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Faecal microbiota and antibiotic resistance genes in migratory waterbirds with contrasting habitat use

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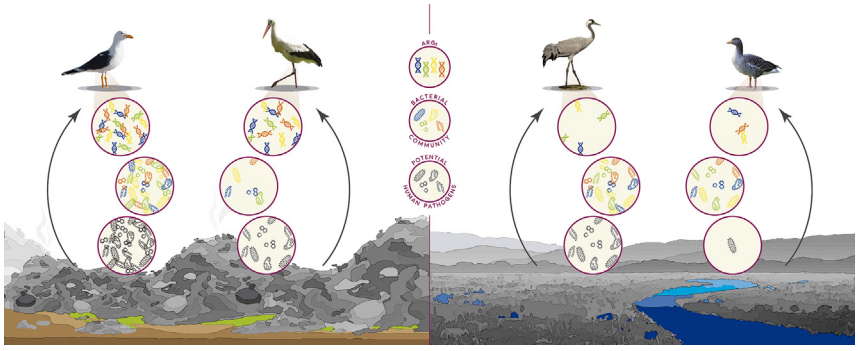
HIGHLIGHTS

- Waterbirds wintering in Spain have distinct bacterial communities in their faeces.
- Storks and gulls carry more antibiotic resistance genes (ARGs) than cranes and geese.
- Gulls carry particularly high loads of bacterial pathogens.
- Gulls carry genes conferring resistance to last-resort antibiotics.
- Waterbirds egest ARGs into urban habitats and those used for food and water supply.

GRAPHICAL ABSTRACT



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ABSTRACT

Migratory birds may have a vital role in the spread of antimicrobial resistance across habitats and regions, but empirical data remain scarce. We investigated differences in the gut microbiome composition and the abundance of antibiotic resistance genes (ARGs) in faeces from four migratory waterbirds wintering in South-West Spain that differ in their habitat use. The white stork *Ciconia ciconia* and lesser black-backed gull *Larus fuscus* are omnivorous and opportunistic birds that use highly anthropogenic habitats such as landfills and urban areas. The greylag goose *Anser anser* and common crane *Grus grus* are herbivores and use more natural habitats. Fresh faeces from 15 individuals of each species were analysed to assess the composition of bacterial communities using 16S rRNA amplicon-targeted sequencing, and to quantify the abundance of the Class I integron integrase gene (*intI1*) as well as genes encoding resistance to sulfonamides (*sul1*), beta-lactams (bla_{TEM}, bla_{KPC} and bla_{NDM}), tetracyclines (*tetW*), fluoroquinolones (*qnrS*), and colistin (*mcr-1*) using qPCR. Bacterial communities in gull faeces were the richest and most diverse. Beta diversity analysis showed segregation in faecal communities between bird species, but those from storks and gulls were the most similar, these being the species that regularly feed in landfills. Potential bacterial pathogens identified in faeces differed significantly between bird species, with higher relative abundance in gulls. Faeces from birds that feed in landfills (stork and gull) contained a significantly higher abundance of ARGs (*sul1*, bla_{TEM}, and *tetW*). Genes conferring resistance to last resort antibiotics such as carbapenems (bla_{KPC}) and colistin (*mcr-1*) were only observed in faeces from gulls. These results show

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that these bird species are reservoirs of antimicrobial resistant bacteria and suggest that waterbirds may disseminate antibiotic resistance across environments (*e.g.*, from landfills to ricefields or water supplies), and thus constitute a risk for their further spread to wildlife and humans.

1. Introduction

Humans are dependent on healthy ecosystems. The "One Health" approach, under which the World Health Organization (WHO) has based the global strategy to tackle the problem of antimicrobial resistance (AMR), is based on the close link between humans, animals and environmental health (Amarasiri et al., 2020; Dafale et al., 2020). Despite the extensive knowledge on the mechanisms and processes ruling the spread and consequences of AMR in clinical settings, less information is available on the key agents and ecological constraints that regulate the accumulation of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in the environment, and how they disseminate across different habitats (Allen et al., 2010; Taylor et al., 2011; Wu et al., 2017). Wildlife can act as reservoirs of ARB and ARGs (Guenther et al., 2011; Wu et al., 2018; Dolejska and Literak, 2019). Since wildlife may also act as vectors of active dissemination of ARB and ARGs across habitats, studies encompassing the ecology of vector species are vital to shed light on how ARB and ARGs can spread (Vittecoq et al., 2016).

Birds are considered key agents in this dissemination since ARB may easily colonize their gut through ingested food or polluted water sources (Franklin et al., 2020), converting them into environmental reservoirs and vectors for ARB and ARG (Bonnedahl, 2011; Bonnedahl and Järhult, 2014; Ahlstrom et al., 2018). The problem is accentuated for migratory birds, which can fly non-stop over hundreds or even thousands of kilometers, enabling dissemination across continents (Guenther et al., 2011; Vergara et al., 2017; Ahlstrom et al., 2021). Furthermore, global change has led many waterbirds to become increasingly dependent on artificial habitats such as wastewater treatment plants and landfills (Murray and Hamilton, 2010; Martín-Vélez et al., 2020), or natural wetlands that are highly polluted by human populations, these being habitats containing high abundances of ARB (Hessman et al., 2018; Wu et al., 2018; Marcelino et al., 2018).

In Europe, several populations of gulls (Laridae) have been found to be reservoirs of resistance to beta-lactams (Dolejska et al., 2007), with the highest prevalence in gulls from Spain (Stedt et al., 2015). In Iberian Peninsula, the studies of the role of birds as vectors of ARB are scarce, except for specific studies on scavengers (Blanco et al., 2007; Molina-Lopez et al., 2011), white storks (Höfle et al., 2020; Migura-Garcia et al., 2020) and some gull species (Stedt et al., 2015; Vergara et al., 2017). Of particular concern is the discovery of the early emergence of colistin resistance gene *mcr-1* in gulls from Spain and Portugal (Ahlstrom et al., 2019). Since colistin is a last resort antibiotic broadly used to treat infections associated with multidrug resistant strains of the family *Enterobacteriaceae* (Li et al., 2020), the identification of gene *mcr-1* in gulls is especially worrisome.

