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Winged resistance: Storks and gulls increase carriage of antibiotic resistance by shifting from paddy fields to landfills

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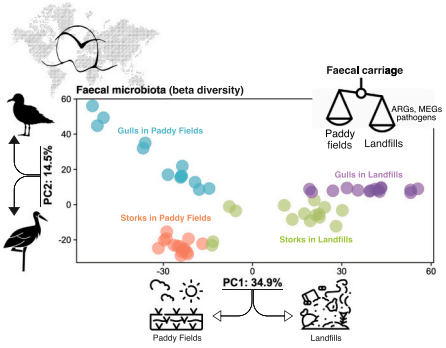
HIGHLIGHTS

- Storks and gulls using landfills become enriched in antibiotic resistance genes.
- Faeces of waterbirds feeding in landfills carry more potential pathogens.
- Potential pathogens as likely hosts for antibiotic resistance genes.

GRAPHICAL ABSTRACT



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ABSTRACT

Waterbirds are vectors for the dissemination of antimicrobial resistance across environments, with some species increasingly reliant on highly anthropized habitats for feeding. However, data on the impact of their feeding habits on the carriage of antibiotic resistance genes (ARGs) are still scarce. To fill this gap, we examined the microbiota (*16S rRNA* amplicon gene sequencing) and the prevalence of ARG (high-throughput qPCR of 47 genes) in faeces from white storks (*Ciconia ciconia*) and lesser black-backed gulls (*Larus fuscus*) feeding in highly (landfill) and less (paddy fields) polluted habitats. Faecal bacterial richness and diversity were higher in gulls

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Faecal bacterial community
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feeding upon landfills and showed a greater abundance of potential pathogens, such as *Staphylococcus*. In contrast, faecal bacterial communities from storks were similar regardless of habitat preferences, maybe due to a less intense habitat use compared to gulls. In addition, birds feeding in the landfill carried a higher burden of ARGs compared to the surrounding soil and surface waters. Network analysis revealed strong correlations between ARGs and potential pathogens, particularly between *tetM* (resistance to tetracyclines), bla_{CMY} (beta-lactam resistance), *sul1* (sulfonamide resistance) and members of the genera *Streptococcus*, *Peptostreptococcus*, and *Peptoclostridium*. Our work demonstrates how transitioning from paddy fields to landfills fosters the carriage of ARGs and potential pathogens in the bird gut, shedding light on the ecological role of these avian vectors in antimicrobial resistance dissemination.

1. Introduction

Wildlife is a sink and source of antibiotic resistance (AR) (Greig et al., 2015; Luo et al., 2022). The main cause is the anthropogenic pollution of their habitats, which intensifies their exposure to antibiotic residues, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs). This exposure offers countless opportunities for ARB to colonize either the external surfaces or the internal organs (*e.g.,* the gut) of exposed animals, offering a variety of conditions and niches where they can proliferate, persist, and mobilise their genes. Many animals have been considered reservoirs of ARB and ARGs, and some are even considered vectors for their spread (Kraemer et al., 2019; Luo et al., 2022).

Among wildlife, migratory waterbirds are of special interest to study the dissemination of AR determinants for several reasons, namely: *i)* many opportunistic species have changed their dietary habits, from natural prey based on invertebrates and small vertebrates (Bécares et al., 2019; Martín-Vélez et al., 2022, 2020a) to human waste (Gilbert et al., 2016) due to the proliferation of anthropogenic habitats such as landfills during the last decades; *ii)* they have gregarious behaviour, which results in a large concentration of individuals, increasing the potential of AR load in recipient ecosystems; and *iii)* their greater capacity for long-distance dispersal of AR compared to other animals, and our increasing understanding of their movement patterns. Storks and gulls are well-known examples of these species and previous studies show that they function as reservoirs of ARB and ARGs due to their use of landfills (Jarma et al., 2021; Ljubojević et al., 2016; Martín-Maldonado et al., 2020; Martiny et al., 2011; Szczepańska et al., 2015; Zeballos-Gross et al., 2021). In this regard, anthropogenic waste is the most likely source of exposure to AR by avian populations (Mukerji et al., 2020). However, studies on the effect of feeding in dumps on both the faecal ARG load and the faecal microbial composition of waterbirds remain scarce, as do studies comparing different bird species inhabiting the same habitats and geographical area.

The microbial diversity in the gut of wild birds depends on intrinsic factors, such as age, sex, health, and the phylogenetic history of the bird species, as well as on extrinsic factors, such as diet, social interactions (gregarious or not), and the environment (Grond et al., 2018). For instance, birds at the same stopover site are exposed to the same environmental bacterial community (Lewis et al., 2017), and in some cases, share a core microbiota (Ryu et al., 2014). Although some intrinsic factors shaping the gut microbial communities of birds are known (Drobniak et al., 2022; Sottas et al., 2021; Waite and Taylor, 2014), the influence of extrinsic factors on the AR carriage has yet to be explored. Studies on bacterial communities from soil and water have shown that shifts in the community composition alter the antibiotic resistome (Cheng et al., 2016; Jia et al., 2015). However, studies dealing with both ARGs and microbiota are scarce for wildlife and even fewer link environmental pollution (*i.e.*, antibiotic residues, heavy metals, and faeces) to the assemblage of bacterial communities and the selection of ARGs (Zhu et al., 2017).

Our study aimed to discern the effects of environmental pollution on the faecal microbiota of waterbirds and the carriage of ARGs and mobile genetic elements (MGEs), the latter enabling the horizontal transfer of ARGs among bacterial cells. For that purpose, we used two well-monitored species, white storks (*Ciconia ciconia*) and lesser black-backed gulls (*Larus fuscus*), in the Guadalquivir Basin (SW Spain). Both are known to switch regularly between two major foraging habitats, paddy fields and a nearby landfill (López-Calderón et al., 2023; van Rees et al., 2021). Our work aimed to answer the following questions: *i)* How does the use of contaminated sites alter the faecal microbiota (composition, diversity, and occurrence of human bacterial pathogens) of two unrelated bird species with different body sizes but similar habitat use? *ii)* Is the faecal load of ARGs and MGEs positively correlated with pollution in the habitats that birds use for feeding? *iii)* Do co-occurrence patterns emerge between ARGs and bacterial pathogens? We hypothesised that environmental pollution severely alters the faecal microbiota of birds, increasing the prevalence of priority bacterial pathogens (*i.e.*, those that commonly cause severe disease and may often be multidrug resistant) and the carriage of ARGs.

2. Material and methods

2.1. Model species and study sites

The lesser black-backed gull (*Larus fuscus*) and white stork (*Ciconia ciconia*) are migratory, generalist waterbirds that have experienced substantial population increases in recent decades, associated with the proliferation of landfills across Europe (Gilbert et al., 2016). The recent change in their foraging behaviour toward more anthropogenic resources (Ramo et al., 2013) makes these bird species interesting models for understanding the impact of human activities on the dispersal of AR at different scales. In addition to long-distance migratory movements, during daily flights gulls and storks often travel between roosting and feeding sites connecting highly polluted systems (where they feed) with wetlands and agricultural habitats (where they rest) (López-Calderón et al., 2023; Martín-Vélez et al., 2020b).

Sampling was conducted in paddy fields (ricefields; 37°12'47.21"N, 6° 9'13.19"O for storks; 37°10'57.57"N, 6°10'36.08"O for gulls) of the Guadalquivir delta (Seville, SW Spain) in the area surrounding Doñana Natural Space, and a nearby landfill (Centro Integral de Tratamiento de Residuos Sólidos Urbanos Montemarta-Cónica, Seville, Spain;) where both target species feed and roost (37°13'32.37"N, 5°52'50.62"O for storks; 37°13'21.14"N, 5°52'41.89"O for gulls) during the wintering period (September-March; Suppl. Fig. S8). Up to 15,000 gulls and about 1000 storks regularly use these paddy fields during this period (Ramo et al., 2013; Rendón et al., 2008). Artificial methods, mainly using nitrogen and phosphorus application, are employed for fertilization in these agroecosystems, excluding the use of manure (Campos et al., 2010). These paddy fields are a key node of high functional connectivity with other habitat types, including landfills (López-Calderón et al., 2023; Martín-Vélez et al., 2020a, 2020b; van Rees et al., 2021). The Montemarta-Cónica landfill is the largest integrated municipal solid waste treatment centre in Andalusia, managing >620,000 tons per year of waste, including organic matter, packaging, construction, biosanitary waste, and leachate. This landfill receives waste from the metropolitan area of Seville, which supports nearly 1.5 million people. Study site selection was based on GPS tracking data and the use of

habitat by birds (see (López-Calderón et al., 2023; Martín-Vélez et al., 2020b; van Rees et al., 2021).

