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High levels of antibiotic resistance genes and opportunistic pathogenic bacteria indicators in urban wild bird feces☆

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ABSTRACT

This study analyzed fresh feces from three common bird species that live in urban environments and interact with human communities. Antibiotic resistance genes (ARGs) encoding resistance to three major classes of antibiotics (i.e., tetracyclines, β-lactams, and sulfonamides) and the mobile genetic element integrase gene (*intI1*) were abundant (up to 10^9, 10^8, 10^9, and 10^{10} copies/g dry feces for *tetW*, bla_{TEM}, *sul1*, and *intI1*, respectively), with relative concentrations surprisingly comparable to that in poultry and livestock that are occasionally fed antibiotics. Biomarkers for opportunistic pathogens were also abundant (up to 10^7 copies/g dry feces) and the dominant isolates (i.e., *Enterococcus* spp. and *Pseudomonas aeruginosa*) harbored both ARGs and virulence genes. ARGs in bird feces followed first-order attenuation with half-lives ranging from 1.3 to 11.1 days in impacted soil. Although residual antibiotics were detected in the feces, no significant correlation was observed between fecal antibiotic concentrations and ARG relative abundance. Thus, other unaccounted factors likely contributed selective pressure for ARG maintenance. These findings highlight the contribution of wild urban bird feces to the maintenance and dissemination of ARGs, and the associated health risks.

1. Introduction

The global spread of antibiotic resistance poses a growing threat to public health (O'Neill, 2014) and requires improved understanding of ARG sources and vectors to mitigate the associated risks (Vikesland et al., 2017; Wu et al., 2018). The resistome carried by wildlife could be an important but underappreciated factor in ARG dissemination (Allen et al., 2010), and urbanization could amplify such risks as it fosters many overlapping habitats and frequent interactions between wildlife populations and humans (Magle et al., 2012).

Urban wild birds tend to have a relatively high population density (1.13–1.18 birds/resident) (Fuller et al., 2009) and opportunities to interact with human communities (Luniak, 2004; Magle et al., 2012) since urban settings generally have abundant food sources, low predator pressure, and a milder microclimate. Urban birds also have a high probability of exposure to water sources contaminated with antibiotics (Xu et al., 2016; Zhang et al., 2018), which may impact the abundance and diversity of ARGs within their gut and fecal microbiomes. Additionally, urban wild birds may serve as vectors for antibiotic resistant bacteria (ARB) derived from anthropogenic sources, as demonstrated by studies revealing clinically important ARB in bird feces (Ahlstrom et al., 2018; Hernandez et al., 2013). These bird-carried ARB and ARGs can be transferred to urban residents through swimming in feces-polluted waters, dermal contact with bird feces (or impacted soil) during outdoor recreational activities (Tsiodras et al., 2008), or inhalation of aerosolized fecal particles (Feddes et al., 1992). Therefore, it is important to investigate the abundance, diversity, and bacterial hosts of ARGs harbored by common urban wild birds.

Previous studies have conducted culture-based identification of ARB in bird feces (Dolejska and Literak, 2019; Wang et al., 2017) or analyzed ARGs harbored by wild birds near ARG hotspots (e.g., wastewater treatment plants (Marcelino et al., 2019) and ARG-polluted rivers (Wu et al., 2018)). However, there is a need to assess the relative abundance, diversity, and seasonal persistence of ARGs and their co-occurrence with virulence genes after transport and deposition by wild birds in densely populated environments to inform the associated risks.

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In this study, fecal samples deposited by common urban wild birds (i.e., ducks, crows, and gulls) were collected from highly frequented sites in Houston metropolitan areas. ARGs and the mobile genetic element (MGE) integrase gene (*intI1*) were measured and compared with those found in poultry and livestock. These ARGs are commonly reported in the environment and were thus selected to facilitate comparison with other studies. Opportunistic pathogen levels were quantified by specific biomarker abundance, and their antibiotic resistance and potential virulence were evaluated by plate assays followed by PCR analyses. The natural attenuation of ARGs in impacted soil was then monitored to assess their persistence over different seasons. Possible selective pressure by residual antibiotics and their co-occurrence and correlation to ARG abundance were also considered.

2. Materials and methods

2.1. Wild bird selection and fecal sample collection

Three common Houston wild bird species (i.e., ducks, crows, and gulls) with diverse foraging and migratory habits were selected (Table 1) (Lockwood and Freeman, 2014; Oberholser et al., 1974) due to their abundance and frequent interactions with humans. We observed when these birds defecated, and fresh fecal samples from a given species were separately collected and homogenized as follows. Freshly deposited feces of each bird species were collected in 20-mL sterilized scintillation vials from two outdoor sites (i.e., nearby parks and beaches) in both summer and winter seasons (Table 1, Fig. S1). Specifically, six evenly distributed sampling locations were designated in each site, and two sites were chosen for each bird species to corroborate results. Thus, following common sample collection strategies (Guo et al., 2018; Hurst et al., 2019; Thames et al., 2012), fresh feces from a given bird species and specific site were pooled and homogenized for overall characterization. Each site was sampled three times per season, making 36 fecal pools in total. Each homogenized fecal pool (about 60–100 g fresh weight) was divided into four aliquots under aseptic conditions. One aliquot was used for culture-based microbial analysis, one for genetic biomarker analysis, one for ARG natural attenuation test, and one for chemical analysis. Detailed sampling information including bird species, sampling sites, and amount of sampled feces are provided in Table S1.

