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Original Article

Wild Birds as Reservoirs of Multidrug-Resistant Enterobacteria in Mulungu, Brazil

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ABSTRACT

Caatinga is a biome unique to Brazil, where degradation caused by anthropogenic actions has led to loss of biodiversity and put many species at risk of extinction. The Ceará state is located within the Caatinga and has a rich avifauna. It comprises 433 species, including 13 species that are in danger of extinction, which are found in the Baturité Massif. The aim of this study was to investigate the frequency and diversity of enterobacteria in wild birds and determine their susceptibility to antimicrobials. Cloacal swab samples were collected from 50 individuals of 28 different species, including the Ceara Gnatheter (Conopophaga cearae) and the Red-necked Tanager (Tangara cyanocephala), which are classified as vulnerable (VU) by the Brazilian Ministry of the Environment. A total of 55 isolates belonging to 14 different species of Enterobacteriaceae were identified. Among them, Pantoea agglomerans and Escherichia coli were the most prevalent species with isolation rates of 36% and 26%, respectively. The highest rate of antimicrobial resistance found was to ampicillin (41.8%), followed by nalidixic acid (36.3%) and amoxicillin associated with clavulanic acid (32.7%). Drugs that presented best efficacy were tobramycin (96.4%), ciprofloxacin (92.6%), and tetracycline (90.9%). Multidrug resistance was observed in 23.5% of the analyzed strains. This research provides important information about the composition of the cloacal microbiota of wild birds in Mulungu, Brazil, as well as their health status. Additionally, these results demonstrate that they harbor multidrug-resistant strains of Enterobacteriaceae.

INTRODUCTION

The Caatinga biome is estimated to harbor 548 species of birds, which are distributed in 74 families and represent 28.6% of the total number of species recorded in Brazil, including 36 endangered species (ICMBio, 2018, 2018; Souza *et al.*, 2020; Ferreira Fernandes *et al.*, 2023). It is characterized by dry landscapes, but it also presents other remarkable phytophysiognomies, such as coastal forests (often associated with extensive mangroves), Cerrado fragments, and remnants of Atlantic Forest and Forest Amazon embedded in the semiarid zone (Bouimetarhan *et al.*, 2018; Machado *et al.*, 2019; Pagano *et al.*, 2019). In mountain areas of the Ceará state, such as the Baturité Massif, typical Cerrado vegetation occurs in lower altitudes, while Atlantic and Amazon Forest characteristics appear in enclaves of humid forest in the more elevated areas (Quinet *et al.*, 2007; Alencar *et al.*, 2022). The Baturité Massif has been under strong anthropogenic pressure since its original occupation, having suffered severe environmental degradation caused by deforestation, fires, introduction of exotic species, landscape fragmentation, predatory hunting, and growth of urban centers, all of which which have been important factors for the alteration of the local biota (Cavalcante, 2005). Moreover, it is home to 13 bird species that are classified as endangered in the Red List of the Brazilian Ministry of Environment (Ceará, 2022). Therefore, the Baturité Massif is a priority area for avian conservation in Northeastern Brazil (Nunes *et al.*, 2015).

Environmental degradation can cause notable negative consequences on wildlife (Biondo *et al.*, 2019). Environmental pollution poses a threat to the conservation of avifauna due to anthropogenic actions, particularly regarding the dissemination of significant pathogens for animal and public health, such as *Salmonella* and other enteropathogens (Batoye *et al.*, 2020; Buelow *et al.*, 2021). Moreover, free-ranging birds may be exposed to residues of antibiotics or resistant microorganisms when interacting with contaminants in their living environment (Carter *et al.*, 2018; Machado *et al.*, 2018). This can impact the health of birds, since factors such as antibiotic ingestion and infection by pathogenic organisms may alter the intestinal microbiota (Banerjee *et al.*, 2018; Umar *et al.*, 2018).

