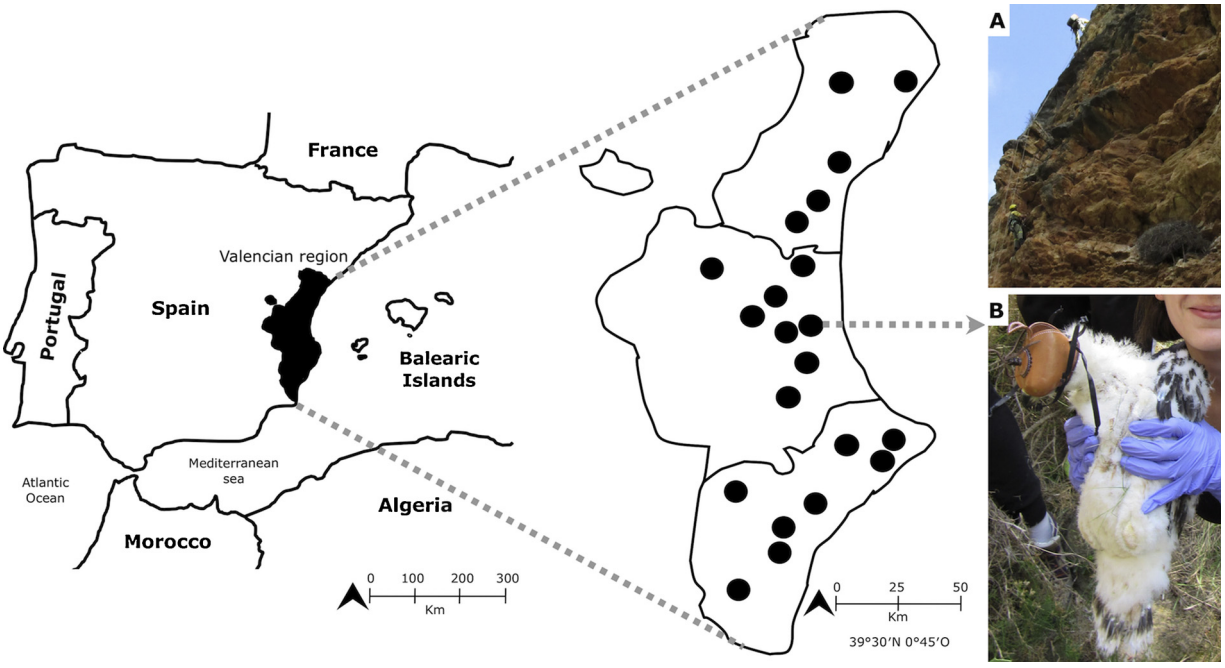

Fig. 1. Map of the study area showing the location of the sampled breeding colonies within the distribution range of Bonelli's eagles (*Aquila fasciata*) in Castellón, Valencia and Alicante provinces, Eastern Spain. Sampling location are represented as black circles. Details of a nest and nestling sampled.