2.2. Quantification of selected ARGs and intI1

One aliquot of fecal samples was dried by lyophilization (Millrock Technology, USA), which is a common strategy in dehydration of soil and manure samples for ARG analysis (He et al., 2014; Luo et al., 2010). Microbial DNA was extracted from the dried samples using the FastDNA Spin Kit for soil (MP Biomedicals, Solon, OH) following the manufacturer's instructions. Phage λ DNA was used as an internal standard to calculate DNA recovery efficiency which was in the range of 89%–95%. Quantitative PCR (qPCR) was performed on CFX96TM Real-Time System to quantify selected ARGs (i.e., *sul1*, *sul2*, *tetW*, *ampC*, and bla_{TEM}), 16S rRNA, and an MGE indicator (*intI1*) (Table S2). The 16S rRNA gene was included to quantify the total bacterial amount and to normalize the abundance of ARGs in different samples. These details are available in the Supporting Information (SI).

2.3. Chemical analyses of antibiotics

To explore the potential selective pressure for ARGs, the concentrations of nine antibiotics were analyzed in lyophilized fecal samples (He et al., 2014; Hurst et al., 2019), including four tetracyclines (tetracycline (TET), chlortetracycline (CTC), doxycycline (DOXY), and oxytetracycline (OTC)), one β-lactam (ampicillin (AMP)), and four sulfonamides (sulfadiazine (SD), sulfadimethoxine (SDM), sulfamethoxazole (SMX), and sulfachloropyridazine (SCP)). The concentrations of these antibiotics were determined by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) following a previously reported protocol (Luo et al., 2011). Briefly, lyophilized fecal samples were first extracted with extraction buffer (15 mL methanol, 5 mL 0.1 M Na_2-EDTA, and 10 mL citrate buffer with pH of 5). The supernatant samples were then pretreated with Strata strong anion exchanger cartridges (Thermo scientific, USA) followed by extraction with Oasis hydrophilic-liphophilic balance (HLB) cartridges (Waters, Watford, UK). The analyte separations were carried out on the Intersil ODS-3 column (GL Sciences, Japan) for HPLC-MS/MS analysis with the gradient program described in the same protocol (Luo et al., 2011). Antibiotic recovery rates were 71–90% and the limit of quantification (LOQ) was 1.8–2.0 ng/g.

2.4. Tracking the natural attenuation and dissemination of fecal ARGs

To track the natural attenuation of ARGs, one aliquot of fresh feces from each homogenized fecal pool was placed in the initial sampling sites and protected by artificial fences to avoid external physical disruption. Aliquots (0.5 g) of feces were collected after one, three, five, and seven days, respectively. The samples were subjected to ARG quantification via qPCR, and total viable cultivable bacteria extracted by Nycodenz gradient centrifugation (Maron et al., 2006) were quantified via plate assays. The attenuation trends were fitted with first-order kinetics (Burch et al., 2014) and the attenuation coefficients (k, day^{-1}) for both ARGs and bacteria were determined using: $k = (\ln(C_0) - \ln(Ct))/t$, where C_0 and Ct represent abundance (copies of ARG/g dry weight, CFU/g dry weight) initially and after t days, respectively.

Table 1
Wild bird selection and fecal sample collection.

Bird name	Binomial name	Feeding	Migrations [a]	Sampling areas	Sampling time
Duck	*Cairina moschata*	Omnivores (grasses, tubers, insect, worms, small fish et al.)	Nonmigratory	Hermann Park George Bush Park	Aug.–Sept., 2018 (Temperature 98/70 °F)> Jan.–Feb. 2019 (Temperature 79/32 °F)
Crow	*Corvus brachyrhynchos*	Omnivores (seeds, grains, nuts, fruit, insects, worms et al.)	Partially migratory	Oyster Creek Lake Chinatown	
Gull	*Larus atricilla*	Opportunistic omnivore (earthworms, insects, snails, crabs, fish, squid et al.)	Partially migratory	Surfside Beach Porretto Beach	

[a] Nonmigratory indicates that all the birds are residents, partially migratory indicates that a fraction of the birds are residents while others fly from the north in winter to Houston (Banks et al., 2004; Lockwood and Freeman, 2014; Oberholser et al., 1974).