Several studies with free-living birds have shown that they may carry strains of bacteria from the Enterobacteriaceae family with resistance to multiple antimicrobials (Skarżyńska *et al.*, 2021; Rybak *et al.*, 2022). Antimicrobial resistance has emerged as a global clinical and public health threat over the course of several decades, posing a challenge to the effective treatment of common infections caused by resistant pathogens, which can lead to treatment failure and increased mortality (Haenssgen *et al.*, 2021; Salam *et al.*, 2023). The development of bacterial resistance may be explained by the natural evolution of microorganisms. However, the widespread use as well as the misuse of antibacterial agents in humans and animals has accelerated this process (Yin *et al.*, 2020; Sulaiman *et al.*, 2021). In recent years, significant evidence has linked the high prevalence of antimicrobial-resistant bacteria in the environment to anthropogenic sources (Hashmi*et al.*, 2023; Scott *et al.*, 2023). In this context, there is a growing interest in research involving the environment, including wildlife, to better understand the effects of pollution and antimicrobial resistance derived from anthropogenic impacts in ecosystems (Ramey *et al.*, 2021).

Anthropogenic effects on wildlife are not well understood, and the degree to which animal populations contribute to the dissemination of antibiotic resistance remains unclear. Therefore, given the limited number of studies examining the interaction between free-ranging birds and multidrug-resistant enterobacteria in Ceará State, which are typically restricted to a few species, further investigation is warranted (Beleza *et al.*, 2021; Ramey *et al.*, 2021).

Hence, this study aimed to investigate the presence of enterobacteria in cloacal swab samples of wild birds captured in the city of Mulungu, Ceará, Brazil, and determine the phenotypic profile of antimicrobial sensitivity of the isolates.

MATERIAL AND METHODS

Characterization of the Study Area

This research was authorized by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) with SISBIO protocol number 31847-6 and approved by the Ethics Committee for the Use of Animals of the State University of Ceará (Protocol number 4832011/2014).

The study was carried out in the city of Mulungu, Ceará, Brazil (Figure 1). Records show that there has been a decline in vegetal cover in the region for the last two decades, indicating that rudimentary agricultural practices may be the cause of this deterioration process (Freire & Souza, 2007). Within the region known as the Baturité Massif, there is an Environmental Protection Area (APA) located approximately 120 kilometers from the state capital, Fortaleza. This APA presents its highest peak at 1,115 m of altitude and is composed by tropical pluvial subdeciduous forest and pluvio-nebular subevergreen forest (average annual temperatures of 24°C to 26°C with average annual rainfall of 1,737.5 millimeters and with hot sub-humid and humid tropical conditions) with trees up to 30 m high, river springs and waterfalls. This region shows a marked contrast to the surrounding semiarid backwoods (*sertão*) in the middle of a hot, dry region. It has high anthropogenic activity, such as agriculture, livestock production and urban growth, and presents mostly altered vegetation (Oliveira *et al.*, 2007; FUNCEME, 2015; IPECE, 2021).

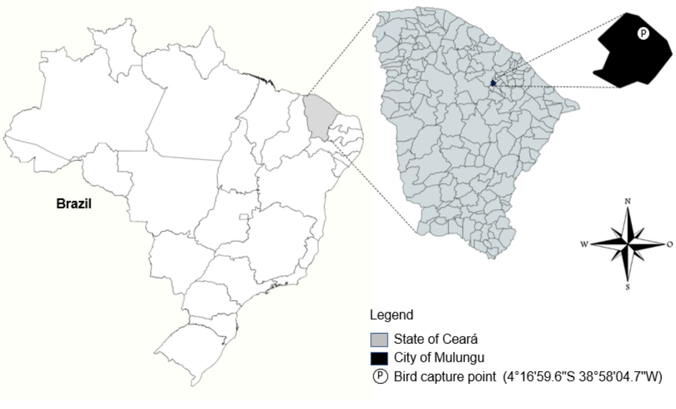

Figure 1 – Location (4°16'59.6"S 38°58'04.7"W) in which wild birds were captured in Mulungu (P), Ceará, Brazil.

Sample collection

Birds were captured during a period of 3 months (october, november and december 2019), using 4 mist nets (Ecotone Mist nets - 1030/12-nailon; length: 12 cm; height: 3.2 m; mesh: 30 x 30 cm; denier: 110/2; 4 bags, fixed with rods at the ends).

Nets were placed 20 cm above the ground in linear transects in the forest. These were opened at dawn and closed at dusk (7:00 am to 5:00 pm), and were checked every 20 min to remove the captured birds.