One key question for research is how AMR depends on ecological traits of the host (Vittecoq et al., 2016). For instance, to what extent do habitat use, diet, and bird movements explain patterns in ARB and ARG abundance? It is therefore important to compare the gut microbiota and the ARG content in waterbird species present in the same region but differing in their foraging and movement ecology, to investigate how these factors influence their potential to disseminate ARB and ARG across habitats and regions. In the current study, we focused on four waterbird species wintering in South-West Spain, namely: the lesser black-backed gull (*Larus fuscus*), the white stork (*Ciconia ciconia*), the common crane (*Grus grus*), and the greylag goose (*Anser anser*). These birds are long distance migrants wintering in Andalusia but with different habitat preferences (Rendón et al., 2008; del Moral et al., 2012). In particular, gulls and storks are largely reliant on landfills for foraging (likely to be a major source for ARGs and ARB), although they also feed in ricefields and other agricultural fields (Martín-Vélez et al., 2020, 2021a). Our working hypothesis was that faeces from gulls and storks would have a higher abundance of ARGs and a higher proportion of potential pathogens than birds roosting and feeding in less polluted environments (*i.e.* cranes and geese).

2. Material and methods

2.1. Study species

We studied four waterbirds wintering in Andalusia, which holds the majority of waterbirds wintering in Spain (del Moral et al., 2012), especially because of the importance of the Doñana wetland system for migratory birds (Rendón et al., 2008; Green et al., 2018). The four species studied were:

1) The white stork, whose wintering population in Andalusia includes a mixture of resident breeders and birds migrating from central Europe (Bécares et al., 2019). Both the resident and wintering populations have experienced an important increase in recent decades, largely due to the use of landfills and ricefields as predictable food sources (Ciach and Kruszyk, 2010; Rojas, 2012; Ramo et al., 2013).
2) The common crane breeding in northern and central Europe. In winter, it feeds extensively in holm oak dehesas (savannah-type habitat) of Central and South Iberian Peninsula (Sánchez Guzmán et al., 1998) since acorns are a principal winter food (Avilés, 2004), but also feeds on waste grain in cereal fields.
3) The lesser black-backed gull, breeding in northern Europe. In Andalusia, it feeds mainly in ricefields and landfills, roosting in reservoirs, rivers, fish ponds and other waterbodies, but also uses marine habitats (Martín-Vélez et al., 2019, 2020).
4) The greylag geese is a herbivore that breeds in northern and central Europe, with an increasingly small proportion of birds migrating to winter in the Iberian Peninsula (Ramo et al., 2015). It feeds largely in the natural marshes of Doñana, but also in ricefields (Rendón et al., 2008).

In the winter of 2019–2020, surveys across Andalusian wetlands counted a total of 90,216 lesser black-backed gull individuals, 4995 white storks, 6871 common cranes and 40,210 greylag goose (data provided by regional government, Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible de la Junta de Andalucía).

2.2. Collection of bird faeces

Freshly-voided faeces (n = 60) were collected from roosting habitats used by monospecific flocks located with binoculars. The birds flew away as we approached, and fresh faeces were readily distinguished from older, dry faeces. Sampling was conducted from 24th until 31th January 2019. Since gull flocks of *L. fuscus* are usually mixed with yellow-legged gulls *Larus michahellis*, they were considered as a mono-specific flock when ≥90% of the individuals were L. *fuscus*. Gull samples were collected from a landfill (Miramundo, Cadiz, 36° 28′ 50″ N, 6° 0′ 54″ W), white stork samples from the Doñana rice fields (37° 4′ 31″ N 6° 0′ 19″ W, see Martín-Vélez et al., 2021b), crane samples from post-harvest wheat fields just outside the Doñana National Park (37°, 04′ 26.3″ N 6° 221′ 23.3″ W) and geese samples from the Cerro de los Ansares dunes in Doñana National Park (36° 55′ 54″ N 6° 25′

28″ W, see Martínez-Haro et al., 2013) (Fig. 1). Faecal samples were collected with sterile spatulas and potential contamination with neighbouring soil was avoided by sampling at the core of the faecal material. We collected 15 droppings for each species. Samples were collected at least two-meters apart to ensure they belonged to different individuals. All the samples were kept in a cool bag on ice until arrival at the laboratory, within 4 h. Afterwards, they were frozen at −20 °C until processing.

2.3. DNA extraction

Faecal samples were thawed at room temperature and DNA was extracted using the FastDNA Spin for Soil kit (MP Biomedical) according to the manufacturer's instructions with a previous mechanical lysing of cells using the MP Biomedicals FastPrep24 (three rounds of 30 s at speed of 5.5). Quality of the final DNA was measured with a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific; Wilmington, DE, USA) by measuring A260/A230 and A260/A280 absorbance ratios. The concentration of DNA in final extracts was measured using a Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA, USA).

2.4. High-throughput sequencing and analyses of sequence datasets

High-throughput multiplexed 16S rRNA gene sequencing with the Illumina MiSeq System (2 × 250 PE) was carried out using primer pair 515f/806r (Caporaso et al., 2011), targeting the V4 region of the 16S rRNA gene complemented with Illumina adapters and sample specific barcodes. Sequencing was performed at the Sequencing and Genotyping Unit of the Genomic Facility/SGIker of the University of the Basque Country. Demultiplexing, quality filtering, clustering into amplicon-sequence variants (ASV) and construction of the ASV table were performed in QIIME2 (Bolyen et al., 2019).