To investigate the dissemination of fecal ARGs in the receiving environments, topsoil (up to 1 inch depth) was collected in the North, South, East, and West directions in four concentric circles at a distance of 0, 1, 2, and 4 ft away from four targeted duck feces (freshly voided) in Hermann Park, Houston, in both summer and winter seasons. Both summer and winter samples were collected in the same region of the park, and no other feces were observed within this area. Grass, branches, and stones were carefully avoided during sampling. Soil samples were sieved through a 2 mm mesh standard test sieve (Fisher Scientific, USA), dried by lyophilization (Millrock Technology, USA), and subjected to DNA extraction and qPCR analyses following the aforementioned protocol.

2.5. Quantification antibiotic resistance indicators and potential opportunistic pathogens

Escherichia coli (*E. coli*) and *Enterococcus* spp. are widely considered as antibiotic resistance indicators since they can easily acquire and transfer ARGs (Berendonk et al., 2015; Radhouani et al., 2012). Species- and genus-specific functional genes (Yu et al., 2018) were chosen as biomarkers to verify and quantify *E. coli* and *Enterococcus* spp. in bird feces by qPCR (Table S3). Potential opportunistic pathogens, including *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Clostridium perfringens*, *Vibrio vulnificus*, and *Salmonella enterica*, were also quantified by species-specific functional genes (Table S3) since they were previously identified in bird cloacal or fecal samples (Fernández-Delgado et al., 2016; Merkeviciene et al., 2017; Shane and Gifford, 1985). *Enterococcus* spp. were also considered as potential opportunistic pathogens in our study since major species in this genus are known to carry virulence genes (Han et al., 2011; Poeta et al., 2005; Sidhu et al., 2014; Song et al., 2019).

2.6. Characterization of antibiotic resistance indicators and opportunistic pathogens

Selective media was used to isolate *E. coli*, *Enterococcus* spp., and *P. aeruginosa* for further characterization due to their high abundance of biomarkers in our primary tests. Fecal bacteria extracted by Nycodenz gradient centrifugation (Maron et al., 2006) were inoculated on specific isolation agar medium, including Eosin methylene blue (EMB) agar (Hardy Diagnostics, USA) (Leininger et al., 2001), Pfizer Selective *Enterococcus* Agar (HIMEDIA, USA) (Liu, 2015), and *Pseudomonas* Isolation Agar (BD Biosciences, USA) (Grobe et al., 1995), for the isolation and cultivation of the three targeted bacterial species (or genus), respectively.

After incubation at 37 °C overnight, well-separated colonies were subjected to colony PCR followed by 16S rRNA gene sequencing to ensure their taxonomy. Potential antibiotic resistance was verified by determining the presence of the five targeted ARGs via PCR analyses and investigating the viability of ARG-positive colony isolates on agar spiked with corresponding antibiotics (Ampicillin 100 mg/L, Tetracycline 10 mg/L, and Sulfanilamide 10 mg/L). The *Enterococcus* spp. and *P. aeruginosa* isolates were subjected to multiplex PCR to assess whether they carried virulence genes. Targeted virulence genes for *Enterococcus* spp. were *gelE* (encoding gelatinase), *asa1* (encoding pheromone-inducible protein), *cad1* (encoding pheromone cAD1 precursor lipoprotein), *fsr* (encoding regulator of *gelE* expression), and *efm* (encoding cell wall adhesin) (Han et al., 2011; Poeta et al., 2005; Sidhu et al., 2014; Song et al., 2019). Targeted virulence genes for *P. aeruginosa* were *lasB* (encoding elastase), *toxA* (encoding exotoxin A), *plcH* (encoding elastase), *exoS* (encoding exotoxin S), and *algD* (encoding alginate) (Fazeli et al., 2014). Details of PCR and multiplex PCR are included in the SI. The proportion of ARG positive, virulence gene positive, and both ARG and virulence gene positive isolates were calculated for both *P. aeruginosa* and *Enterococcus* spp.

2.7. Statistical analysis

ANOVA analysis was performed to compare the differences between various fecal samples in ARG abundance and degradation rate. Pearson correlation analysis was also conducted to further characterize the relationship between antibiotic concentrations and ARG relative abundance, using SPSS 26.0 software. Differences were considered to be significant at the 95% confidence level ($p < 0.05$).

3. Results and discussion

3.1. High levels of ARGs and intI1 were present in urban wild bird feces

ARGs encoding resistance to tetracycline, β-lactam, and sulfonamide antibiotics were detected in all bird fecal pools (n = 36) of the three bird species in both summer and winter. Their absolute abundance was (in copies/g dry feces) up to 10^9 for *tetW*, 10^{10} for *ampC*, 10^8 for bla_{TEM}, 10^9 for *sul1*, and 10^9 for *sul2* (Fig. S2). The corresponding relative abundance varied from 10^{-3} to 10^{-2} copies/16S rRNA for ducks, 10^{-5} to 10^{-2} for crows, and 10^{-5} to 10^{-1} for gulls. Notably, the relative abundance of ARGs, especially in the feces from ducks and gulls, were comparable to those found in the fresh feces of poultry (e.g., chicken (Cheng et al., 2013; Le Devendec et al., 2016; Lin et al., 2017; Mu et al., 2015)) occasionally fed with antibiotics (Fig. 1). Considering their high population (1.13–1.18 birds/resident) (Fuller et al., 2009) and mobility, this finding highlights the role of wild birds as urban ARGs reservoir and potential vectors.