Species were identified according to the Avis Brasilis field guide (Sigrist *et al.*, 2014) and by consulting the list of birds in Brazil provided by the Brazilian Committee for Ornithological Records - CBRO (Pacheco *et al.*, 2021).

Biological samples were obtained using sterile cloacal swabs, which were stored in Stuart medium at room temperature, transported and sent within 48 h to the Ornithological Studies Laboratory, State University of Ceará (LABEO/UECE) for further microbiological processing. After sampling, individuals were marked by clipping a secondary feather from the right wing before being released back to the wild.

Microbiological procedure

Once at the Ornithological Studies Laboratory (LABEO), samples were transferred from Stuart media to 5 mL of 1% Peptone Water (Kasvi®) and were cultured. The incubation conditions were standardized at 37°C/24h for all the steps of the microbiological procedure. Aliquots of 0.5mL were collected from the peptone water samples and transferred to tubes containing Brain-Heart Infusion (Kasvi®) (BHI) and Selenite-Cystine (Kasvi®) (SC) enrichment broths. Additionally, aliquots of 0.05mL were collected and transferred to Rappaport-Vassiliadis broth (Kasvi®) (RP). After incubation, a loopful was collected from each broth and streaked on plates containing Brilliant Green agar (Himedia®), Salmonella-Shigella agar (Himedia®) and MacConkey agar (Kasvi®), following incubation. Different colonies were collected from each plate and were inoculated into tubes containing Triple Sugar Iron Agar (Kasvi®). To identify the enterobacteria, biochemical tests were used, including SIM Medium (Himedia®), lysine-decarboxylase (LIA) (Kasvi®), ornithine-decarboxylase (Himedia®), methyl red (VM), Voges-Proskauer (VP) (Himedia®), urea (Dynamic Formula®), Simmons citrate agar (Himedia®), arginine decarboxylase (Exodus Cientifica®), malonate broth (Himedia®), lactose (Merck®), sucrose (Dinâmica®), mannitol (Dinâmica®), arabinose (Dinâmica®), raffinose (Dinâmica®), rhamnose (Dinâmica®), dulcitol (Dynâmica® ®), adonitol (Dinâmica®), inositol (Sigma®), and sorbitol (Sigma®) (Koneman *et al.*, 2018).

Antimicrobial susceptibility profile

Isolates were submitted to an antimicrobial susceptibility test using the Kirby-Bauer disk diffusion technique (Bayer *et al.*, 1966), and the inhibition zones were compared to the standards established by the Clinical and Laboratory Standards Institute-CLSI (CLSI *et al.*, 2019). Eleven antimicrobials of 7 pharmacological classes were tested: Quinolones (nalidixic acid, 30 µg); Fluoroquinolones (ciprofloxacin, 5 µg); Aminoglycosides (gentamicin, 10 µg and tobramycin, 10 µg); Tetracyclines (tetracycline, 30 µg); Macrolides (azithromycin, 15 µg); Sulfonamides (sulfamethoxazole + trimethoprim, 25 µg); Beta-lactams (penicillin: ampicillin, 10 µg and amoxicillin + clavulanic acid 10 µg, Cephalosporins: ceftriaxone, 30 µg and Carbapenems: meropenem 10 µg); (All antimicrobials from Oxoid Ltd., Cambridge, UK). Isolates expressing resistance or intermediate phenotypes were considered resistant. Bacteria were considered resistant to multiple drugs (RMD) when resistance occurred to at least three classes of antibiotics (Magiorakos *et al.*, 2012). The *Escherichia coli* ATCC 25922 strain was used as a control sample. To perform the test, isolates were cultured in tubes containing 5 mL of Brain-heart Infusion broth (BHI), and placed in a bacteriological incubator at 37°C for 24 h. Subsequently, aliquots of the broth were seeded onto MacConkey agar plates and incubated again. Afterwards, two to three colonies were selected and placed in 5mL tubes of saline solution. Then, a swab was moistened in the turbid saline solution (which contained a turbidity of 0.5 according to the Mcfarland Nephelometric scale) and streaked on the surface of a plate containing Mueller-Hinton agar (Kasvi®), upon which antimicrobial disks were placed. After the incubation of the plates at 37°C for 24h, the inhibition zones were measured, and results were interpreted as sensitive or resistant.