The analysis yielded 9,707,808 total reads of high quality (80% of reads averaged ≥ Q35 scores) distributed across 60 samples with a minimum and maximum number of reads per sample of 31,140 and 283,130, respectively. Deblur algorithm (Amir et al., 2017) was used to build the ASV table (7451 ASV in total). Representative ASV sequences were aligned against Greengenes v12_08 (DeSantis et al., 2006). The *feature-classifier* script implemented in QIIME2 was employed for taxonomic assignment using the SILVA reference database v. 138 (Quast et al., 2012). Before downstream analysis of alpha and beta diversity, the ASV table was filtered to remove spurious ASVs appearing in less than 9 samples (≈15% of total) and with less than 100 counts across all samples. 500 ASVs were retained after filtration (6.7% of original ASV), comprising up to 86.2% of the unfiltered reads (8,367,862). This indicates that filtered ASVs were extremely rare across samples.

For community analysis (alpha and beta diversity), the filtered ASV table was rarefied by randomly selecting a subset of 24,800 sequences per sample to minimize bias due to different sequencing depth across samples. Alpha and Beta diversity analyses were performed in the Microbiome Analyst (MA) platform (Chong et al., 2020, *Nature Protocols*, 15:729–821). Alpha diversity was assessed using richness (Chao1) and diversity (Shannon) indices and compared through a Kruskal-Wallis pairwise test. Beta diversity was assessed by computing Bray–Curtis distance between samples and nonmetric multidimensional scaling (nMDS) ordination. A stress level < 0.20 was considered acceptable for nMDS ordination. Permutational multivariate analysis of variance (PERMANOVA) between communities based on their taxonomic

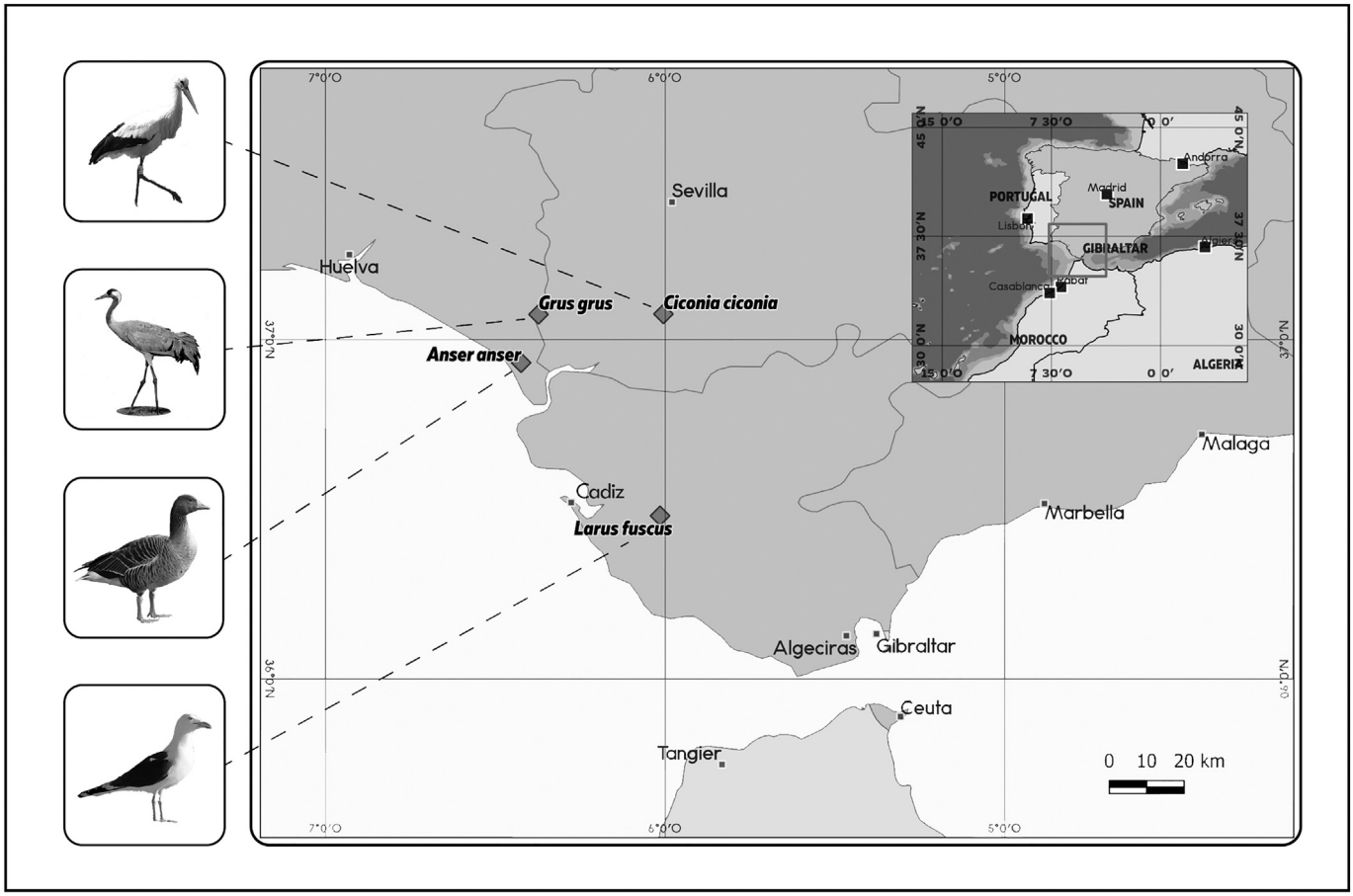

Fig. 1. Sampling sites for the study species in Andalusia, Southern Spain. Boundaries between provinces (*e.g.* Sevilla, Cádiz, Huelva) are shown.
Bird illustrations were adapted from photos by Andreas Trepte (2013). License CC BY-SA 2.5, Andrej Chudý (2019) & Bernard Dupont (2016). License: CC-BY-SA-2.0, David Iliff (2006). License: CC BY-SA 3.0.

composition explored differences between bird species. Lastly, we compared relative abundance of potential human pathogens defined as priorities for the WHO through analysis of composition of microbiomes (ANCOM) (Mandal et al., 2015), implemented in QIIME 2. ANCOM compares differences in the relative abundances at family and genus levels between bird species.