The MGE indicator *intI1* was also detected in all fecal pools with relative abundance ranging from 10^{-3} to 10^{-1} copies/16S rRNA for all three wild bird species. *IntI1* levels in the feces of gulls were over five times more abundant than reported for farm animals (Fig. 1), indicating the potential for horizontal transfer of ARGs in bird gut and receiving environments. Note that *intI1* concentrations were positively correlated with *sul1* (Pearson correlation coefficient $r = 0.80$, $p < 0.05$) and total of β-lactam resistance gene concentrations (*ampC* plus bla_{TEM}, $r = 0.92$, $p < 0.05$), which corroborates previous suggestions of their close genetic link in environmental settings (Ma et al., 2017). High correlation ($r = 0.95$, $p < 0.05$) between *intI1* and total ARG abundance suggests that *intI1* could be an important indicator of ARGs in urban bird feces.

Variations in fecal ARG profile were observed among bird species, probably due to differences in their ecological niches, foraging preference (Lockwood and Freeman, 2014; Oberholser et al., 1974), and gut microbiome (Grond et al., 2018; Marcelino et al., 2019). Interestingly, crow feces harbored a significantly lower level of ARGs in the summer compared to ducks and gulls. In addition, crow feces were the only ones presenting a significant seasonal difference in ARG abundance ($p < 0.05$), possibly due to seasonal variance of suitable food resources (Dolejska and Literak, 2019; Luniak, 2004). Crows may forage more in highly populated areas and waste disposal sites during the winter due to scarcity of their natural substrates (e.g., seeds, grains, fruit, insects, and worms) (Luniak, 2004; Preininger et al., 2019), and therefore probably ingest more frequently food contaminated with ARB (Heringa et al., 2010) and resistance-inducing substances (Lu et al., 2018). Overall, these results suggest that urban wild birds may facilitate ARG dissemination in urban environments through their feces. Given the short-term (one-year) sampling period and that other seasonal

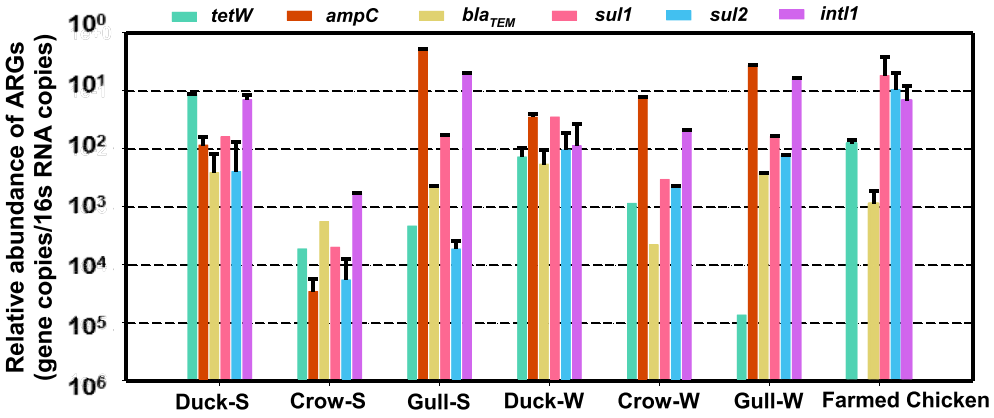

Fig. 1. Similar relative abundance of ARGs in the feces of urban wild birds with that reported for the fresh feces of farmed poultry that are occasionally fed antibiotics. ARGs abundance (copies of ARGs/copies of 16S rRNA gene) was the mean of six fecal pools from two sampling sites, with error bars representing ± one standard deviation. The abundance of ARG and *intI1* reported for poultry was calculated by averaging the mean values from multiple studies for each gene. The standard deviation was calculated based on the mean values reported in each study.

factors (e.g., bird populations) might affect sample variability, further long-term investigation may be required to corroborate the seasonal ARG profile differences in the targeted bird feces.