2.2. Sample collection

Faeces from both bird species and from both habitats were collected between November and December 2020. Fresh bird faeces were sampled when monospecific flocks were observed in the study area for at least 30 min. Samples were collected in sterile conditions and at least 1 m apart to ensure that each sample corresponded to an individual bird, and to avoid pseudo-replication. The analysis of faecal bacterial communities was conducted at the individual level ($n = 15$ faecal samples per species and habitat). In contrast, the quantification of ARGs by high-throughput qPCR (HT-qPCR, see below) was conducted on composite faecal samples for each bird species after pooling an equal amount of fifteen individual samples per species and habitat ($n = 6$ composite samples per species and habitat). By using pooled faecal samples we were able to reduce the variability among samples (Clasen et al., 2016). The composition of faecal bacterial communities was also determined in the same pooled samples to infer potential associations between bacteria and ARGs. Besides, we collected faeces from both bird species to analyse their diets at each study site ($n = 27$ for storks per habitat, $n = 32$ for gulls per habitat) to infer the potential sources of ARG exposure.

On the same day of faecal sampling, soil samples ($n = 6$ in the paddy field, $n = 3$ in the landfill) were collected and placed in 15 mL sterile Falcon tubes. Similarly, samples ($n = 3$) from surface water were collected from paddy fields using sterilized glass bottles (500 mL). To assess the pollution status of bird habitats, soil and water samples were also collected for analysis of antibiotic residues and heavy metals. All samples were stored in a portable icebox (4 °C) until arrival at the laboratory (< 6 h after collection). Once in the laboratory, the soil samples were immediately frozen at −20 °C until analysis. For molecular analysis, 100 mL of the collected water samples were filtered through 0.22 μm pore diameter nylon membranes (Whatman, Maidstone, UK) using a sterilized filtration device and a vacuum pump. The filters were stored at −20 °C until DNA extraction.

2.3. Analysis of dietary habits from faecal samples

Faecal samples kept at 4 °C were rehydrated and sieved through a 200 μm mesh. The retained material was placed in a sterile Petri dish and observed under a stereomicroscope (LEICA EZ4). Remains from fed organisms (*e.g.,* crayfish, insects, snails, among others) were identified to the lowest taxonomic level possible and quantified. Artificial items were also separated and classified into ceramics, plastic paint, metals (lead and aluminium), plastics, and glass (Suppl. Table S1).

The compositional dataset of faecal samples grouped by bird species and habitat was loaded into R (R Core Team, 2021) to construct a non-metric multidimensional scaling using the package *vegan* v2.6-2 (Oksanen et al., 2020). Significant differences between species were assessed by two-way permutational multivariate analysis of variance (PERMANOVA) in *vegan* using species and habitat as fixed factors. Subsequently, pairwise comparisons were conducted using the package *RVAideMemoire* v0.9-81-2 (Hervé, 2021). The effects of bird species and habitat (fixed factors) and their interaction on community richness and diversity, the presence of plastic and the presence of crayfish on diet were tested using generalised linear models (GLM) with Poisson distribution and log link function for community richness, a linear model with normal distribution for the Shannon Index, and negative binomial with logit link function for presence of plastics and crayfish, using the package *glmmTMB* v 1.1.4 (Brooks et al., 2017). When the interaction was not significant, it was excluded from the final model.

2.4. Measurements of antibiotics and heavy metals in soil, water, and faecal samples

After collection, the samples were freeze-dried in a Cryodos-50 freeze-dryer (Telstar, Terrasa, Spain), homogenized in a mortar, and sieved (particle size <100 μm). Details of the standards for antibiotics or metabolites tested here and the internal standards can be found in Mejías et al. (2022). The structures of the target compounds and their pK_a and log K_{ow} values are presented in Suppl. Table S7. Pre-treated faecal samples (0.5 g dry weight (DW)) were spiked with the internal standards at 100 $ng{\cdot}g^{-1}$ DW. The samples were extracted three times with 5 mL of methanol (MeOH) by sonication in an ultrasonic bath (25 °C, 80 kHz) for 10 min. After each extraction, solid-liquid separation was performed by centrifugation for 10 min at 2900 ×*g*. The liquid phases were combined into a clean centrifuge tube containing 0.8 *g* of C18 for dispersive solid-phase extraction (d-SPE) clean-up. The tubes were shaken and centrifuged for 10 min at 2900 ×*g*. The liquid phase was then transferred to another tube and evaporated to dryness under a gentle nitrogen stream. The extract was reconstituted in 0.5 mL of MeOH:water (1:1, *v*/v) and filtered through a 0.22-μm cellulose syringe filter. A 2 μL aliquot of the filtered extract was injected into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument (Agilent 1260 Infinity II chromatograph coupled to a 6495 triple quadrupole mass spectrometer) according to Mejías et al. (2022).

For the analysis of metals, we carried out a pre-treatment of samples. Briefly, samples were freeze-dried in a Cryodos-50 freeze-dryer (Telstar, Terrasa, Spain), homogenized in a mortar, and passed through a 2 mm sieve discarding the fraction >2 mm and eliminating stones, roots, and fragments of plastic and metal. Finally, the material was sieved mechanically to obtain a fraction of <63 μm. The final materials were stored in plastic bottles at room temperature until further analysis. Selected metals were determined by ICP-MS/MS Agilent 8800. A subsample of 0.5 g of dry sample (< 63 μm) was weighed directly in a dried, cleaned PTFE digestion vessel, and 5 mL HNO_3 (65 % *w*/w) and 15 mL HCl (35.5 % w/w) were added. Subsequently, the digestion vessel was placed in the chamber of a microwave system. The digestion conditions were optimized at different times and powers in a microwave system. Maximum recoveries were obtained at a power of 60 % (400 W) for 20 min. The solution was filtered through Whatman No. 42 filter paper, quantitatively transferred to a 25 mL calibrated flask, and diluted with deionized water.

2.5. Composition of faecal bacterial communities

The bacterial community in faecal samples was used as a proxy for the gut microbiota of the bird species, as previously described (Yan et al., 2019). All samples (composite and individual faecal samples, soil, and water) were subjected to DNA extraction using the FastDNA Spin Kit for soils (MP Biomedicals; Santa Ana, CA). Sequencing was performed at the Sequencing and Genotyping Unit of the Genomic Facility/SGIker of the University of the Basque Country (Leioa, Spain) following Illumina guidelines for library preparation and high-throughput multiplexed sequencing of the V4 region of the *16S rRNA* gene using the universal primer pair 515f/806r (Apprill et al., 2015; Caporaso et al., 2011; Parada et al., 2016). Sequence datasets were deposited in the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA875258.

Sequence reads were processed using QIIME2 release 2020.8 (Bolyen et al., 2019). First, paired-end sequences were demultiplexed, and then dada2 was used to denoise and merge paired reads, filter chimaeras, and dereplicate sequences. Number of reads per sample ranged from 26,109 to 151,180 (see Suppl. File S1 for further details). Sequences were aligned to the SILVA database (release 138, pre-trained Naive Bayes classifier from the 515f/806r region) for taxonomic assignment (Suppl. File S1). The resulting Amplicon Sequence Variant (ASV) table and taxonomy were then imported into R v4.1.1 (R Core Team, 2021). The

dataset was filtered to retain ASVs with a minimal proportional abundance in a sample of 0.1 % and a prevalence of each feature in all samples of 5 % using the *CoDaSeq* package v0.99.6 (Gloor et al., 2017). To rigorously deal with the compositional nature and sparsity of microbiome datasets (Gloor et al., 2017), we estimated the 0 count values using the *zCompositions* package v1.4.0-1 (Palarea-Albaladejo and Martín-Fernández, 2015) before applying centred log-ratio transformation of the data.

Taxonomy bar charts between environmental matrices (faeces, soil, and water), species (storks and gulls) and habitats (paddy field and landfill) were plotted in R using *phyloseq* v1.36.0 (McMurdie and Holmes, 2013) and *qiime2R* v0.99.4 (Bisanz, 2018). Alpha diversity indices (ASV richness and Shannon diversity index) were calculated in R using *vegan*. Two-way analysis of variance (ANOVA) was computed using the package *car* v3.1-0 (Fox et al., 2021) to assess differences in diversity metrics among species (*Ciconia vs. Larus*), habitats (paddy field *vs.* landfill), and the interaction between both factors, followed by pairwise comparisons for any significant interaction. For beta diversity, compositional biplots were generated using centred log-ratio transformed ASV values with 0 replacement using the package *propr* v4.2.6 (Quinn et al., 2017). Significant differences among faeces, soil, and surface water were assessed using a two-way PERMANOVA in *vegan*, including source and habitat as fixed factors as well as their interaction. Pairwise comparisons were subsequently conducted for the interaction using the package *RVAideMemoire*.

We also conducted a differential analysis to compare the relative abundance of priority human pathogens defined by the World Health Organization (2017) using the ALDEx2 test from the *ALDEx2* package v1.24.0 (Fernandes et al., 2014, 2013) and the ANCOM test from the *ANCOMBC* package v1.2.2 (Lin and Peddada, 2020). These analyses allowed us to identify bacterial genera that were differentially abundant between the two habitats for both species according to both approaches. Dissimilarities among species and habitats were visualised using bubble charts in *ggplot2* v3.3.6 (Wickham, 2016), using data generated in *phyloseq*.