2.5. Quantification of antibiotic resistance genes

Quantitative PCR (qPCR) was used to quantify copy numbers of seven genes encoding resistance to the main antibiotic families used in clinical and veterinary contexts, such as b-lactams (bla_{TEM}, bla_{KPC}, bla_{NDM}), fluoroquinolones (*qnrS*), sulfonamides (*sul1*), tetracyclines (*tetW*), and colistin (*mcr-1*). Copy numbers of the class 1 integron-integrase gene (*intI1*) were also quantified as a proxy for anthropogenic pollution and horizontal gene transfer (Gillings et al., 2015; Stalder et al., 2014). Copy numbers of each target ARG in a sample were normalized to the copy numbers of the 16S rRNA gene, which was used as a proxy for the bacterial abundance in that sample. All genes were quantified using primers and conditions provided in Supplementary Table S1. All qPCR standard curves were obtained after serial dilutions of DNA extracts containing a known concentration of the target gene ranging from 10^8 to 10^2 gene copies per μL. All qPCR assays were performed using SYBR green detection chemistry on a MX3005 system (Agilent Technologies; Santa Clara, CA, USA). Samples were analysed in duplicate with a standard curve and a negative control included in each run. Differences in the abundance of target genes were analysed using one-way ANOVA with species as a fixed factor. The Kruskal-Wallis test was used for data not following parametric conditions. All analyses were performed in R software (version 4.0.2).

3. Results

3.1. Composition of gut bacterial communities

The composition of bacterial communities inhabiting the gut varied between bird species (Fig. 2). The gut microbiota of storks was dominated by orders *Fusobacteriales* (38%), *Lactobacillales* (24%) *Campylobacteriales* (23%), *Enterobacterales* (7%), and *Clostridiales* (6%), while in gulls, orders *Lactobacillales* (69%), *Enterobacterales* (6%), *Peptostreptococcales* (6%), *Pseudomonadales* (7%) and *Staphyloccocales* (4%) dominated. In geese, the most abundant orders were *Lactobacillales* (50%), *Clostridiales* (14%), *Fusobacteriales* (11%), *Campylobacteriales* (10%) and *Peptostreptococcales* (5%). In contrast, the gut microbiota of cranes showed a higher proportion of *Lactobacillales* (59%), *Enterobacterales* (14%), *Mycoplasmatales* (12%), *Pseudomonadales* (2.75%), and *Staphyloccocales* (3%). Interestingly, genera encompassing potential bacterial pathogens were identified in faeces from all bird species but showing differential abundances (Table 1 and Suppl. Table S3). Particularly, *Acinetobacter* spp. were only identified in faeces from gulls, whereas *Campylobacter jejunii* and *Helicobacter* spp. were detected only in cranes and storks, respectively. Gut bacterial communities from gulls, storks and cranes hosted potential pathogens such as the genera *Enterococcus* and *Pseudomonas,* and the family *Enterobacteriaceae*, which were absent in faeces collected from geese. *Staphylococcus* spp. were only identified in gulls and cranes whereas *Streptococcus* spp. were found in gulls, storks and geese (Table 1). Comparison of alpha diversity estimators between bird species revealed that faecal bacterial communities from gulls were richer and more diverse than those from the other bird species (Kruskal-Wallis, $p < 0.001$, Fig. 3A and Suppl. Table S2). Crane samples were the second richest community, while geese and storks showed the lowest values.

The nMDS ordination of samples according to their similarity in community composition (Bray–Curtis distance) showed a clear and significant segregation according to bird species (PERMANOVA, $p < 0.001$; Fig. 3B and Suppl. Table S2). However, bacterial communities in faeces collected from species feeding in landfills (gulls and storks) were more similar than those from birds feeding in less anthropized habitats (geese and cranes) (Fig. 3B and Suppl. Table S2).

3.2. Abundance of gene intI1 and ARGs

All bird faeces analysed showed a marked content of the genes studied, although their abundances significantly differed between species (Fig. 4, Table 2 and Suppl. Table S4). High concentrations of gene *intI1* were measured in gulls, storks and cranes, but it was below the limit of detection in geese (Fig. 4, Table 2 and Suppl. Table S4).

Regarding ARGs, the highest diversity was detected in faeces from gulls (Fig. 4, Table 2 and Suppl. Table S4). For genes conferring resistance to carbapenems (bla_{NDM} and bla_{KPC}), we only detected bla_{KPC} and only in faeces from gulls. Resistance to beta-lactams (bla_{TEM}) were found in all species, but its abundance was significantly higher in gulls and storks than in geese and cranes ($p < 0001$). For fluoroquinolones resistance, higher concentrations of *qnrS* gene were found in gulls and storks in comparison to cranes and geese. The concentration of gene *sul*, conferring resistance to sulfonamides, was significantly higher in faeces from gulls ($p < 0.001$) and storks ($p < 0.01$) whereas no significant differences were observed between cranes and geese. Similarly, resistance to tetracyclines (gene *tetW*) was significantly different between species ($p < 0.05$), with higher concentrations in gulls and storks than in geese and cranes. Resistance to colistin (*mcr-1*) was only detected in faeces from two gull individuals, suggesting that resistance to this last-resort antibiotic was rare among the four bird species.

4. Discussion

We found that four waterbird species, including white stork, lesser black-backed gull, common crane and greylag goose, differed in their faecal microbiota, in the relative abundance of potential pathogenic bacteria, and in the diversity and abundance of targeted ARGs. Here, we report that bird species that feed regularly in landfills (*i.e.* white stork and lesser black-backed gull) (Bécares et al., 2019; Martín-Vélez et al., 2019, 2020) harbour higher abundance of ARGs in their faeces. These species are also the only ones that use urban habitats. Storks breed in urban areas, whereas gulls use ports, coastal towns, and beaches with a high human density, as well as urban areas close to farmland (Martín-Vélez et al., 2021b). On the other hand, the waterbird with the fewest ARGs and almost no potential pathogens was the goose, a strictly herbivorous bird that feeds in habitats with lower human impact (Ramo et al., 2015). These results provide support to our initial hypothesis that those species that use the most anthropogenic habitats will present higher abundance of potentially pathogenic bacteria, and of genes that confer resistance to several antibiotics. This is likely to be owing to the transfer of ARGs and bacteria from human waste.