3.2. Presence of residual antibiotics in bird feces may partially contribute but does not fully explain high abundance of ARGs

To explore whether the selective pressure exerted by residual antibiotics could account for the high level of ARGs observed in bird feces, the concentrations of their corresponding antibiotics were measured. Average concentrations of tetracyclines, β-lactams, and sulfonamides in fecal pools were 35.18, 4.33, and 8.85 ng/g dry feces, respectively (Table S5), which were generally 1–3 orders of magnitude higher than those in urban rivers, lakes, and seawater (Arpin-Pont et al., 2016; Batt et al., 2016). The detected antibiotics concentration are below their minimum inhibitory concentrations (MIC) (about 0.01%–0.1% of standard MIC values (CLSI, 2019; EUCAST, 2019)), which generally contributes selective pressure to sustain high levels of ARGs in bird gut microbiomes and excreted feces (Gullberg et al., 2011; Liu et al., 2011; Lundström et al., 2016; Wistrand-Yuen et al., 2018). However, Pearson analysis revealed no significant correlation between the concentrations of tetracycline or β-lactam antibiotics and the total relative abundance of their corresponding ARGs ($r = -0.49$, $p = -0.162$ for tetracyclines; $r = -0.16$, $p = -0.488$ for β-lactams). A stronger correlation was observed between sulfonamides and their resistance genes ($r = 0.70$), but this was not statistically significant either ($p = 0.704$) (Fig. 2, Table S6). These results suggest that residual antibiotics may have contributed but were not fully responsible for the high abundances of ARGs in bird feces, and underscores the need to discern the importance of other potential etiological factors such as host diet, age, idiosyncratic microbiome structure, and other stressors.

Urban wild birds can be exposed to antibiotics in their food and water from anthropogenic sources (Batt et al., 2016; Boonsaner and Hawker, 2013), which may also contain ARG co-selecting elements

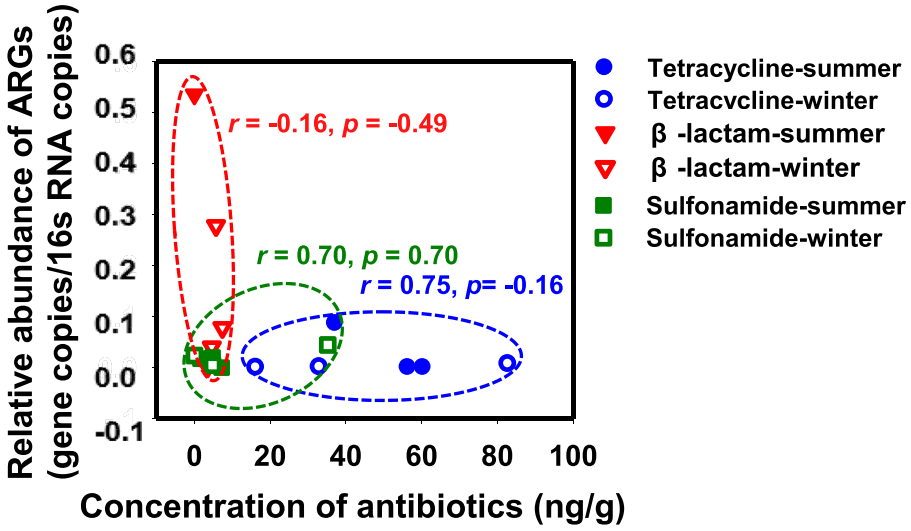

Fig. 2. Insignificant correlation between the antibiotic concentrations and the total relative abundance of corresponding ARGs. The dashed red circle highlights the relationship between β-lactams and the sum of bla_{TEM} and *ampC*, green squares for sulfonamides and the sum of *sul1*and *sul2*, and blue dots for tetracyclines and *tetW*. Solid symbols represent summer feces from three bird species, while hollow ones for winter feces. *J* is the Jaccard similarity index (which was significant in all cases), and *r* is Pearson correlation coefficient (not significant). Each symbol is the average level of antibiotic and ARG in six fecal pools. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

such as heavy metals (Baker-Austin et al., 2006; Li et al., 2018), disinfectants (e.g., dibromoacetic acid) (Lv et al., 2014), and antimicrobial chemicals (e.g., triclosan) (Lu et al., 2018) that are capable of inducing and promoting antibiotic resistance. Additionally, other bird substrates such as surface water (Xu et al., 2016), grass (Yan et al., 2019), insects (Zurek and Ghosh, 2014), and fish (Abgottspon et al., 2014) could also be contaminated or colonized by ARB and therefore influence bird gut resistome. Moreover, common toxin-antitoxin systems are known to enhance plasmid maintenance, along with co-located ARGs and other genes, providing a potential mechanism by which ARGs may be maintained in the absence of selective pressure by residual antibiotics (Yang and Walsh, 2017).