RESULTS AND DISCUSSION

During the study, a total of 50 birds of 28 different species distributed in 13 families (Thraupidae, Tyrannidae, Columbidae, Trochilidae, Passerellidae, Dendrocolaptidae, Turdidae, Furnariidae, Picidae, Conopophagidae, Icteridae, Thamnophilida, and Hirundinidae) were captured. The most frequent species was Pectoral Sparrow (*Arremon taciturnus*), with a total of 5 individuals, followed by Yellow-bellied Elaenia (*Elaenia flavogaster*) and Ruddy Ground-Dove (*Columbina talpacoti*), both with 4 individuals. Two rare species classified as endangered (EN) were also collected, which were the Ceara Gnateater (*Conopophaga cearae*) and the Red-necked Tanager (*Tangara cyanocephala*) (Instituto Chico Mendes de Conservação da Biodiversidade, 2018; Ceará; 2022).

A total of 55 strains distributed in 14 different bacterial species were detected in the analyzed samples. The prevalence of positive birds for at least one bacterial species was 52.0%. *Pantoea agglomerans* and *Escherichia coli* were the most prevalent ones, occurring in 36.0% (18/50) and 26% (13/50) of the investigated birds. *Serratia rubidaea* was the third most isolated bacterial species, followed by *Hafnia alvei*, which presented isolation rates of 14.0% (7/50) and 10.0% (5/50), respectively (Table 1).

Negative samples were obtained from birds of the Hirundinidae family. On the other hand, all species of the Dendrocolaptidae, Turdidae, Picidae, Conopophagidae, Icteridae and Thamnophilidae family had at least one bacterial isolate. In the Trochilidae family, there was only one (1/6 species) bird that presented bacterial growth, which was a Rufous-breasted Hermit (*Glaucis hirsutus*) that was positive for *Pantoea agglomerans*. Another family with a low number of positive birds was Thraupidae, presenting only two birds positive for enterobacteria (2/10 species). An Orange-headed Tanager (*Thlypopsis sordida*) was positive for *Hafnia alvei* and *Pantoea agglomerans*, whereas a Bananaquit (*Coereba flaveola*) was positive for *Pantoea agglomerans* and *Escherichia coli*. The Tyrannidae family had the same number of positive birds (2/8 species), two Yellow-bellied Elaenia (*Elaenia flavogaster*) individuals from which *Pantoea agglomerans* and *Serratia rubidaea* were isolated from one sample, and *Escherichia coli* was isolated from the other. Lafresnaye's Woodcreeper (*Xiphorhynchus guttatoides eytoni*) was the species with the highest number of isolated enterobacteria (*Enterobacter cloacae*, *Serratia rubidaea*, *Escherichia coli*, *Edwardsiella tarda*, *Hafnia alvei* and *Arizona* spp). The Red-necked Tanager species (*Tangara cyanocephala*), classified as endangered (EN), had no isolates, while the Ceara Gnateater (*Conopophaga cearae*), classified as vulnerable (VU), was positive for *Proteus mirabilis* and *Pantoea agglomerans* (Table 2).

Table 1 – Absolute and relative frequencies of enterobacteria per bird family in cloacal samples of wild birds captured from October to December 2019 in the city of Mulungu, Ceará, Brazil