2.6. High-throughput qPCR

DNA extracts from composite samples ($n = 6$ for both bird species and habitats), soil ($n = 3$ for both paddy field and landfill), and water samples ($n = 3$ for paddy field) were analysed using the SmartChip qPCR system at Resistomap facilities (Helsinki, Finland; https://www.resistomap.com/). All DNA samples analysed yielded a minimum concentration of 5 ng/μL and a minimum 260/280 absorbance ratio of 1. The relative abundance of selected ARGs and MGEs was assessed in each sample using customised primer sets for 47 genes selected according to their prevalence in natural environments and clinical relevance (Suppl. Table S6). The abundance of the *16S rRNA* gene was used to normalise the abundance of the target genes. A threshold cycle (CT) of 27 was used as the detection limit, as previously described (Muziasari et al., 2017, 2016; Wang et al., 2014; Zhu et al., 2013). Briefly, the 33 samples plus three blanks were analysed with a 48 × 36 SmartChip (48 genes × 36 samples) using volumes and qPCR conditions previously described (Wang et al., 2014). The mean CT of three technical replicates for each qPCR reaction was used to calculate the ΔCT values. Genes detected in only one of the three technical replicates were discarded for downstream analysis. The $2^{-\Delta CT}$ method (where ΔCT = CT-detected gene – CT *16S rRNA* gene) was used to calculate the relative abundance of the detected gene in proportion to the *16S rRNA* gene in each sample (Schmittgen and Livak, 2008).

To visualise differences in ARG abundance between sample types (faeces, soil and water), species (gulls *vs.* storks) and habitats (paddy field *vs.* landfill), gene abundance data were imported into R, log-transformed, and then used to build a heatmap and stacked bar plot using *ggplot2*. One- or two-way ANOVA in R was used to detect differences in gene abundances among species, habitats, or the interaction between both factors, followed by pairwise comparisons. The non-parametric test equivalent was run when the data did not comply with normality assumptions.

2.7. Microbiota and ARG correlations

To infer potential relationships between the faecal microbiota and the antibiotic resistance profile for both bird species, we integrated centred log-ratio-transformed ASV and ARG data from pooled faecal samples using a sparse Partial Least Squares or Projection to Latent Structures (sPLS) model with a leave-one-out cross-validation method using the package *mixOmics* v6.19.1 (Rohart et al., 2017). We first discarded those samples and features (*i.e.,* genes) with high rates of missing values (*e.g.,* 36 out of the 48 genes analysed were not detected in sample LFp24). We used the *network* function and the *igraph* package v1.3.4 (Csardi and Nepusz, 2006) to generate correlation structures among variables and exported them to Cytoscape v3.9.1 (Shannon et al., 2003) for editing.

3. Results

3.1. Landfills as a source of anthropogenic materials for bird guts

Bird faeces contained both partially digested aquatic organisms and many fragments of synthetic origin (plastics, aluminium, glass, ceramic, among others; Suppl. Table S1). Among the former, faecal samples mainly contained remains of the invasive red swamp crayfish *Procambarus clarkii* and plant material. Maximum occurrence of *P. clarkii* remnants was found in faeces from gulls and storks collected in paddy fields (91 % and 100 %, respectively), while the presence of crayfish material in landfill samples was lower (16 and 52 %, respectively). Vegetal material mainly corresponded to rice plants (*Oryza sativa*) in gull faeces (78 % of occurrence in landfill samples and 59 % in paddy field samples) and seeds (detected in 38 % of paddy field samples, and none in the landfill). In turn, stork faeces showed a higher occurrence of plant material unidentifiable owing to fragmentation (33 % in the landfill and 67 % in the paddy field). Overall, higher richness and diversity were measured in faecal samples from paddy fields, where they were higher in storks than gulls. In contrast, in landfill samples richness and diversity were higher in gulls (Richness interaction term, $p = 0.018$; Shannon interaction term, $p = 0.003$).

Although artificial items represented <1 % of the sample volume in both bird species and habitats (mainly plastic and glass), their occurrences were higher in the landfill (44 % and 91 % for storks and gulls, respectively) than in paddy fields (11 % and 6 %, respectively; Suppl. Table S1). Gull faeces from the landfill also contained aluminium, ceramic, plastic paint, and lead (<10 % in each case). Remarkably, 4 % of faecal samples from storks collected at the landfill also contained polystyrene (Suppl. Table S1).

Ordination of faecal samples according to their composition showed overlap between bird species and habitats, with a significant interaction between both factors (PERMANOVA, $p < 0.001$; Fig. 1). Overall, these results indicate that birds that fed in landfills had a diet enriched in synthetic materials, whereas those feeding in paddy fields had a diet mainly enriched in aquatic organisms but that also contained anthropic debris. Subsequent pairwise comparisons revealed that the diet composition of both bird species differed between each other and between habitats ($p < 0.05$ for all comparisons). Plastic materials prevailed in samples from landfills and contributed the most to differentiating between habitats, whereas faeces collected in the paddy field were characterized by the presence of red swamp crayfish *P. clarkii* remains. The GLM for the presence of plastics revealed significant differences between species ($p < 0.001$) and habitats ($p < 0.0001$) (with no significant interaction). The occurrence of plastics was significantly higher in gulls than storks, and in samples from the landfill than from paddy fields.

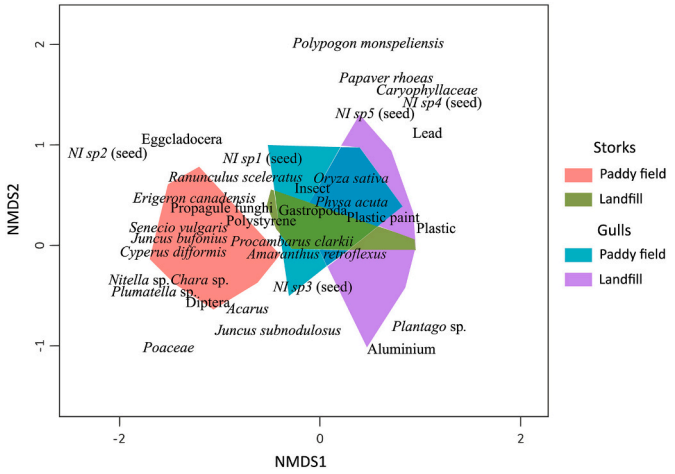

Fig. 1. Landfills as a source of anthropogenic materials for bird guts: Clustering of faecal samples using a non-metric multidimensional scaling ordination by bird species and habitat type (stress = 0.14; See also Suppl. Table S1).

3.2. Antibiotic and heavy metal concentrations in soil, water, and bird faeces

The characterization of the pollution levels by antibiotic residues and heavy metals was only possible in paddy fields, due to logistic and administrative constraints to sampling at the landfill. In the paddy fields, the concentrations of different antibiotic residues identified were higher in water than in the surrounding soil, with the major presence of residues belonging to tetracyclines (oxytetracycline, 62 ng/L), fluoroquinolones (ciprofloxacin, 590 ng/L; norfloxacin, 205 ng/L; ofloxacin, 108 ng/L), and sulfonamides (sulfadiazine 484 ng/L; sulfamethoxazole 319 ng/L, trimethoprim 98 ng/L). Neither beta-lactams (such as amoxicillin and penicillin) nor carbapenems were detected (Suppl. Table S2).

We also detected some specific antibiotics or derived metabolites (oxytetracycline, >30 ng/g dw; erythromycin, > 28 ng/g dw; *N*-desmethyl clarithromycin, > 0.02 ng/g dw) in bird faeces collected at the landfill, but no antibiotic residues in paddy field faeces. The 4-epi-tetracycline was solely detected in stork faeces (average 19.3 ng/g dw), and trimethoprim only in gull faeces (average 6.3 ng/g dw). Ofloxacin (average 36.6 ng/g dw) and sulfamethoxazole (average 50.5 ng/g dw) were ubiquitous in faeces from both bird species (Suppl. Table S3).

The most abundant metals in the studied samples were (by decreasing order): Zn (380-1399 mg/Kg dw), Mn (162-472 mg/Kg dw), Cu (27-103 mg/Kg dw), Ni (6-25 mg/Kg dw), and Pb (7-19 mg/Kg dw), with As, Co and Cd having the lowest concentrations (< 9 mg/Kg dw). Sb and Ti were solely detected in soil (< 0.5 mg/Kg dw). Interestingly, for all the metals studied, the concentrations were much higher in bird faeces than in the surrounding water but lower than in soil (Suppl. Table S4).

3.3. Birds feeding in landfills show distinct faecal microbiota

After denoising and filtering the sequence dataset, we obtained 4,962,726 reads with a sample depth ranging from 26,109 to 151,180 reads per sample. For all samples, the accumulation curves of the amplicon sequence variants (ASV) reached a plateau phase (Suppl. Fig. S1), thus suggesting that we captured most of the ASV richness. A total of 13,582 ASVs were recovered. Of these, 4165 ASVs were recovered from gull faeces, 1472 from stork faeces, 8949 from soil samples, and 1886 from surface water.