3.3. Moderate persistence and dissemination of fecal ARGs in natural environments

Natural attenuation (e.g., hydrolysis and photolysis (Strickler et al., 2015)) of three representative ARGs (i.e., *tetW*, bla_{TEM}, and *sul1*) and *intI1* in bird feces followed first-order kinetics ($R^2 > 0.75$) with attenuation coefficients (k) between 0.058 and 0.535 day^{-1} (Table 2). The corresponding half-lives ranged from 1.3 to 11.1 days (Table 2), which are generally higher than those of decaying fecal bacteria (1.2–1.3 days in the summer, 1.4–2.2 days in the winter (Table S7)). Thus, ARGs and *intI1* released in these bird feces exhibited moderate persistence in the impacted environments. ANOVA revealed that half-lives were not significantly different among gene types ($p > 0.05$), but were affected by seasons and bird species. The persistence of fecal ARGs and *intI1* showed significant seasonal variance for ducks (stronger in winter) and gulls (stronger in summer) ($p < 0.05$), but no significant difference for crows.

Given their moderate persistence, bird fecal ARGs and *intI1* may increase the local resistome in the receiving urban environments. The abundance of the representative ARGs and *intI1* in topsoil decreased with increasing distance from targeted duck feces (within a 4-ft radius) (Fig. 3), indicating an enlarged region of influence beyond the bird feces. ARGs and *intI1* abundance in topsoil was generally higher in the summer, probably due to higher temperature and the consequential elevated metabolic activities including horizontal gene transfer (Dijkstra et al., 2011).

3.4. Characterization of AR indicators and potential opportunistic pathogenic bacteria

E. coli and *Enterococcus* spp., which are commonly used as general indicators of antibiotic resistance in surveillance efforts (Berendonk et al., 2015; Radhouani et al., 2012), were abundant in wild bird feces with concentrations ranging from 10^5 to 10^7 copies/g dry feces (Table 3). PCR analyses showed that 24.2% *E. coli* and 22.9% *Enterococcus* spp. isolates carried at least one of the targeted ARGs (Table 4). The MGE *intI1* was observed in all *E. coli* and *Enterococcus* spp. isolates (Table S8), suggesting its importance for horizontal ARG transfer.

Despite presumed differences in foraging ranges and living habits (Lockwood and Freeman, 2014; Oberholser et al., 1974), feces from the three common bird species shared similar opportunistic pathogen profiles with *Enterococcus* spp. and *P. aeruginosa* being dominant species (or genus) at up to 10^7 copies/g dry feces (Table 3). These findings are noteworthy as *Enterococcus* spp. is primarily involved in urinary tract infections, sepsis and endocarditis (Osman et al., 2016), and *P. aeruginosa* is a major cause of respiratory infections and notorious for biofilm formation (Limoli and Hoffman, 2019). Although *C. perfringens* was not a dominant pathogen, it was detected in all the winter fecal pools. This species is a spore-forming opportunistic pathogen associated with food poisoning (Augustin, 2011). Relative to the summer feces, winter feces generally harbored more diverse and abundant opportunistic pathogens, possibly due to lower sunlight inactivation (Goyal et al., 1977; Sinton et al., 2007). Also, seasonal differences in moisture levels, temperature and other factors are likely to affect bacterial abundance and diversity (Bharathi et al., 2019).

ARGs and virulence genes were characterized for *Enterococcus* spp. and *P. aeruginosa* isolated from bird feces. All ARG positive isolates exhibited viability on agar medium spiked with antibiotics. The co-existence of ARGs and virulence genes was observed for almost all fecal samples (Table 4). Notably, such co-existence was highest in the summer gull feces with 52.5% of isolated *Enterococcus* spp. and 45.0% of *P. aeruginosa* containing both ARGs and virulence genes. This corroborates previous reports that bird feces can carry antibiotic resistant pathogens (Ahlstrom et al., 2019; Merkeviciene et al., 2017; Ngaiganam et al., 2019; Yahia et al., 2018). The acquisition of ARGs by major opportunistic pathogens harbored by bird feces underscores the need to mitigate potential exposure pathways.

4. Conclusions

This study provides a profile of ARGs carried by wild birds in urban areas, and vital information to guide further characterization and mitigation of the associated health risks. Wild birds are important carriers of ARGs (some of which are harbored by opportunistic pathogens), as indicated by the high relative

Table 2
First-order attenuation of representative ARGs and *intI1* in the bird feces. [a]