Isolated Enterobacteriaceae	Total number of birds (n=50)	Absolute and relative frequencies												
		THR (n=10)	TYR (n=8)	COL (n=6)	TRO (n=6)	PAS (n=5)	DEN (n=5)	TUR (n=3)	FUR (n=2)	PIC (n=1)	CON (n=1)	ICT (n=1)	THA (n=1)	HIR (n=1)
Pantoea agglomerans	18 (36.0%)	2 (20.0%)	1 (25.5%)	2 (33.3%)	1 (16.6%)	3 (60.0%)	3 (60.0%)	1 (33.3%)	1 (50.0%)	1 (100.0%)	1 (100.0%)	1 (100.0%)	1 (100.0%)	0 (0%)
Escherichia coli	13 (26.0%)	1 (10.0%)	1 (25.5%)	3 (50.0%)	0 (0%)	2 (40.0%)	3 (60.0%)	2 (66.6%)	1 (50.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Serratia rubidaea	7 (14.0%)	0 (0%)	1 (25.5%)	2 (33.3%)	0 (0%)	1 (20.0%)	1 (20.0%)	1 (33.3%)	0 (0%)	1 (100.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Hafnia alvei	5 (10.0%)	1 (10.0%)	0 (0%)	0 (0%)	0 (0%)	1 (20.0%)	2 (40.0%)	1 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Enterobacter gergoviae	2 (4.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20.0%)	0 (0%)	1 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Edwardsiella tarda	2 (4.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100.0%)	0 (0%)
Klebsiella pneumoniae	1 (2.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Proteus vulgaris	1 (2.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Proteus mirabilis	1 (2.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100.0%)	0 (0%)	0 (0%)	0 (0%)
Cronobacter sakazakii	1 (2.0%)	0 (0%)	0 (0%)	1 (16.6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Enterobacter cloacae	1 (2.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Arizona spp	1 (2.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Yersinia enterocolitica	1 (2.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Shigella spp	1 (2.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (50.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Positive samples	26 (52.0%)	2 (20.0%)	2 (25.0%)	4 (66.6%)	1 (16.6%)	4 (80.0%)	5 (100.0%)	3 (100.0%)	1 (50.0%)	1 (100.0%)	1 (100.0%)	1 (100.0%)	1 (100.0%)	0 (0%)

THR= Thraupidae, TYR= Tyrannidae, COL=Columbidae, TRO=Trochilidae, PAS= Passerellidae, DEN=Dendrocolaptidae, TUR=Turdidae, FUR=Furnariidae, PIC=Picidae, CON= Conopophagidae, ICT= Icteridae, THA= Thamnophilidae, HIR= Hirundinidae.

Table 2 – Bacterial species isolated from free-living wild birds captured in the city of Mulungu, Ceará, Brazil.