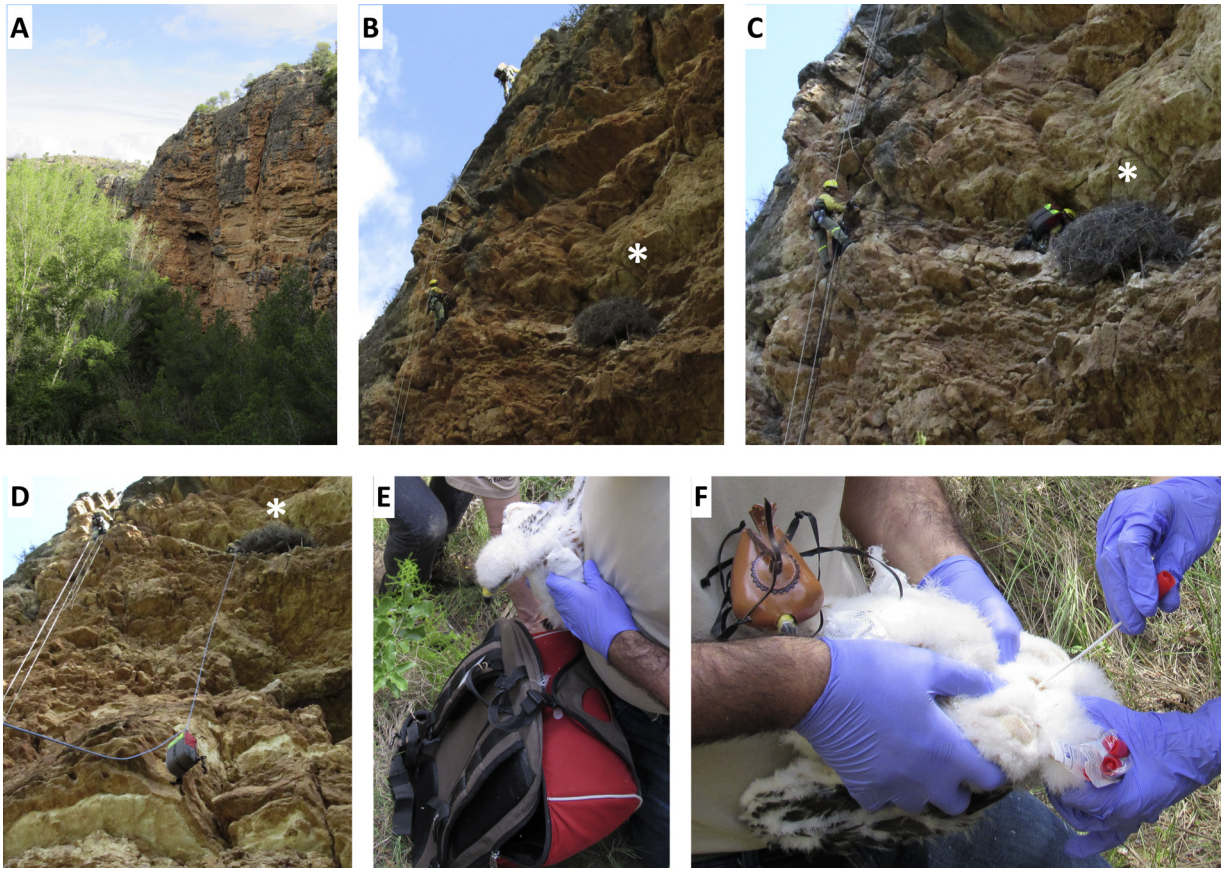

Fig. 2. Representation of the sampling. (A) Cliff example where the Bonelli's eagles (*Aquila fasciata*) usually nests in Spain. (B, C and D) Cliff descent of the Regional Ministry staff for the collection of samples. The nest is represented by a white star. (E) Nestlings recovery after the descent. (F) Cloacal swab sample collected from the nestling recovery.

2.4. *Campylobacter* isolation and identification

Bacteriological culture was performed based on the European official method ISO 10272-1:2006 for *Campylobacter* spp. [38]. All samples were analysed by direct culture, and the pre-enriched sample was plated if the direct culture was negative. Cloacal swabs were directly streaked onto two selective agar mediums: modified charcoal cefoperazone deoxycholate agar (mCCDA, AES laboratories, Bruz Cedex, France) and Preston Agar (AES laboratories, Bruz Cedex, France). Both were incubated at 41.5 ± 1 °C for 44 ± 4 h in a microaerobic atmosphere. For the pre-enriched, the original sample was pre-enriched in Bolton Broth (OXOID, Dardilly, France) in 1:10 vol/vol proportion, and was incubated at 37 ± 1 °C. After 5 h of incubation, sample was incubated at 41.5 ± 1 °C for 43 ± 1 h. Then, if the direct culture was negative, 10 µL of mixing were cultured on the same two selective agar plates (mCCDA and Preston agar) and incubated as reported above (41.5 ± 1 °C for 44 ± 4 h). Characteristic *Campylobacter* spp. colonies were purified on blood agar and identified to species level with the standard procedure: hippurate hydrolysis test.