Bird species	Genes	Attenuation coefficient (k)		Half-life ($t_{1/2}$) [b]		R^2	
		summer	winter	summer	winter	summer	winter
Duck	*tetW*	0.19 ± 0.04	0.24 ± 0.05	3.75 ± 0.90	2.94 ± 0.71	0.77 ± 0.04	0.91 ± 0.07
	bla_{TEM}	0.37 ± 0.07	0.14 ± 0.02	1.87 ± 0.39	4.95 ± 0.73	0.81 ± 0.03	0.90 ± 0.01
	sul1	0.38 ± 0.02	0.13 ± 0.01	1.83 ± 0.11	5.32 ± 0.24	0.73 ± 0.01	0.97 ± 0.02
	intI1	0.54 ± 0.03	0.17 ± 0.01	1.30 ± 0.08	4.21 ± 0.14	0.92 ± 0.04	0.95 ± 0.02
Crow	*tetW*	0.17 ± 0.02	0.20 ± 0.01	4.06 ± 0.42	3.52 ± 0.13	0.90 ± 0.03	0.86 ± 0.01
	bla_{TEM}	0.29 ± 0.02	0.06 ± 0.02	2.41 ± 0.21	11.14 ± 3.18	0.92 ± 0.02	0.98 ± 0.13
	sul1	0.16 ± 0.02	0.19 ± 0.01	4.36 ± 0.69	3.74 ± 0.15	0.98 ± 0.01	0.98 ± 0.01
	intI1	0.35 ± 0.06	0.28 ± 0.09	2.00 ± 0.38	2.51 ± 1.32	0.97 ± 0.01	0.93 ± 0.16
Gull	*tetW*	0.12 ± 0.01	0.18 ± 0.01	5.58 ± 0.14	3.97 ± 0.01	0.85 ± 0.04	0.93 ± 0.01
	bla_{TEM}	0.07 ± 0.02	0.19 ± 0.01	10.27 ± 3.30	3.74 ± 0.18	0.87 ± 0.36	0.75 ± 0.06
	sul1	0.11 ± 0.03	0.17 ± 0.02	6.10 ± 1.61	4.21 ± 0.50	0.91 ± 0.12	0.93 ± 0.04
	intI1	0.40 ± 0.01	0.49 ± 0.01	1.75 ± 0.05	1.42 ± 0.03	0.91 ± 0.03	0.93 ± 0.01

[a] Values (mean ± SD) are calculated from six fecal pools.
[b] Half-life values were calculated as $t_{1/2} = \ln(2)/k$.

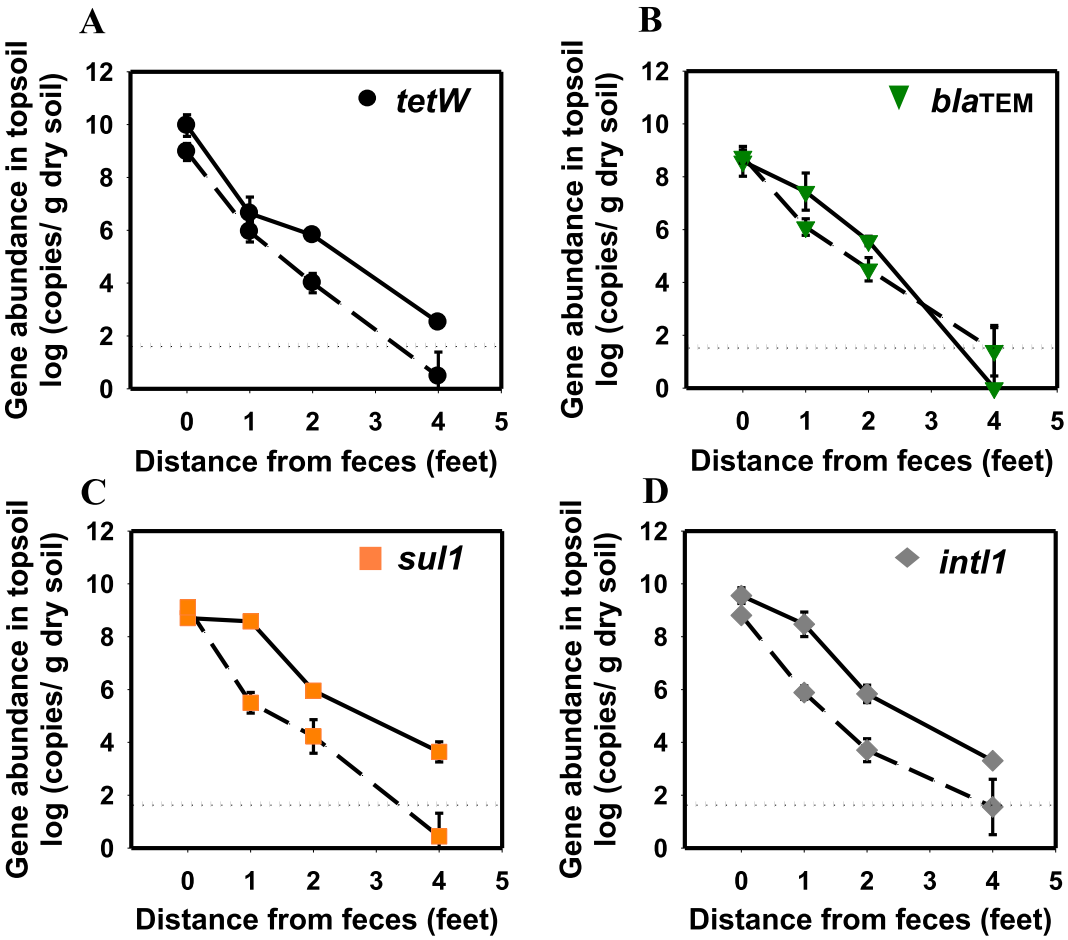

Fig. 3. Absolute abundance of three representative ARGs (A–C) and *intI1* (D) in duck feces (0 ft) and surrounding soil (1, 2, and 4 ft). Solid lines represent summer samples while dashed lines represent winter samples. Limits of quantification (50 gene copies/g) for qPCR analysis are indicated by the horizontal dashed line. Error bars represent ± one standard deviation from the mean of four samples (n = 4).