Family	Common and scientific name	Isolated species of Enterobacteriaceae
Thraupidae	Burnished-buff Tanager (*Stilpnia cayana*)	-
	Bananaquit (*Coereba flaveola*)	-
	Palm Tanager (*Thraupis palmarum*)	-
	Red-necked Tanager (*Tangara cyanocephala*)	-
	Palm Tanager (*Thraupis palmarum*)	-
	Palm Tanager (*Thraupis palmarum*)	-
	Guira Tanager (*Hemithraupis guira*)	-
	Orange-headed Tanager (*Thlypopsis sordida*)	*Hafnia alvei / Pantoea agglomerans*
	Bananaquit (*Coereba flaveola*)	*Pantoea agglomerans / Escherichia coli*
Tyrannidae	Gray Elaenia (*Myiopagis caniceps*)	-
	Gray Elaenia (*Myiopagis caniceps*)	-
	Mouse-colored Tyrannulet (*Phaeomyias murina*)	-
	Short-crested Flycatcher (*Myiarchus ferox*)	-
	Yellow-bellied Elaenia (*Elaenia flavogaster*)	*Pantoea agglomerans/ Serratia rubidaea*
	Yellow-bellied Elaenia (*Elaenia flavogaster*)	-
	Yellow-bellied Elaenia (*Elaenia flavogaster*)	*Escherichia coli*
	Yellow-bellied Elaenia (*Elaenia flavogaster*)	-
Columbidae	Gray-fronted Dove (*Leptotila rufaxilla*)	*Pantoea agglomerans/Serratia rubidaea*
	Gray-fronted Dove (*Leptotila rufaxilla*)	-
	Ruddy Ground-Dove (*Columbina talpacoti*)	*Escherichia coli*
	Ruddy Ground-Dove (*Columbina talpacoti*)	*Pantoea agglomerans/ Escherichia coli*
	Ruddy Ground-Dove (*Columbina talpacoti*)	-
	Ruddy Ground-Dove (*Columbina talpacoti*)	*Escherichia coli/ Cronobacter sakazakii/ Serratia rubidaea*
Trochilidae	Ruby-topaz Hummingbird (*Chrysolampis mosquitus*)	-
	Woodnymph (*Thalurania furcata*)	-
	Rufous-breasted Hermit (*Glaucis hirsutus*)	-
	Rufous-breasted Hermit (*Glaucis hirsutus*)	-
	Rufous-breasted Hermit (*Glaucis hirsutus*)	*Pantoea agglomerans*
	Woodnymph (*Thalurania furcata*)	-
Passerellidae	Pectoral Sparrow (*Arremon taciturnus*)	*Escherichia coli/Pantoea agglomerans*
	Pectoral Sparrow (*Arremon taciturnus*)	*Escherichia coli*
	Pectoral Sparrow (*Arremon taciturnus*)	*Pantoea agglomerans/Hafnia alvei/Serratia rubidaea/ Klebsiella pneumoniae*
	Pectoral Sparrow (*Arremon taciturnus*)	*Enterobacter gergoviae/Pantoea agglomerans*
	Pectoral Sparrow (*Arremon taciturnus*)	-
Dendrocolaptidae	Straight-billed Woodcreeper (*Dendroplex picus*)	*Escherichia coli*
	Straight-billed Woodcreeper (*Dendroplex picus*)	*Pantoea agglomerans*
	Planalto Woodcreeper (*Dendrocolaptes platyrostris*)	*Hafnia alvei/ Pantoea agglomerans*
	Lafresnaye's Woodcreeper (*Xiphorhynchus guttatoides eytoni*)	*Enterobacter cloacae/ Serratia rubidaea / Escherichia coli / Edwardsiella tarda / Hafnia alvei / Arizona spp*
	Red-billed Scythebill (*Campylorhamphus trochilirostris*)	*Pantoea agglomerans / Yersinia enterocolitica / Escherichia coli*
Turdidae	Pale-breasted Thrush (*Turdus leucomelas*)	*Proteus vulgaris*
	Rufous-bellied Thrush (*Turdus rufiventris*)	*Pantoea agglomerans/ Serratia rubidaea/ Escherichia coli / Enterobacter gergoviae*
	Rufous-bellied Thrush (*Turdus rufiventris*)	*Hafnia alvei / Escherichia coli*
Furnariidae	Pale-legged Hornero (*Furnarius leucopus*)	*Pantoea agglomerans/Shigella spp./Escherichia coli*
	Pale-legged Hornero (*Furnarius leucopus*)	-
Picidae	Green-barred Woodpecker (*Colaptes melanochloros*)	*Pantoea agglomerans / Serratia rubidaea*
Conopophagidae	Ceara Gnateater (*Conopophaga cearae*)	*Proteus mirabilis/Pantoea agglomerans*
Icteridae	Variable Oriole (*Icterus pyrrhopterus*)	*Pantoea agglomerans*
Thamnophilidae	Great Antshrike (*Taraba major*)	*Pantoea agglomerans/Edwardsiella tarda/Serratia rubidaea*
Hirundinidae	Southern Rough-winged Swallow (*Stelgidopteryx ruficollis*)	-

Considering the total of evaluated strains, the highest rate of antimicrobial resistance occurred to ampicillin 47.3% (26/55). Even after the exclusion of intrinsic resistance (*Klebsiella pneumonia* and *Hafnia alvei*), the rate of 41.8 % (23 strains) was still the highest result. After excluding cases of intrinsic resistance (*Hafnia alvei*), the second and third antimicrobials with the highest resistance rates were nalidixic acid with a rate of 36.3% (20/55) and amoxicillin associated with clavulanic acid with 32.7% (18/55). Tobramycin, ciprofloxacin, and tetracycline showed the best efficacy rates of 96.4%, 92.6%, and 90.9%, respectively. Meropenem and gentamicin also performed well (85.5% and 81.8% efficacy, respectively). Considering *E. coli*, the highest resistance rate was also detected to ampicillin, 53.8% (7/13). In contrast, all strains were sensitive to ciprofloxacin. Regarding strains of *Cronobacter sakazakii* and *Enterobacter cloacae*, both showed resistance only to nalidixic acid (Table 3).

Among birds classified as vulnerable (VU) by the list of the National Official List of Fauna Species Endangered with Extinction (Instituto Chico Mendes de Conservação da Biodiversidade, 2018), it was not possible to detect resistance in samples collected from the Red-necked Tanager (*Tangara cyanocephala*), since there was no bacterial isolation. However, the *Proteus mirabilis* strain that was isolated from a Ceara Gnateater (*Conopophaga cearae*) was resistant to azithromycin, ceftriaxone, and nalidixic acid. Moreover, the *Pantoea agglomerans* strain that was isolated from the same individual was resistant to eight out of twelve tested antibiotics (gentamicin, nalidixic acid, ceftriaxone, amoxicillin + clavulanic acid, ciprofloxacin, ampicillin, ciprofloxacin, and meropenem), which correspond to five of the seven antimicrobial classes.