4.1. Gulls and storks — bird species regularly feeding in landfills and urbanized areas

The white stork and lesser black-backed gull have overlapping habits in the study area, with both species relying on dumps for feeding during the end of wintering period (January–February), but also feeding in ricefields in early winter (October–December) (Rendón et al., 2008; Martín-Vélez et al., 2020, 2021a; Bécares et al., 2019). This may explain why the clusters of these species in beta diversity analysis are closer to each other compared with the bacterial communities of geese and cranes, which form two clearly separated clusters. Nevertheless, faecal bacterial communities of storks and gulls showed marked and distinctive clusters. The dissimilarities in bacterial composition of their microbiota and profile of ARGs might be a product of differences between species in habitat use for roosting during the winter, or in their migration routes from distinct breeding areas. The lesser black-backed gull exploits a more heterogeneous variety of habitats, including marine

Fig. 2. Relative abundance of bacterial orders in the gut microbiota for each of the four bird species studied.

Table 1
Relative abundance of bacterial genera and families encompassing potential human pathogens (according to WHO) identified in gut bacterial communities of waterbird species. The ANCOM "W" statistic is the number of cases where the ratio of the frequency of a given amplicon sequence variant (ASV) to the frequency of another ASV was significantly different between bird species.

	Larus fuscus	*Ciconia ciconia*	*Grus grus*	*Anser anser*	*w*
Acinetobacter spp.	1.20%	–	–	–	207
Campylobacter jejunii	–	–	0.27%	–	215
Helicobacter spp.	–	1.73%	–	–	217
Enterococcus spp.	2.08%	0.63%	0.06%	–	179
Enterobacteriaceae	2.70%	0.04%	4.16%	–	122
Pseudomonas spp.	3.70%	0.30%	2.60%	–	217
Staphylococcus spp.	3.27%	–	2.74%	–	212
Streptococcus spp.	1.20%	0.23%	–	3.70%	213

ones (Martín-Vélez et al., 2020). Hence, this species is exposed to different bacterial environments, as shown by the higher alpha diversity found in gull faecal samples compared to storks. After wintering, lesser black-backed gulls migrate to breed in Northern Europe. The ARGs detected in our study might be carried to higher latitudes and discharged on urban built-up and green areas or agricultural lands, all common habitats used in the north (Baert et al., 2018; Spelt et al., 2019).

4.2. Cranes and geese — species inhabiting more natural, less-polluted sites

Cranes and geese also showed distinct faecal microbiotas. During the winter, the common crane feeds largely in dehesas, savannah-type ecosystems maintained by the grazing of cattle (Sánchez Guzmán et al., 1998), with acorns as its main food resource (Avilés, 2003, 2004). Similarly, the goose is a herbivore that exploits agricultural ecosystems, particularly croplands and grasslands (Ramo et al., 2015;