For both bird species, the composition of faecal bacterial communities varied between habitats (Fig. 2). Faeces from gulls feeding in the landfill were dominated by sequences affiliated with the order Lactobacillales (78 %), mostly ascribed to the genus *Lactobacillus*, whereas the remaining 22 % were distributed among several bacterial orders at relative abundances lower than 7 % (Suppl. Table S5). Lactobacillales was also the most prevalent bacterial order (53 %) in gull faeces collected in paddy fields, although these ASVs were mainly assigned to the genus *Catellicoccus*. The faecal bacterial community of gulls also contained 15 % of sequences affiliated to Erysipelotrichales (Fig. 2), mostly assigned to the genus *Breznakia*, and other bacterial orders at abundances ranging from 6 % (Fusobacteriales) to 3 % (Propionibacteriales) (Suppl. Table S5).

Faecal samples from storks collected at the landfill also contained a higher proportion of sequences affiliated to Lactobacillales (52 %), mainly *Lactobacillus*, and several other bacterial orders that include well-known human pathogens, namely: Campylobacterales (11 %), Peptostreptococcales-Tissierellales (10 %), Clostridiales (8 %), Enterobacterales (7 %), and Staphylococcales (5 %). Stork faeces from paddy fields showed higher proportions of Clostridiales (38 %), mostly attributed to the genus *Clostridium*, Lactobacillales (36 %, mainly ascribed to genera *Lactobacillus* and *Catellicoccus*), Fusobacteriales (19 %), and Peptostreptococcales-Tissierellales (4 %).

Soil and surface water samples had bacterial communities distinct from those in bird faeces, with a high proportion of groups usually found in terrestrial and aquatic environments (Fig. 2). In particular, the soil samples contained members of the order Propionibacteriales (29 % and 5 % in the landfill and paddy fields, respectively), Cyanobacteriales (19 % and 5 %), Nitrosophaerales (4 % and 11 %), and Burkholderiales (5 % and 8 %), among others (Fig. 2). The surface water collected in the paddy field was characterized by a higher proportion of Burkholderiales (48 %), Corynebacteriales (6 %), Rhizobiales (6 %), Sphingomonadales (5 %), Bacillales (5 %), and Micrococcales (3 %) (Suppl. Table S5).

A comparison of alpha diversity estimators between bird species and habitats revealed that gulls hosted richer faecal bacterial communities than storks (ANOVA, $p < 0.001$; Fig. 3A). Gull faeces also showed significant differences in bacterial diversity between habitat types (landfill *vs.* paddy field, $p < 0.001$; Fig. 3B), a difference that was not observed for storks ($p = 0.320$; Fig. 3B).

Analysis of beta diversity revealed a significant interaction between sample type and habitat (PERMANOVA, $p = 0.001$), with limited differences between faecal and soil/water samples. Pairwise comparisons yielded significant differences between the composition of faecal bacterial communities and the surrounding environment ($p < 0.05$ for all comparisons). Moreover, the ordination of faecal samples showed clear and significant segregation according to bird species and habitat (PERMANOVA, $p = 0.001$; Fig. 3C). Further pairwise comparisons revealed that bacterial communities differed significantly between bird species and between habitats ($p < 0.001$ for all comparisons).

Differential abundance analyses showed that birds feeding in the landfill carried a higher proportion of priority pathogens. In particular, the genera *Enterococcus* and *Staphylococcus* were prevalent in landfill samples from gulls (mean relative abundances of 5.9 and 3.6 %, respectively) compared to their paddy field samples (0.9 and 0.05 %, respectively) (Fig. 4A). The opposite trend was observed for the mean abundance of the genera *Streptococcus* (9.6 % and 3.9 % in paddy fields and landfill) and *Campylobacter* (0.2 % and 0.04 %). Both the ALDEx2 and ANCOM tests yielded significant differences between habitats for the relative contents of *Staphylococcus* and *Campylobacter* in faecal samples from gulls (ALDEx2, corrected *p*-values <0.05, and <0.01, respectively; ANCOM-corrected *p*-values <0.01, and < 0.001). For storks, landfill samples contained higher relative abundances of several priority pathogens such as *Helicobacter*, *Escherichia-Shigella*, and *Staphylococcus* (10.5 %, 6.7 %, and 4.8 %, respectively) compared to their abundances in paddy fields (<0.01 %, <0.01 %, and 0.02 %, respectively) (Fig. 4B) although they were not.

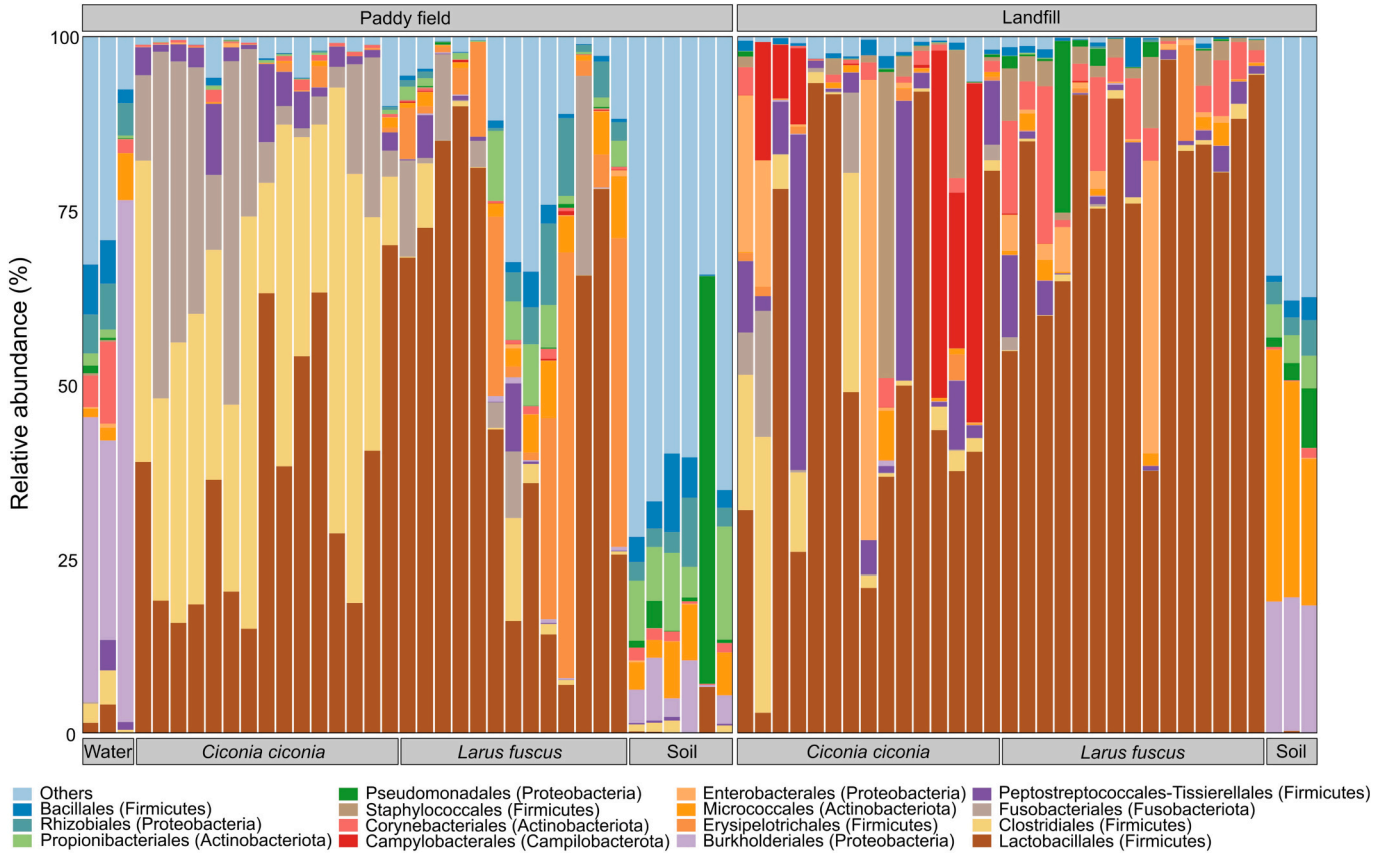

Fig. 2. Birds feeding in landfills show distinct faecal bacterial orders: Relative abundance of bacterial orders in faecal samples from two bird species and the surrounding environment (water and soil) in two habitats (See also Suppl. Table S5).