As expected, when considering the bacterial species that have intrinsic resistance mechanisms, resistance to at least one of the tested antimicrobials was observed in all the strains. However, when considering only acquired resistance, 10 isolates (18.2%) were sensitive to all of the investigated drugs. Multidrug resistance (acquired cases) was observed in 13 isolates (23.5%), and three strains were resistant to seven antibiotics. From the total of 13 *Escherichia coli* strains, 2/13 (7.7%) presented multidrug resistance and 4/13 (30.8%) of the strains were sensitive to all the studied antimicrobials (Table 4).

In this study, more than half of the samples were positive to some of the investigated enterobacteria. Despite the isolation of fourteen different species of bacteria, birds were not necessarily suffering from

Table 3 – Absolute and relative frequencies of antimicrobial-resistant enterobacteria isolated from cloacal swab samples of wild birds captured from October to December 2019 in the city of Mulungu, Ceará, Brazil

Enterobacteriaceae	GEN	AZI	TOB	AMP	CEF	AMO+AC. CLA	CIP	TET	SUL	AC. NAL	MER
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Pantoea agglomerans, n=18	4 (22.2%)	5 (27.7%)*	-	7 (38.9%)	3 (16.6%)	9 (50%)	1 (5.5%)	-	-	7 (38.9%)	2 (11.1%)
Escherichia coli, n=13	1 (7.7%)	2 (15.4%)*	1 (7.7%)	7 (53.8%)	2 (15.4%)	2 (15.4%)	-	1 (7.7%)	2 (15.4%)	2 (15.4%)	1 (7.7%)
Serratia rubidae, n=7	2 (28.6%)	2 (28.6%)*	-	4 (57.1%)	2 (28.6%)	1 (14.3%)	1 (14.3%)	1 (14.3%)	-	3 (42.8%)	2 (28.6%)
Hafnia alvei, n=5	-	2 (40.0%)*	-	2 (40%)*	1 (20%)	2 (40%)*	-	1 (20%)	2 (40%)	2 (40%)	-
Enterobacter gergoviae, n=2	1 (50%)	-	-	1 (50%)	-	1 (50%)	-	-	-	-	-
Edwardsiella tarda, n=2	-	2 (100%)*	-	1 (50%)	-	2 (100%)	1 (50%)	-	-	-	-
Klebsiella pneumonia, n=1	1 (100%)	-	-	1 (100%)*	1 (100%)	1 (100%)	1 (100%)	-	-	1 (100%)	1 (100%)
Proteus vulgaris, n=1	-	1 (100%)*	-	-	1 (100%)	1 (100%)	-	1 (100%)*	-	1 (100%)	1 (100%)
Proteus mirabilis, n=1	-	1 (100%)*	-	-	1 (100%)	-	-	-	-	1 (100%)	-
Cronobacter sakazakii, n=1	-	-	-	-	-	-	-	-	-	1 (100%)	-
Enterobacter cloacae, n=1	-	-	-	-	-	-	-	-	-	1 (100%)	-
Arizona spp, n=1	-	-	-	1 (100%)	-	1 (100%)	-	-	-	-	-
Yersinia enterocolitica, n=1	-	1 (100%)*	-	1 (100%)	-	-	-	-	-	-	-
Shigella spp, n=1	1 (100%)	1 (100%)	1 (100%)	1 (100%)	-	-	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)
Acquired resistance	10 (18.2%)	1 (1.8)	2 (3.6%)	23 (41.8%)	11 (20%)	18 (32.7%)	5 (9.1%)	4 (7.8)	5 (9.1%)	20 (36.3%)	8 (14.5)
Total resistance (acquired + intrinsic)	10 (18.2%)	17 (30.9)*	2 (3.6%)	26 (47.3%)*	11 (20%)	20 (36.3%)	5 (9.1%)	5 (9.1%)*	5 (9.1%)	20 (36.3%)	8 (14.5)