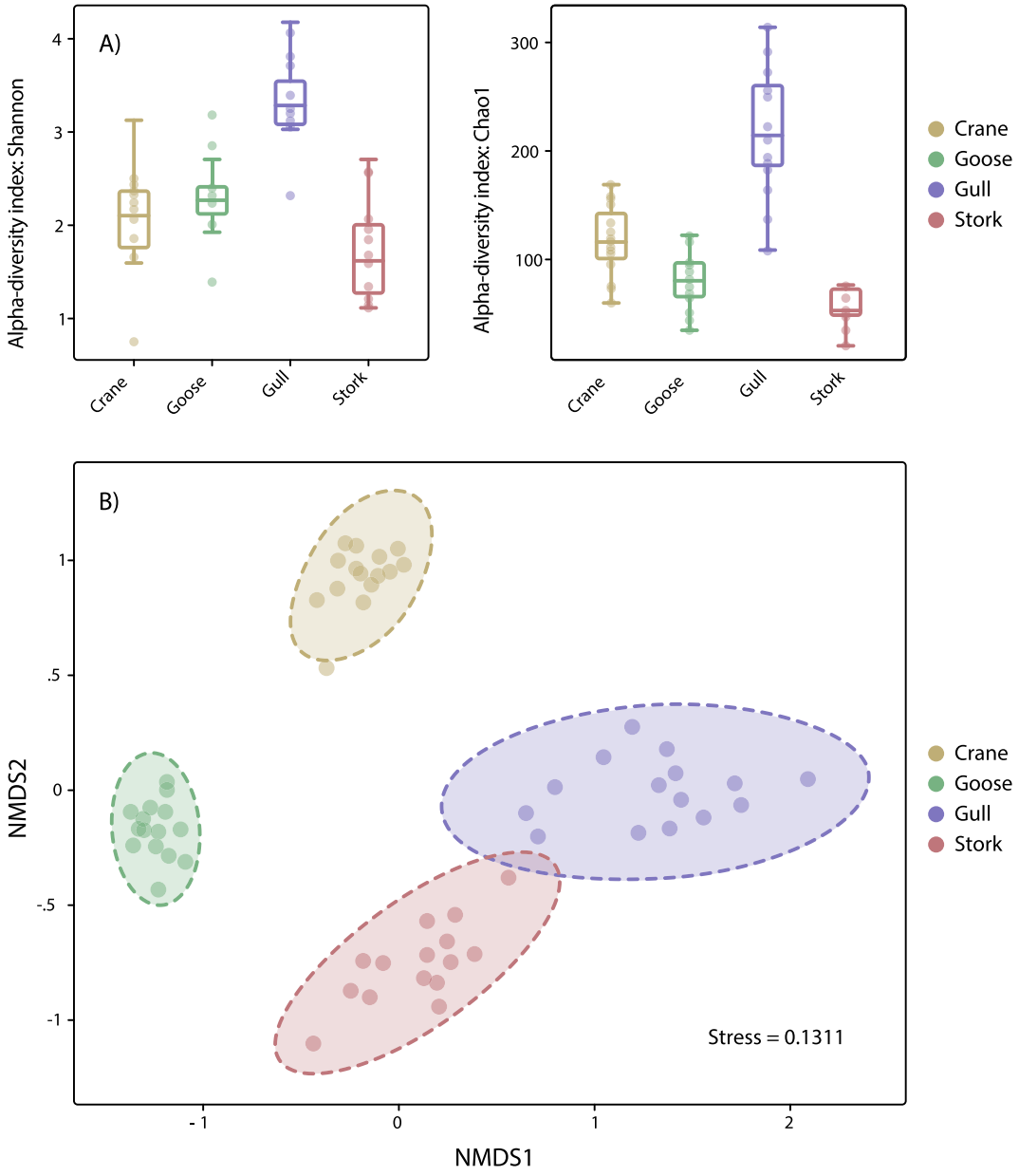

Fig. 3. A) Comparison of alpha diversity estimators (Shannon diversity index and Chao1 richness) from gut bacterial communities of the four bird species. The lower and upper edges of each boxplot are the first and third quartiles, the mid-line shows the median, and the whiskers extend from the minimal and maximal values. B) NDMS ordination of faecal bacterial communities corresponding to the 60 individuals sampled and grouped by bird species.

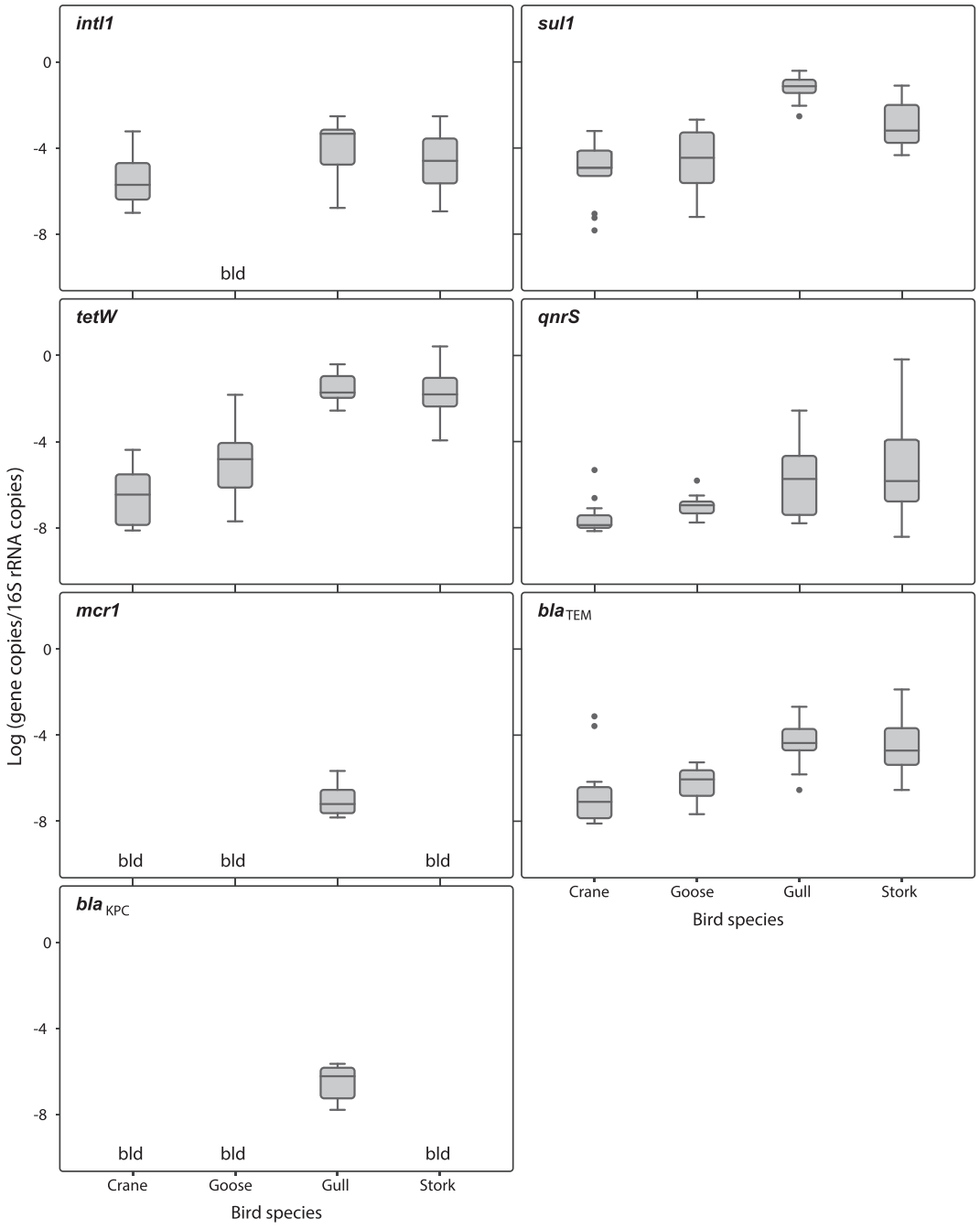

Fig. 4. Boxplots showing the relative concentration (normalized by copies of 16S rRNA) of target genes. bdl, below limit of detection. The lower and upper edge of each boxplot are the first and third quantiles, the mid line shows the median, and the whiskers extend between the minimal and maximal values.