3.4. Birds feeding in landfills are enriched in ARGs and MGEs

Faecal samples from the landfill carried a higher burden of ARGs and MGEs (Fig. 5; Suppl. Fig. S2). Among the 47 ARGs targeted (Suppl. Table S6), the genes conferring resistance to colistin (*mcr1* and *mcr2*), macrolide-lincosamide-streptogramin B (*mphA*), trimethoprim (*dfrG*), and vancomycin (*vanA* and *vanB*) were below detection limits (Suppl. Fig. S3). Genes conferring resistance to phenicol (*catB2*, *catB3*, and *catB8*), quinolones (*qepA*, *qnrB*, and *qnrS*), and MDR (*cfr*) were detected in only two or three samples (Suppl. Fig. S4). MGEs, integron integrases, and genes conferring resistance to tetracyclines, beta-lactams, sulfonamides, and aminoglycosides were ubiquitous both in bird faeces and in samples from the surrounding environment (Suppl. Fig. S3).

Genes conferring resistance to tetracyclines (*tetA* and *tetM*) differed between habitats and bird species, but both genes showed higher relative abundances in faeces of either species collected at the landfill (-1 $\log_{10}$ *tetM* copies/*16S rRNA* copies for storks and -2 $\log_{10}$ for gulls; -4 $\log_{10}$ *tetA* copies/*16S rRNA* copies for storks and -5 $\log_{10}$ for gulls). In addition, the relative abundance of *tetA* was higher in stork faeces (Suppl. Fig. S5A). Regarding genes conferring resistance to beta-lactams, 13 genes were detected in only a few samples or were below the detection limit of the assay (Suppl. Fig. S5A). The genes bla_{CMY}, bla_{TEM}, and *pbp5* were more prevalent in landfill faeces for both bird species, with storks carrying higher abundances of bla_{TEM} and *pbp5* (-3 and -1 $\log_{10}$ gene copies/*16S rRNA* copies, respectively; Suppl. Fig. S5A).

Genes conferring resistance to carbapenems, such as bla_{VIM} and bla_{KPC}, were only detected in faecal samples from landfill gulls (-5 and -4 $\log_{10}$ gene copies/*16S rRNA* copies, respectively). Regarding resistance to sulfonamides, genes *sul1* and *sul2* were exclusively detected in landfill faeces (from -3 to -5 $\log_{10}$ gene copies/*16S rRNA* copies) and in a few soil and water samples. The gene *acc(3)-iid_iia*, which confers resistance to aminoglycosides, was only detected in a few landfill samples (both bird faeces and soil) collected (Suppl. Fig. S5A).

Regarding MGEs, the genes *tnpA* (encoding a transposase) and the insertion sequences *IS6100* and *ISEfm1* were transversally detected across birds and habitats, but their relative abundances were consistently higher in landfill samples (from -1 to -3 $\log_{10}$ gene copies/*16S rRNA* copies; Suppl. Fig. S5B). Regarding genes encoding integron integrases, we only detected *intI2* in landfill faeces, with stork samples having the highest abundance (-3 $\log_{10}$ gene copies/*16S rRNA* copies).

3.5. Potential hosts of ARGs and MGEs

Before any correlation analysis, we pre-processed and filtered the data to avoid spurious results as described in the Materials and methods section. These steps reduced the original dataset to 23 samples (*i.e.,* six stork faecal samples from paddy fields and six from the landfill, plus five gull samples from paddy fields and six from the landfill), 12 genes, and 480 ASVs. Optimisation of the sPLS model resulted in the selection of one latent component, consisting of a linear combination of 11 genes and 20 ASVs, explaining 51 % of the total variance for the ARG data and 33 % for the microbiota data. We also used a second latent component for visualization. The generated sample plot showed a clear segregation of samples between paddy fields and landfill) (Suppl. Fig. S6A). In contrast to samples from paddy fields, landfill samples were distributed unevenly along the first component, suggesting a large variability between them not explained by bird species. Then, we generated an arrow plot to assess the level of agreement between the two datasets (*i.e.*, bacterial composition and ARG abundance). Most samples were scattered similarly across the space, spanned by the first two latent components according to each of the datasets (faecal microbial composition and ARG abundance; Suppl. Fig. S6B). Subsequently, we constructed a

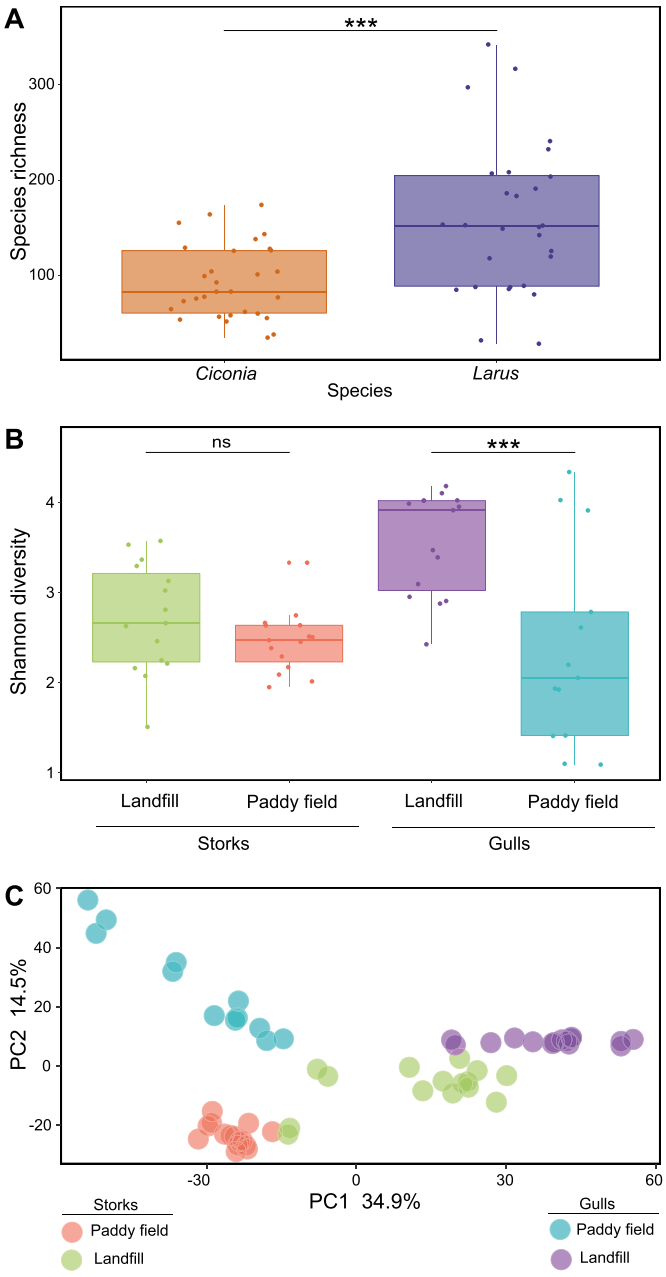

Fig. 3. Birds feeding in landfills show distinct faecal microbial structure: Comparison of (A) species richness and (B) diversity of bacterial communities in faecal samples of two bird species (*C. ciconia* and L. *fuscus*) collected in two habitats. (C) Ordination of faecal bacterial communities according to Aitchison distance, coloured by bird species and habitat. ns: non-significant; *** *p*-value <0.001.

correlation network showing only those ASVs that were positively correlated (Pearson correlation approximation ≥0.7) to ARGs in the first latent component. The *tetM* (tetracycline resistance), bla_{CMY} (beta-lactam resistance), *sul1* (sulfonamide resistance), and *IS6100* (MGE) genes were positively correlated with 11 ASVs, including one ASV taxonomically assigned to the genus *Streptococcus* and three assigned to genera *Peptostreptococcus*, *Peptoclostridium*, and *Peptoniphilus* (class Clostridia), all of them identified as potential pathogens (Fig. 6). The co-occurrence of ASVs and ARGs in both bird species feeding in the landfill suggests the potential hosts for these ARGs (Suppl. Fig. S7).

4. Discussion

To properly address the emergence, transmission, and persistence of AR in the environment, efforts must be focused on the compartments that act as AR reservoirs, with special attention to potential vectors that may contribute to their spread. So far, waterbirds have been overlooked as dispersal agents of microorganisms across biomes (Green et al., 2023). We studied the faecal microbiome and the faecal ARG carriage of two abundant wintering waterbirds in South-west Spain —the white stork and the lesser black-backed gull— in two major habitats differing in their roles as a source (landfill) and sink (paddy fields) of AR (López-Calderón et al., 2023; Martín-Vélez et al., 2020a). We also studied bird diet and pollution status of these two habitats. To the best of our knowledge, this is the first study comparing AR determinants of these two species in landfills and paddy fields, thus allowing the comparison of faecal carriage of ARGs and potential pathogens in the context of environmental pollution and dietary habits. Our working hypothesis that feeding in more polluted sites (*i.e.*, landfills) would increase the faecal burden of ARG and bacterial pathogens was broadly supported by our findings.