2.5. Antimicrobial agent susceptibility testing

Antimicrobial susceptibility was tested according the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [39]. The source for zone diameters used for interpretation of the test was: http://www.eucast.org/clinical_breakpoints/. One *Campylobacter* and one *Salmonella* strain per positive nestling/nest was tested. Each strain was tested for antibiotic susceptibility using the Kirby–Bauer disk diffusion method [39], and following the antimicrobial concentrations recommended by the European Committee on Antimicrobial Susceptibility Testing. *Salmonella* strains were streaked onto Mueller-Hinton agar to form a bacterial lawn and plates were incubated at 37 °C for 24 h. *Campylobacter* strains were streaked to form a bacterial lawn onto Mueller-Hinton agar supplemented with 5% defibrinated sheep blood and then incubated with antimicrobial disks at 37 °C for 48 h under microaerobic conditions. The antibiotics selected were those set forth in Decision 2013/653 [40], including two quinolones: ciprofloxacin (CIP, 5 µg) and nalidixic acid (NA, 30 µg); three b-lactams: ampicillin (AMP, 10 µg), cefotaxime (CTX, 30 µg) and ceftazidime (CAZ, 30 µg); one phenicol: chloramphenicol (C, 5 µg); one potentiated sulfonamide: trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg); one polymyxin: colistin (COL, 10 µg); one macrolide: azithromycin (AZM, 15 µg); one glycylcycline: tigecycline (TGC, 15 µg); one aminoglycoside: gentamycin (GN, 10 µg), and one pyrimidine: trimethoprim (TM, 5 µg). MDR was defined as acquired resistance to at least one agent in two or more antimicrobial classes [41].

2.6. Statistical analysis

We tested whether occurrence of bacterium was related to sampling point. To do so, we fitted a generalised linear model (GLM) where occurrence of *Salmonella* and *Campylobacter* was the response variable and the sampling point (nest and nestlings), sample collected (faecal samples and cloacal swabs) and their interaction, sex (female and male), age (35–40, 41–45 and > 45 days of age) and province (Valencia, Castellón and Alicante) were fixed effects. For this analysis, the error was designated as having a binomial distribution and the probit link function was used. Binomial data for each sample were assigned a 1 if *Salmonella* and *Campylobacter* was isolated or a 0 if not. In addition, we tested whether occurrence of *Salmonella* was related to the number of

nestlings per nest, using a GLM as previously. To do so, we fitted GLM where occurrence of *Salmonella* was the response variable, and number of nestlings per nest (1 or more than 1) was the fixed effect. A P value < 0.05 was considered to indicate a statistically significant difference. Analyses were carried out using a commercially available software application (SPSS 21.0 software package; SPSS Inc., Chicago, IL, 2002).

3. Results

A total of 28 Bonelli´s eagle nests with 45 nestlings from the Valencia Region (Eastern Spain) were sampled (province of Valencia [n = 11], Castellón [n = 7] and Alicante [n = 10]), 11 with 1 nestling and 17 with two nestlings. Sex identification revealed that the nestlings were 20 females and 25 males and ranged between 35 and 50 days of age. Diarrhea was not observed in the Bonelli's nestlings and nest sampled. From nests, *Salmonella* was isolated in 61 ± 9.2% (17/28) of samples, while *Campylobacter* was isolated in 11 ± 5.8% (3/28) of samples ($p < 0.05$). From nestlings, *Salmonella* and *Campylobacter* were isolated in 36 ± 7.1% (16/45) and 11 ± 4.7% (5/45) of the animals sampled ($p < 0.05$), respectively. Otherwise, there were no statistical differences between the microorganism isolated and the sampling methods. For *Salmonella* detection, faecal samples were positive in 71 ± 11.1% (12/17) of samples, while 53 ± 12.1% (9/17) of the cloacal swabs showed positive results ($p > 0.05$). Nevertheless, for *Campylobacter* detection, it is important to highlight that *C. jejuni* was not isolated from faecal samples, being all positive samples isolated from cloacal swabs. Moreover, statistical differences were found between the number of nestlings present in the nest and the bacteria shedding. For *Salmonella*, 65 ± 11.6% of positive nests contained two nestlings (11/17), while 35 ± 11.6% of the positive nests had only one nestling (6/17, $p < 0.05$). In 7 of the 11 *Salmonella* positive nests, both nestlings were shedding *Salmonella* simultaneously. Likewise, in 2 of the 3 *Campylobacter* positive nests, both nestlings present were shedding *Campylobacter* simultaneously. In 1 nest, the nestlings were shedding *Salmonella* and *Campylobacter* at the same time. Moreover, no statistical differences were found on age, sex or province where they inhabit ($p > 0.05$).