Table 2
Summary of the relative abundance of gene *intI1* and studied ARGs (median and 95% confidence intervals) and percentage of potential pathogens identified in sequence datasets for the four waterbird species, namely: the lesser back blacked gull (*Larus fuscus*) and the white stork (*Ciconia ciconia*) that use anthropized and urban areas; and the common crane (*Grus grus*) and the greylag goose (*Anser anser*) that inhabit natural and naturalized habitats. *b.l.d.*: below detection limit.

	Gull	Stork	Crane	Goose
intI1	4.1×10^{-4} (1.6×10^{-6}–8.2×10^{-4})	7.6×10^{-5} (2.0×10^{-6}–2.1×10^{-4})	6.2×10^{-6} (3.5×10^{-7}–1.9×10^{-5})	9.1×10^{-7} (5.4×10^{-7}–1.6×10^{-6})
sul1	7.0×10^{-2} (3.0×10^{-2}–3.0×10^{-2})	1.9×10^{-3} (1.1×10^{-4}–1.0×10^{-2})	1.6×10^{-5} (4.8×10^{-6}–6.4×10^{-5})	9.9×10^{-5} (3.3×10^{-7}–3.7×10^{-4})
bla_{TEM}	4.9×10^{-5} (1.4×10^{-5}–1.4×10^{-4})	6.2×10^{-5} (2.8×10^{-6}–2.0×10^{-4})	1.7×10^{-7} (1.6×10^{-8}–4.4×10^{-7}	1.1×10^{-6} (1.5×10^{-7}–$2.2 \times 10^{-6)}$
bla_{KPC}	3.8×10^{-7} (6.1×10^{-8}–1.2×10^{-6})	*b.l.d.*	*b.l.d.*	*b.l.d.*
tetW	2.0×10^{-2} (1.0×10^{-2}–9.0×10^{-2})	2.0×10^{-2} (4.7×10^{-3}–6.0×10^{-2})	1.3×10^{-6} (1.7×10^{-8}–3.1×10^{-6})	4.5×10^{-5} (2.2×10^{-7}–1.0×10^{-4})
qnrS	4.4×10^{-6} (4.5×10^{-8}–2.3×10^{-5})	1.1×10^{-4} (1.8×10^{-7}–1.8×10^{-4})	2.1×10^{-8} (1.5×10^{-8}–4.4×10^{-8})	1.3×10^{-7} (5.9×10^{-8}–1.8×10^{-7})
mcr1	9.4×10^{-8} (3.4×10^{-8}–3.7×10^{-7})	*b.l.d.*	*b.l.d.*	*b.l.d.*
% pathogens	14.15	2.93	9.83	3.70

Olsson et al., 2017). However, the cranes and goose flocks we sampled were feeding in cereal fields and natural seasonal marshes, respectively. Flocks of both species move to wetlands (including reservoirs) for resting, and often these same wetlands are exposed to waste from livestock and human populations in the catchment, or are used for extraction of water for drinking or irrigation (Gorham and Lee, 2016). For example, the goose population winters mainly in the marsh of the Doñana National Park, the edges of which receive important discharges from urban waste waters (Paredes et al., 2021).

Our results identify correlations with habitat use that may have alternative explanations. Telemetry studies have provided very detailed information about movements and habitat use of the Andalusian gull and stork populations (Martín-Vélez et al., 2020, Bécares et al., 2019) but more research into the movements of geese and cranes would help to identify potential sources for their ARGs.

4.3. Comparison of potential human pathogens between bird species

The four studied species differed in faecal microbiota composition of potential pathogens. The gull showed the highest loads of well-known bacterial pathogens of concern such as *Staphylococcus* spp., *Pseudomonas* spp. and *Acinetobacter* spp. We are unaware of previous data on the presence of *Acinetobacter* spp. in faeces from lesser black-backed gull. This includes the pathogenic *Acinetobacter baumannii*, a human opportunistic pathogen ranked first in the list of priority pathogens by the WHO (World Health Organization, 2017) and the ECDC (European Centre for Disease Prevention and Control, 2017). Although we have not detected these bacterial strains with direct techniques such as culture based methods, specific or quantitative PCRs, these potential pathogenic genera identified in gull faeces are especially relevant due to their co-occurrence with ARGs, especially for mechanisms such as resistance to carbapenems (bla_{KPC}) or colistin (*mcr-1*).

Human potential pathogens were recorded from white storks, although the abundance of most of these microorganisms was higher in gulls and cranes. An exception was *Helicobacter* spp., only identified in stork faeces, this genus being common in the intestinal tract and oral cavity of humans and other mammals (Brock et al., 2003), with some species (*e.g. Helicobacter pylori*) being responsible for gastrointestinal infections. Finally, we identified *Campylobacter jejunii*, another species of concern due to its presence in cranes. Our results are consistent with earlier studies in cranes from North America (Lu et al., 2013) and India (Prince Milton et al., 2017). Moreover, this species hosts a significant abundance of *Enterobacteriaceae* (4.16%). Some bacteria species from this family are pathogens of global concern such as *Salmonella* spp., or opportunistic bacteria such as some strains of *Klebsiella* spp. and *Escherichia coli*. The presence of these bacteria in cranes, along with the ARGs to tetracyclines, beta-lactams, sulfonamides and quinolones, indicates a potentially serious risk from this waterbird. These results regarding potential pathogens should be treated with caution since they are exclusively based on sequence similarity in small DNA fragments (250 base pairs). Although our results ought to be considered as an indicator for potential presence of human pathogenic bacteria, further confirmatory tests (*e.g.*, specific virulence markers or bacterial cultures) are required.

4.4. Relation between ARG content and bird ecology

Previous studies have identified the role of migratory and waterbird species as reservoirs for ARGs, especially in North America and Asia (Cao et al., 2020; Lin et al., 2020; Ahlstrom et al., 2021). The novelty of our study includes the evaluation of ARGs in the lesser black-backed gull for the first time, and the addition of valuable information about the antibiotic resistance in white storks (Szczepańska et al., 2015; Gómez et al., 2016; Höfle et al., 2020; Martín-Vélez et al., 2020), cranes and geese in Europe (Garmyn et al., 2011; Hamarova et al., 2017).