4.1. Comparison of pollution levels between study sites

Although we did not measure antibiotics and heavy metals in the landfill, previous studies have demonstrated that landfills are highly polluted with antibiotic residues and other compounds that also select for AR (Wu et al., 2015; You et al., 2018; Yu et al., 2016). Landfills also accumulate huge amounts of diapers and sanitary napkins that are known to contain high concentrations of coliform bacteria, which are positively correlated with the abundance of ARGs in landfills (Sun et al., 2016). Our soil samples from the landfill encompassed a higher diversity of ARGs than those collected at the paddy fields, agreeing with results by Wu and co-workers, who found significant correlations between the concentration of ARGs and those of antibiotics and heavy metals in landfill leachates (Wu et al., 2015).

The paddy fields showed lower concentrations of both antibiotic residues and heavy metals in soil and water than those we measured in bird faeces and from those reported in landfill leachates (Wu et al., 2015; You et al., 2018; Yu et al., 2016). Our results thus confirm that the paddy fields were less polluted than the landfill and that both bird species are exposed to pollution sources due to their movements and their sporadic or regular visits to polluted habitats (*i.e.,* landfills). In soils, antibiotics interact with organic matter and organisms and are subject to biodegradation (Song and Guo, 2014). In contrast, the higher concentrations of metals measured in bird faeces collected from both habitat types may reflect the higher abundances of metals in the food resources of these birds, such as rice and crayfish (Alcorlo et al., 2006; Meharg et al., 2023).

4.2. Habitat use by bird species

The main goal of our study was to demonstrate that feeding in more polluted sites influences the faecal load of both ARGs and potential bacterial pathogens (see below). Dietary analysis allowed us to understand the intensity of habitat use by the two bird species. Diet varied between individuals using different habitats, with the faeces of gulls and storks collected at the landfill being highly enriched in plastic and glass debris, suggesting that they mainly feed on anthropogenic waste. Similarly, Lopes et al. (2021) found a higher proportion of anthropogenic debris in gulls from urban and landfill sites. Thanks to GPS tracking, we know that both species undergo regular movements between landfills and paddy fields during the winter (López-Calderón et al., 2023; Martín-Vélez et al., 2020a). Although some birds we sampled in one habitat had recently been feeding in the other habitat (likely explaining the presence of crayfish in faeces from landfills), the diet analysis confirmed that gulls in the first place and then storks had

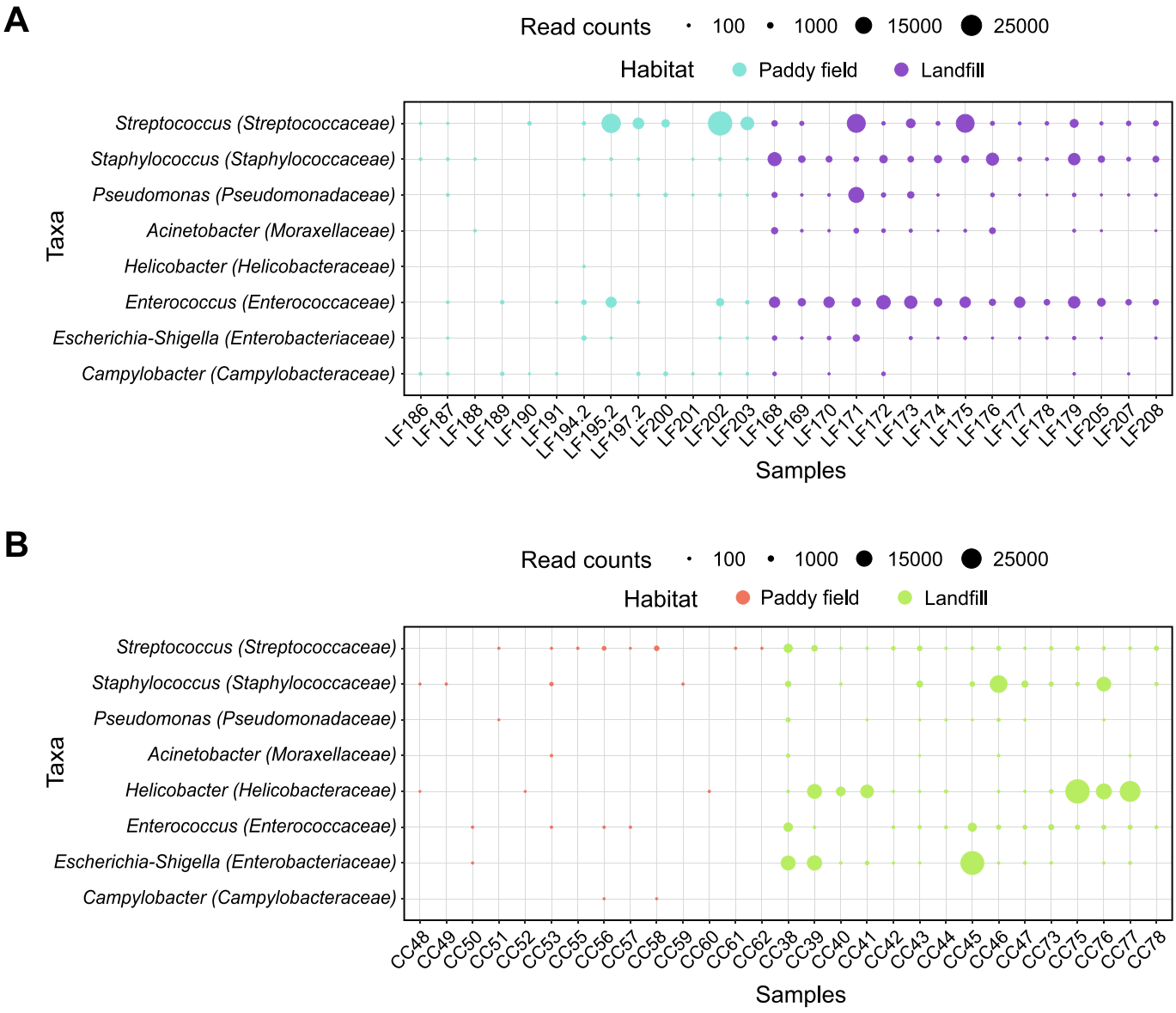

Fig. 4. Birds feeding in landfills show an enrichment of potential pathogens: Read counts assigned to priority bacterial pathogens identified in faecal samples from: (A) *L. fuscus* and (B) *C. ciconia* in two habitats. The diameter of circles is proportional to read counts. Circles are coloured according to habitat type.

been intensively foraging in the habitat where faeces were sampled. In this regard, future studies could benefit from investigating a wider variety of potential food sources, like different habitat types, to enhance our overall understanding of ARG carriage by waterbirds.

4.3. Landfills influence bird faecal microbiota

Host species and the environment are two major drivers determining the diversity and composition of the avian gut microbiome, although the diet also contributes (Cockerham et al., 2019; Dong et al., 2019; Drobniak et al., 2022; Hird et al., 2014; Liu et al., 2020; Waite and Taylor, 2014). We observed a high prevalence of sequences assigned to the order Lactobacillales in faeces from gulls from either habitat, whereas storks showed a more even faecal bacterial community, with prevalence of orders Lactobacillales, Clostridiales, and Fusobacteriales, especially in paddy field samples. These community profiles agree with those reported by Jarma et al. (2021) for the same bird species in the same region two years before our sampling.

Foraging in landfills seemed to alter the composition of the faecal microbiota of both species, although differences were more pronounced for gulls. Bacterial communities in gull faeces collected from landfills had a higher diversity compared to those collected from paddy fields, a result that disagrees with those from Fuirst et al. (2018), who found higher microbial diversity in herring gulls from less urban colonies that visit a wider variety of foraging sites. Gull faeces contained a higher presence of plastic debris than those from storks, suggesting a higher foraging activity in landfills and possibly an indirect effect of plastic particles on bacterial diversity by providing additional carbon sources and substrates for colonization (Amaral-Zettler et al., 2020; Sheridan et al., 2022). On the other hand, the greater microbial richness in the herring gull study is likely to be due to a greater diversity of feeding habitats in both continental and marine ecosystems (Klaassen et al., 2012), whereas our lesser black-backed gulls were only feeding in two habitats (paddy fields, or landfills, see also Martín-Vélez et al., 2020b).