Salmonella serovars isolated (n = 28) were: *S*. Enteritidis (4/28), *S*. Typhimurium (4/28), *S*. Houston (4/28), *S*. Cerro (3/28), *S*. Manhattan (1/28), *S*. Carnac (1/28), *S*. Tomegbe (1/28) and *S*. Schleissheim (1/28). From all the strains serotyped, 9 serotypes were indeterminate. Only one *Campylobacter* species (*C. jejuni*) was identified (5/5).

Regarding the antibiotic resistance patterns, 7 strains from the 19 *Salmonella* isolates were resistant to ampicillin (36.8%) and one strain was also resistant to tigecycline (5.3%). The remaining *Salmonella* strains were susceptible to all antibiotics. All the serovars isolated and their resistance patterns are described in Table 1. Of the five *Campylobacter* isolates, only one could be recovered for antimicrobial susceptibility testing. This isolate was found to be multidrug resistant with resistance to ciprofloxacin, ampicillin, nalidixic acid, trimethoprim-sulfamethoxazole, colistin and azithromycin (Table 1).

4. Discussion

Our study assessed the presence of *Salmonella* and *Campylobacter* in wild Bonelli's eagles. To our best knowledge, this is the first study in the scientific literature to evaluate a considerable sample size to healthy wild Bonelli's eagle nestlings. Besides, due to the wide range of hosts that *Salmonella* spp. and *Campylobacter* spp. can colonise, Bonelli's eagles can serve as a reservoir of these bacteria.

Differences between faecal samples and cloacal swabs, collected directly from the nests and nestlings, could be partly explained due to the intermittent excretion of these microorganisms in faeces and the survival period of them in the environment [42,43]. Moreover, for *Salmonella* spp. faecal samples could be contaminated not only by the nestlings, but also by other sources such as parents' faeces or remains of prey. In contrast, *Campylobacter* spp. were not isolated from faecal samples, probably due to the poor survival of these bacteria in the environment [43,44].

Salmonella spp. showed a higher percentage of positive nestlings than those obtained in previous studies carried out with different species of raptors, such as in Central Spain (prevalence of 4.2%) [34], Andalusia (prevalence of 4.6%) [11], or Catalonia and the Basque Country (Prevalence of 4.7% and 8.5%, respectively) [14,45]. This fact could be explained by several hypotheses, such as the type of raptor studied, the age of the animals sampled, the kind or number of samples collected or the climatological conditions of the area. Specifically, in Bonelli's eagles, Reche et al. [34] did not detect *Salmonella* positive samples in the seven animals examined. In addition, the percentage of *Campylobacter* spp. in Bonelli's nestlings was higher compared to the 1% obtained in the same region (Eastern Spain) in vultures [6] or the 2.3% obtained in Andalusia in different raptor species [11]. Some studies suggest a seasonality for both genera, so that *Salmonella* is more prevalent from March to August while *Campylobacter* is more prevalent from May to October [46,47].

The *Salmonella* serovars most frequently detected in this study were *S*. Enteritidis, *S*. Typhimurium and *S*. Houston. All of these serovars having recently been published in free-living bird studies [1,6,7,40,48], and also in domestic animals (poultry and pigs) and human outbreaks [27]. In addition, *S*. Typhimurium has been reported as a multidrug antimicrobial resistance bacteria and the most frequent serovar involved in subclinical and clinical infections in birds, such as pigeons, an important feed source for Bonelli's eagles [9,49]. Some strains isolated in this study were resistant to ampicillin and tigecycline. Resistance to ampicillin has also been described before in wild birds by other authors [10], but to the best of our knowledge there are no previous records of tigecycline resistance strains in wild raptors. Both resistances have been previously reported in pigs; specifically, the European Food Safety Authority reported in 2016 that 44.7% of ampicillin resistance and

Table 1
Antimicrobial resistance of *Salmonella* and *Campylobacter* isolates from wild Bonelli's eagles.