The lack of detection of gene *intI1*, encoding for the integrase of Class I integrons, in goose faeces agrees with its ecology since this species live and feeds in habitats with low anthropic pollution. Class I integrons are well known as gene capture platforms carrying distinct gene cassettes conferring resistance to different antibiotics and disinfectants (Gillings, 2018). Therefore, the lack of detection of *intI1* also agrees with the low prevalence of ARGs in the source environment. In geese, we mainly observed ARGs providing resistance to sulfonamides and tetracyclines. Studies performed in wastewater effluent in the Doñana wetlands have detected sulfonamide-type antibiotics in concentrations of 0.15 μg L^{-1} (Camacho-Muñoz et al., 2010a, 2010b) and also found them to be accumulated in alien crayfish *Procambarus clarkii* tissues (Kazakova et al., 2018). Quinolone resistance was also detected in crayfish, which is an important prey for the stork and the gull (Martín-Vélez et al., 2021a). Strains resistant to tetracycline have been found in Doñana lynxes (Gonçalves et al., 2013). These studies together with our results show that the resistance to these three antibiotics is moving among different elements of the Doñana ecosystems and food webs.

The crane is mainly herbivorous, with a diet based on leaves, grains and bulbs from croplands (Avilés et al., 2006), and particularly acorns in southern Spain (Avilés, 2003, 2004). Cranes spend a large part of the winter in Dehesas shared with pigs and cattle, where they may be readily exposed to livestock faeces. Our results showed the presence of genes related with tetracyclines and beta-lactams in crane faeces. Previous studies isolated *Enterobacteriaceae* from cranes with low resistance to tetracyclines (*tetA* and *tetB*) and beta-lactams (bla_{TEM} and bla_{KPC}) (Kitadai et al., 2012; Hamarova et al., 2017). Tetracyclines are one of the most widely used antibiotics for the treatment of infections in pigs in Spain (De Briyne et al., 2014), so a transfer of these resistant genes between farm animals and cranes seems likely.

The main difference between the gull and white storks in ARG content was related with the exclusive presence of resistance to carbapenems (bla_{KPC}) and colistin (*mcr-1*) in gulls. Our findings of resistance to beta-lactams in white storks coincides with previous isolation of *E. coli* strains with extended-spectrum beta-lactamases (Höfle et al., 2020). That same study also found that some stork colonies in central Spain present resistance to colistin, a feature not detected in our study from South-West Spain. The detected resistance to last-resort antibiotics such as carbapenemases and colistin (*i.e.* bla_{KPC} and *mcr-1*, respectively) in gulls is worrisome. A possible source of AMR acquisition might be their feeding sites, both rice fields and landfills. Indeed, the gulls are more dependent on landfills after the rice harvest finishes in December (Martín-Vélez et al., 2020). In January, these birds feed in landfills, where the antibiotic resistance detected in this species might mainly be acquired. The presence of these ARGs in migratory species is evidence for possible long-distance dispersal along migration routes. *mcr-1* has recently been detected in wildlife in the Iberian Peninsula (Ahlstrom et al., 2019; Höfle et al., 2020; Migura-Garcia et al., 2020) as well as in cranes and geese in China (Cao et al., 2020). Our detection only in the gull suggests that *mcr-1* may not yet be as widespread in the wildlife of the Iberian Peninsula as in Asian countries.

Finally, the abundance of these species in the study region should also be taken into account when assessing the dissemination risk of ARGs and potential bacterial pathogens. In Andalusia for the 2019 wintering season, eighteen times more gulls were counted than storks, thirteen times more than cranes and twice more than geese. Overall, the risk of release of ARGs and pathogens into the environment is much higher for the lesser black-headed gull than for the other studied species.

4.5. Future work

This work on Andalusian wildlife advances our understanding of how antibiotic resistance may disseminate between different environmental compartments. Future studies are required to determine the effects of the discharge of ARGs into the bacterial communities inhabiting bird

habitats, as well as to define sources and sinks of ARGs and dissemination rates of ARB and ARGs by birds. Furthermore, pathogenic and non-pathogenic strains should be studied with cultivation-based methods to identify the resistance mechanisms associated with each bacterial species, and their phylogenetic relatedness to clinically relevant resistance genes. In addition, the overall resistome and mobilome should be characterized to establish the linkage between both, since ARGs carried in mobile genetic elements pose higher risk (Martínez et al., 2015). Finally, it is vital to identify the optimal conditions that favour the capture and maintenance of ARGs in environmental bacteria (Karkman et al., 2018; Serwecińska, 2020), and to determine the factors that contribute most to boosting horizontal gene transfer events in natural habitats (*e.g.*, concentration of antibiotic residues, disinfectants, heavy metals).

5. Conclusions

Overall, our study suggests that the different feeding habits, habitat use and life strategies of bird species affect their bacterial community composition and structure, and also their exposition and acquisition of antibiotic resistance. There is great potential for waterbirds to spread ARGs into habitats where exposure to humans is a risk (*e.g.*, from landfills into rice production, or into towns). Different bird species show different ARGs, the lesser black-backed gull being the species with the most diverse pool of resistance genes. Therefore, this gull could be an excellent model to understand how antibiotic resistance determinants can be maintained in animal reservoirs, and how they can easily be disseminated over long distances due to the migratory behaviour.

CRediT authorship contribution statement

Dayana Jarma: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft. **Marta I. Sánchez:** Conceptualization, Methodology, Funding acquisition, Resources, Writing – review & editing, Supervision. **Andy J. Green:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Juan Manuel Peralta-Sánchez:** Formal analysis, Data curation, Writing – review & editing. **Francisco Hortas:** Methodology, Writing – review & editing. **Alexandre Sánchez-Melsió:** Methodology, Validation. **Carles M. Borrego:** Conceptualization, Formal analysis, Supervision, Resources, Funding acquisition, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146872.

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