Differences in the composition of faecal bacterial communities were more pronounced between habitats than between species, an observation that suggests that ecological factors (*e.g.*, feeding ecology) can be preponderant in determining microbial communities over biological traits (*e.g.*, phylogeny, size). However, the habitat use of white storks is entirely terrestrial (Bécares et al., 2019; López-Calderón et al., 2023),

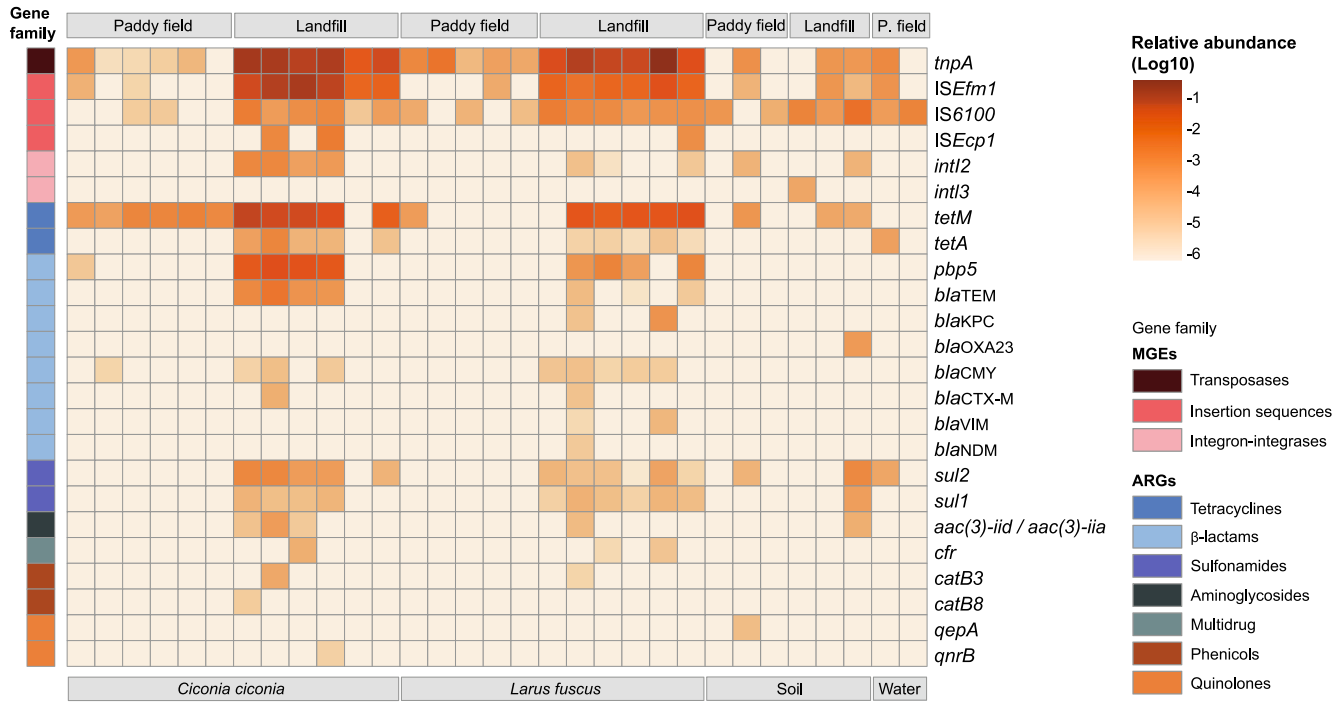

Fig. 5. Birds feeding in landfills are enriched in ARGs and MGEs: Heatmap showing the relative abundance (log scale) of ARGs, MGEs and integrons in faecal samples from L. *fuscus* and *C. ciconia*, and the surrounding environment (soil, surface water) in two habitats. Genes are grouped according to the antibiotic family they confer resistance to. All genes related to gene mobilization and capture were grouped as mobile genetic elements (MGEs) (See also Suppl. Fig. S2, S3, S4 and S5, and Suppl. Table S6).

and they can feed in fewer habitats compared to lesser-black backed gulls, which use both marine and inland habitats (Martín-Vélez et al., 2020a). The greater diversity of habitats explored by gulls likely exposes this species to a broader range of microorganisms and pollutants (Cockerham et al., 2019), explaining the higher richness of their bacterial communities compared to white storks. For example, when feeding in paddy fields, only the gulls sometimes roost in the Guadalquivir River (van Rees et al., 2021), where they are likely exposed to different bacteria.

Besides the dominance of Lactobacillales, the other prevalent orders in faeces collected at the landfill (*e.g.*, Staphylococcales, Campylobacterales, Enterobacterales) include well-known human and animal bacterial pathogens. Although both birds hosted species-specific microbiota, highly polluted habitats seemed to standardise the bacterial communities of these two avian species, as landfill samples were closer in the PCA plot.

4.4. Faecal carriage of ARGs and MGEs in landfill samples

Gulls and storks feeding in the landfill showed a greater diversity and higher abundance of ARGs, particularly genes conferring resistances to tetracyclines (*tetM*, *tetA*), beta-lactams (*pbp5*, bla_{TEM}, bla_{CMY}) and sulfonamides (*sul1*, *sul2*), agreeing with the high content in antibiotic residues (particularly tetracyclines —doxycycline and oxytetracycline— and sulfonamides —sulfamethoxazole—) measured in faecal samples. However, no beta-lactam residues were detected in faeces probably due to their chemical instability (Liu et al., 2018; Wang et al., 2020). For this reason, weak correlations are usually measured between the environmental concentration of beta-lactams and those of ARGs conferring resistance to them (Wang et al., 2020). Jarma et al. (2021) also found greater diversity and abundance of ARGs in these two species compared to two other birds that do not use landfills.

The repertoire of ARGs measured in the analysed samples has often been reported in faeces from both gulls (*tetA*, bla_{CTX-M} and bla_{TEM} in lesser black-backed gulls, Carroll et al., 2015; Stedt et al., 2015) and, especially, white storks (*tetM* and *tetA*, Poeta et al., 2005; Lozano et al., 2016; Skarżyńska et al., 2021; bla_{TEM}, bla_{CTX-M}, bla_{CMY}, and bla_{NDM}, Höfle et al., 2020; Migura-Garcia et al., 2020; Skarżyńska et al., 2021; Loucif et al., 2022; and the gene *aac(3)-iia* that confers resistance to aminoglycosides, Skarżyńska et al., 2021). Of special concern was the detection of two carbapenemases (bla_{NDM}, bla_{VIM}) and a class A beta-lactamase (bla_{KPC}) in two landfill faecal samples from gulls, confirming that these birds carried bacteria resistant to a wide range of beta-lactams, including last-resort antibiotics such as carbapenems and 4th generation cephalosporines (Poole, 2004). Interestingly, these genes were not detected in faecal samples from storks or in soil or water samples.

In comparison to a previous study in the same region comparing the carriage of ARGs by both bird species (Jarma et al., 2021), we measured higher concentrations of bla_{KPC} (in gulls) and bla_{TEM} (in storks) and lower concentrations of *sul1* (in both gulls and storks). However, genes conferring resistance to tetracyclines were the most abundant in both studies and for both species (*tetM* in the present study; *tetW* in Jarma et al., 2021). In turn, using the HT-qPCR approach we were unable to detect some ARGs that have been consistently measured in faecal samples from gulls and storks, such as the quinolone resistance gene *qnrS* (Jarma et al., 2021; Skarżyńska et al., 2021), the carbapenemase gene bla_{OXA48} (Bouaziz et al., 2018; Loucif et al., 2022), or the colistin resistance gene *mcr1* (Jarma et al., 2021; Loucif et al., 2022).

MGEs (*e.g.*, plasmids) associated with some ARGs have previously been described in bacteria isolated from wild birds (Höfle et al., 2020; Migura-Garcia et al., 2020; Tarabai et al., 2019), and this can facilitate transference of ARGs from one species to another. In our study, faecal samples from both bird species collected at the landfill also contained MGEs (*i.e.*, transposase and insertion sequences). Although we do not know the genetic context of the associated ARGs, the co-occurrence with MGEs may facilitate ARG transmission. Indeed, transposable elements including insertion sequences may be the main disseminating agents of ARGs, in addition to strong antibiotic selection pressure (Ebmeyer et al., 2021).