Table 3
Abundance (log copy number/g dry weight) of opportunistic pathogens in wild bird feces sampled in different seasons. [a]

Opportunistic pathogens	Duck		Crow		Gull	
	Summer	winter	summer	winter	summer	winter
Escherichia coli [b]	5.70 ± 0.25	6.80 ± 0.12 [c]	4.83 ± 0.22	7.52 ± 0.11 [c]	5.80 ± 0.08	7.99 ± 0.08 [c]
Enterococcus spp.	7.13 ± 0.20	7.19 ± 0.13	6.63 ± 0.09	7.12 ± 0.14 [c]	7.70 ± 0.16	7.69 ± 0.10
Pseudomonas aeruginosa	4.87 ± 0.17	7.63 ± 0.08 [c]	4.35 ± 0.08	6.15 ± 0.07 [c]	4.40 ± 0.12	5.65 ± 0.06 [c]
Clostridium perfringens	0.00	3.45 ± 0.10 [c]	2.54 ± 0.24	4.08 ± 0.13 [c]	2.47 ± 0.33	3.50 ± 0.19 [c]
Salmonella enterica	0.00	3.12 ± 0.13 [c]	0.00	5.04 ± 0.13 [c]	0.00	1.92 ± 0.03 [c]
Vibrio vulnificus	2.00 ± 0.19	2.58 ± 0.24	2.52 ± 0.12	2.54 ± 0.16	2.16 ± 0.46	2.48 ± 0.19
Aeromonas hydrophila	2.61 ± 0.26	3.28 ± 0.15 [c]	2.48 ± 0.65	1.95 ± 0.11	2.70 ± 0.41	2.01 ± 0.16

[a] Abundance was determined by qPCR targeting species-specific pathogen indicator genes with limits of quantification as 50 gene copies/g. Values (mean ± SD) are calculated from six fecal pools.
[b] *Escherichia coli* is included as a common indicator of antibiotic resistance status in environmental systems (Berendonk et al., 2015).
[c] Indicates significantly higher abundance in winter.

abundance of ARGs in their feces at levels comparable to those of antibiotic-fed poultry and livestock. Antibiotic residues, known to exert selective pressure for ARGs, only partially explained the observed ARG levels in bird feces. Therefore, more research is needed to discern other factors that may explain this observation, to inform potential strategies to minimize this resistome. The presence of high levels of ARGs in urban wild bird feces may present a significant risk of acquisition by human pathogens, since ARGs persisted with half-lives as long as several days. Although a clear etiology between ARG propagation from wild urban birds and antibiotic resistant human infections has not been established, these results underscore the need to limit exposure to bird feces and proactively manage the associated risks.

CRediT authorship contribution statement

Huiru Zhao: Investigation, Formal analysis, Writing - original draft. **Ruonan Sun:** Investigation, Formal analysis, Writing - original draft. **Pingfeng Yu:** Conceptualization, Methodology, Formal analysis, Writing - original draft. **Pedro J.J. Alvarez:** Supervision,

Table 4
Prevalence of ARGs and virulence genes in potential opportunistic pathogens isolated from wild bird feces (n = 40[a]).

Bird species	Opportunistic pathogens	ARG positive		Virulence gene positive		ARG + virulent gene positive	
		Summer	winter	summer	winter	summer	winter
Duck	*Escherichia coli* [b]	15.0%	17.5%	–	–	–	–
	Enterococcus spp.	20.0%	15.0%	95.0%	92.5%	17.5%	10.0%
	Pseudomonas aeruginosa	12.5%	10.0%	77.5%	85.0%	10.0%	5.0%
Crow	*Escherichia coli*	0%	7.5%	–	–	–	–
	Enterococcus spp.	2.5%	5.0%	92.5%	97.5%	2.5%	7.5%
	Pseudomonas aeruginosa	0%	10%	90.0%	85.0%	0%	7.5%
Gull	*Escherichia coli*	60.0%	45.0%	–	–	–	–
	Enterococcus spp.	57.5%	37.5%	95%	90.0%	52.5%	35.0%
	Pseudomonas aeruginosa	50.0%	47.5%	82.5%	87.5%	45.0%	42.5%

[a] There were 36 fecal pools in total, and 40 colonies were isolated for each opportunistic pathogen from each pool.
[b] *Escherichia coli* is included as a common indicator of antibiotic resistance in environmental systems (Berendonk et al., 2015).

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Declaration of competing interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115200.

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