Species	Serovars	n	AMP	CTX	CAZ	GM	NA	CIP	CST	CAM	AZM	TGC	SXT	TMP
Salmonella	Enteritidis	4	2 (50%)	0	0	0	0	0	0	0	0	0	0	0
	Typhimurium	4	2 (50%)	0	0	0	0	0	0	0	0	1 (25%)	0	0
	Houston	4	1 (25%)	0	0	0	0	0	0	0	0	0	0	0
	Cerro	3	0	0	0	0	0	0	0	0	0	0	0	0
	Manhattan	1	1 (100%)	0	0	0	0	0	0	0	0	0	0	0
	Carnac	1	0	0	0	0	0	0	0	0	0	0	0	0
	Tomegbe	1	0	0	0	0	0	0	0	0	0	0	0	0
	Schleissheim	1	1 (100%)	0	0	0	0	0	0	0	0	0	0	0
Campylobacter	*jejuni*	1	1 (100%)	0	0	0	1 (100%)	0	1 (100%)	0	1 (100%)	0	0	0

The resistance was determined by disk diffusion. AMP: ampicillin (10 μg); CTX: cefotaxime (30 μg); CAZ: ceftazidime (30 μg); GM: gentamycin (10 μg); NA: nalidixic acid (30 μg), CIP: ciprofloxacin (5 μg); CST: colistin (10 μg); CAM: chloramphenicol (5 μg); AZM: azithromycin (15 μg); TGC: tigecycline (15 μg), SXT: trimethoprim-sulfamethoxazole (25 μg); TMP: trimethoprim (5 μg).

1.7% of tigecycline resistance came from fattening pigs [27]. Eastern Spain is a region with a high presence of pig farms throughout the countryside. One hypothesis that may explain the fact that strains isolated from Bonelli's eagles nestlings are resistant to ampicillin could be that fattening farms attract birds and other wild animals for feed. Birds, such as pigeons, could acquire resistant bacteria, and then disseminate the resistant bacteria in the environment [50], however further studies are needed to stablish the relationship between resistant strains isolated from eagles and those isolated from pig farms. In the same line, for *Campylobacter*, only one strain could be recovered to analyse the antimicrobial susceptibility, which showed a multidrug resistant phenotype to at least five antibiotics. It is important to highlight that the strain was resistant to colistin, and to the best of our knowledge, this is the first report on colistin-resistant *Campylobacter* in wild raptors. Wild birds not only act as a reservoir for *Campylobacter*, but can also contribute notably to the dissemination of antibiotic resistance, as previously reported in seabirds [13]. As reported above for ampicillin and tigecycline resistance, colistin was also widely used in poultry and swine production to prevent and treat colibacillosis across EU countries [51]. Indeed, more studies are needed to confirm the source of nestlings' infection with resistant and multiresistant strains.

In conclusion, our results indicate that *Salmonella* serovars and *Campylobacter* species are present in the wild Bonelli's eagles population in Eastern Spain. Many isolates are resistant to antimicrobial agents. In addition, faecal samples from nests were most reliable for *Salmonella* detection, while cloacal swab from nestlings were most reliable for *Campylobacter* detection. Further studies should be undertaken in other geographical areas to confirm our results. Moreover, we emphasise the need for continuous local surveillance programmes to identify the potential risk of dissemination of these pathogens to wildlife and the environment.

CRediT authorship contribution statement

Bárbara Martín-Maldonado: Formal analysis, Writing - original draft. **Laura Montoro-Dasi:** Data curation. **Maria Teresa Pérez-Gracia:** Data curation, Methodology. **Jaume Jordá:** Data curation. **Santiago Vega:** Conceptualization, Funding acquisition. **Francisco Marco-Jiménez:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. **Clara Marin:** Conceptualization, Data curation, Funding acquisition, Writing - original draft.

Declaration of Competing Interest

All authors declare no competing interests.

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