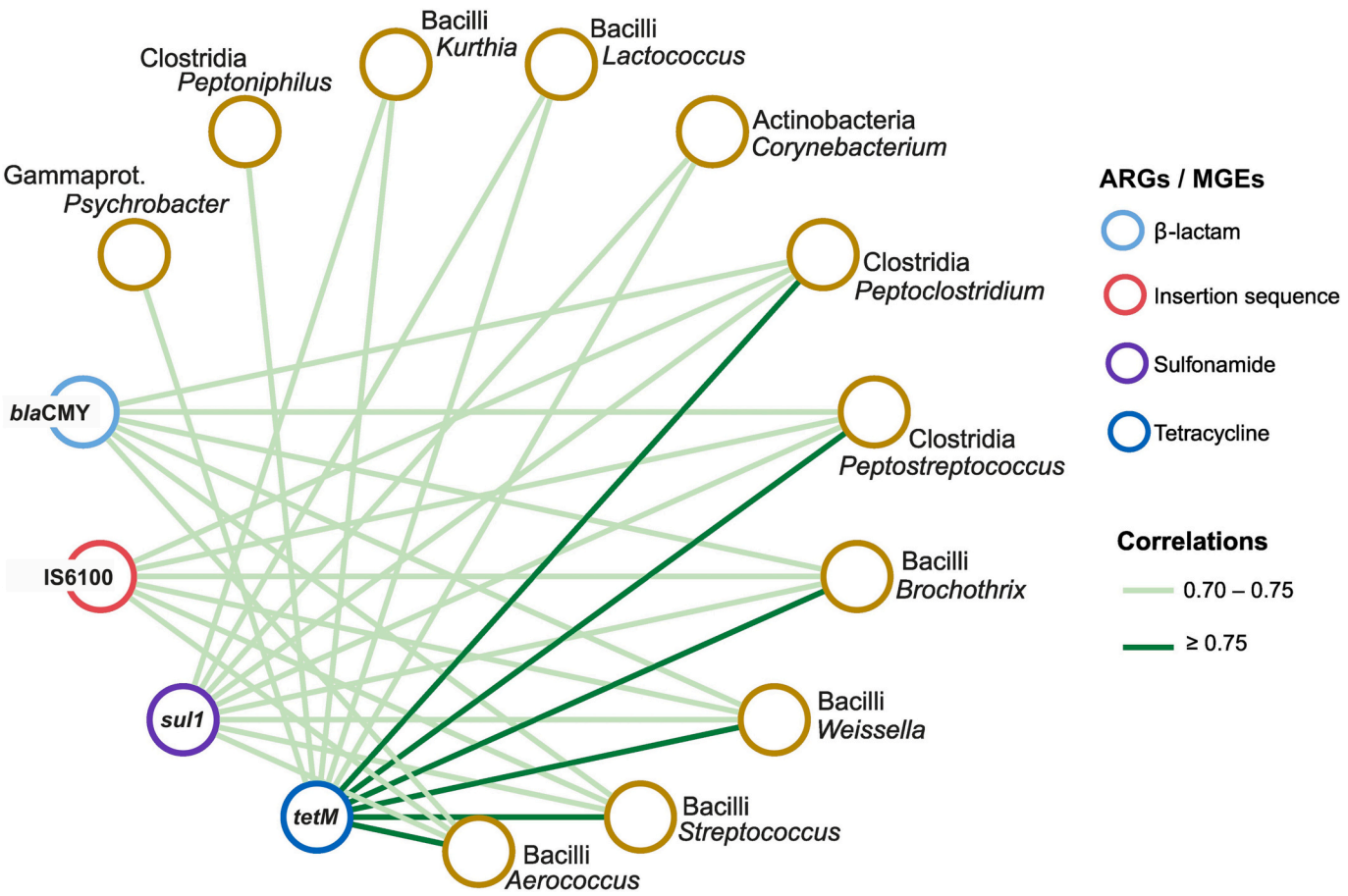

Fig. 6. Potential hosts of ARGs and MGEs: Network representation of sPLS performed on microbiota and ARG data from faecal samples from L. *fuscus* and *C. ciconia*, in two habitats. The network is bipartite, where each edge links an ASV to an ARG node, according to a similarity matrix. Only correlations above 0.7 from the first latent component are shown (See also Suppl. Fig. S6 and S7).

4.5. Landfills as a source of potential bacterial pathogens and associated ARGs

Faeces from both bird species, especially those collected at the landfill, contained sequences potentially assigned to bacterial pathogens, sequences assigned to the genus *Streptococcus* being particularly important in gull faeces (Ryu et al., 2012). However, being solely based on sequence similarity, our results must be regarded with caution and only as indicative of the potential presence of such pathogens. Additional confirmatory tests (identification of specific virulence gene markers or isolation of pathogens using cultivation-based techniques) would be necessary to confirm our gene-based observations and to unequivocally assign the true pathogenicity to a given ASV.

Our results point to the human waste accumulated in the landfill as the likely source of potentially pathogenic bacteria, agreeing with previous studies on (Alm et al., 2018; Dolejska et al., 2016). When feeding in these polluted habitats, birds may become colonised by bacteria carrying ARGs, including potential pathogens, as previously demonstrated for resistant *E. coli* in ring-billed gulls and mallards (Franklin et al., 2020; Sandegren et al., 2018). The avian gut could then act as a melting pot for ARG exchange through horizontal gene transfer, even from phylogenetically distant bacteria, as reported in mammals (Shterzer and Mizrahi, 2015).

We tried to identify potential hosts for the detected ARGs using co-occurrence network analysis (Li et al., 2015) Our hypothesis posited that non-random co-occurrence patterns among 11 bacterial genera and ARGs might provide information about the potential hosts for these ARGs. Notably, representatives of the Bacilli and Clostridia classes exhibited higher correlation values with specific ARGs. This agrees with previous studies showing that members of the Firmicutes are the second dominant host for resistance genes (Rice et al., 2020) and that gene *tetM* is commonly carried by members of *Streptococcus*, *Corynebacterium*, *Lactococcus*, *Peptostreptococcus*, and *Peptoniphilus*, whereas *sul1* is carried by *Corynebacterium* (Forslund et al., 2013; Zhang et al., 2009). These results, however, should be treated with caution because they are solely based on the correlation between recovered sequences (ARGs and ASVs) and their confirmation would require additional steps such as culturing and whole-genome sequencing of resulting isolates.

4.6. Dissemination of ARGs by waterbirds and future perspectives

Landfills are known to be key nodes in the connectivity networks of gulls (Martín-Vélez et al., 2020b) and storks (López-Calderón et al., 2023). We have shown that both avian species are reservoirs of ARGs with the potential to disseminate them to other habitats. Recently López-Calderón et al. (2023) showed that after feeding in landfills, storks move to a range of habitats (including paddy fields, other agricultural fields, and saltpans) for resting, with implications for human health as these are habitats used for food production. In this regard, further studies will have to consider the impact of the time spent by birds in each habitat type on the acquisition and prevalence of ARGs and ARB.

The increase in the numbers of storks and gulls in recent decades is of concern considering their interaction with urban areas, the high carriage of antibiotic-resistant determinants (both ARB and ARGs) in their faeces, and their migratory behaviour. In comparison to white storks, lesser black-backed gulls could be considered of more concern since they are

approximately ten times more abundant in the paddy fields (Rendón et al., 2008). Human-associated microorganisms, including pathogens, can be spread by gulls through their migration routes from dumps to beaches, reservoirs, ports, or other places where they may pose a risk to human health (Alm et al., 2018; Fuentes-Castillo et al., 2023; Martín-Vélez et al., 2020a).

Overall, environmental containment strategies for managing food waste and decreasing gull and stork numbers in landfills are required to control the dissemination of AR determinants or other microbial-related risks (*e.g.*, avian-flu; Alexander, 2000). These strategies may include: *i*) improving food waste collection policies to limit inputs to landfills; *ii*) covering landfill waste to limit the access of wild birds to garbage, as already applied in some landfills; and *iii*) implementing bird deterrent measures in the landfills (*e.g.*, through falconry). A recent study showed positive outcomes of urban waste management/collection policy on the breeding population of yellow-legged gulls (Coccon et al., 2022).

In future, epidemiology studies are needed to infer which resources from landfills act as real sources of AR transferred to birds. More studies are also needed to experimentally assess the carriage time and horizontal transmission of ARB and ARGs. This information is key to knowing how birds can disseminate bacteria at different geographical scales. The few existing studies on microbial retention time show that aquatic birds can carry ARB for up to one month after infection (Girdwood et al., 1985; Palmgren et al., 2006; Sandegren et al., 2018) and mediate rapid transmission between individuals, allowing the persistence of resistant bacteria (Sandegren et al., 2018). Previous studies have also suggested that gulls are not long-term reservoirs of antibiotic-resistant bacteria; rather, they act as host bridges that are transiently populated through drinking water or food (Franklin et al., 2020). Furthermore, waterbirds may also "ingest" ARB and ARGs in other forms which do not colonize the gut, but instead are egested in faeces quickly afterwards as are other propagules (Green et al., 2023) and modelized in flies' gut (Inamine et al., 2018). However, further research is needed to establish how long microbes can persist in the bird gut.

4.7. Conclusions

Our findings collectively suggest that the extensive utilization of landfills by overwintering gulls and storks in SE Spain may contribute to the enrichment of ARGs and potential bacterial pathogens within their guts. This phenomenon raises concerns regarding the potential threat at the human/animal interface. Investigating the role of birds as vectors for the spread of antibiotic resistance determinants across different spatial scales is thus a promising avenue within the 'One-Health' framework.

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CRediT authorship contribution statement

Oriol Sacristán-Soriano: Formal analysis, Methodology, Visualization, Writing – original draft. **Dayana Jarma:** Conceptualization, Formal analysis, Methodology, Visualization, Writing – original draft. **Marta I. Sánchez:** Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing. **Noelia Romero:** Formal analysis, Methodology, Writing – review & editing. **Esteban Alonso:** Formal analysis, Methodology, Writing – review & editing. **Andy J. Green:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Alexandre Sànchez-Melsió:** Methodology, Validation. **Francisco Hortas:** Methodology, Resources, Writing – review & editing. **José Luis Balcázar:** Visualization, Writing – review & editing. **Juan Manuel Peralta-Sánchez:** Methodology, Resources, Writing – review & editing. **Carles M. Borrego:** Conceptualization, Funding acquisition, Methodology, Resources, 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.

Data availability

Sequence datasets were deposited in the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA875258. Part of the code is available in the Supplementary File S1

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