GEN-Gentamicin; AZI-Azithromycin; TOB-Tobramycin; AMP-Ampicillin; CEF-Ceftriaxone; AMO+AC.CLA- Amoxicillin associated with Clavulanic Acid; CIP-Ciprofloxacin; TET-Tetracycline; SUL- Sulfonamide; AC. NAL- Nalidixic Acid; MER-Meropenem; *- Intrinsic Resistance

Table 4 – Absolute and relative frequencies of resistance to multiple drugs of Enterobacteriaceae strains isolated from cloacal swabs from free-living birds captured in the city of Mulungu, Ceará, Brazil

Number of antibiotic classes	Frequency of resistant Escherichia coli (%)	Resistant enterobacteria	
		Only acquired resistance (%)	Total resistance (intrinsic + acquired) (%)
0	4 (30.8%)	10 (18.2%)	-
1	5 (38.4%)	20 (36.4%)	10 (18.2%)
2	2 (15.4%)	12 (21.9%)	22 (40%)
3	1 (7.7%)	5 (9.1%)	13 (23.7%)
4	-	2 (3.6%)	3 (5.4%)
5	1 (7.7%)	3 (5.4%)	3 (5.4%)
6	-	-	1 (1.9%)
7	-	3 (5.4%)	3 (5.4%)

any pathological condition. In addition to the low frequency of isolation, some of these microorganisms may occur naturally in these birds, considering that these strains have been previously isolated from healthy birds, either in the wild or in cages (Santos *et al.*, 2010; Horn *et al.*, 2015; Lopes *et al.*, 2015; Murugaiyan *et al.*, 2015; Vaz *et al.*, 2017; Beleza *et al.*, 2019). As a member of the Enterobacteriaceae family, *Escherichia coli* do not belong to the intestinal microbiota of granivorous caged pet birds, since feeds composed exclusively of seeds have been shown to provoke an inhibitory effect of this bacterial species (Glünder *et al.*, 2002). Therefore, the detection of Enterobacteriaceae in cloacal samples of granivorous birds should be observed with caution, as it suggests favorable conditions for the development of potential pathologies (Di Francesco *et al.*, 2018). However, it is important to note that the bird species that were captured in this study have an omnivorous diet. This may explain the natural presence of enterobacteria, since the occurrence of these microorganisms in the digestive tract is influenced by the composition of their nutrition (Glünder *et al.*, 2002). Other factors may also have influenced the isolation of these bacteria, such as direct or indirect contact with domestic animals, as well as environmental contamination by human action. Several species of enterobacteria also occur in their natural environment, as they are ubiquitous in soil or water. Thus, Enterobacteriaceae have been isolated mainly from omnivorous, piscivorous, and healthy carnivore birds (Bangert *et al.*, 1988; Glünder *et al.*, 2002; Aruji *et al.*, 2004; Dobbin *et al.*, 2005; Gibbs *et al.*, 2007).

The most prevalent bacterial species isolated from birds in this study was *Pantoea agglomerans*. This microorganism can rarely cause infections, while it normally acts as a commensal species that colonizes the intestinal microbiota of birds. Biodiversity studies report the isolation of *Pantoea agglomerans* in the microbiota of several plants and insects (Segado-Arenas *et al.*, 2012; Walterson *et al.*, 2015), which serve as a food source for several of the captured bird species. *Escherichia coli* was also among the most isolated microorganisms (23.2%) and is likewise a ubiquitous organism, being found in soil, water and vegetation (Ewers *et al.*, 2012; Riley, 2014). Despite this bacterial species commonly being found in environmental samples, the low rate of isolation frequency in Passeriformes found in this study may be considered a normal occurrence. According to some studies, elevated rates of isolation of this microorganism in passerines may be related to stress conditions, such as those found in illegal wildlife trade, for example (Braconaro *et al.*, 2015; Cunha *et al.*, 2016). Although its presence is not necessarily a sign of illness, this bacterium can cause pathologies in humans, animals and birds on some occasions, such as when they acquire virulence genes (Lopes *et al.*, 2015). The prevalence of *E. coli* in studies involving free-ranging birds is quite varied. Saviolli (2010) describes the presence of the microorganism in 60.0% of the samples from Magnificent Frigatebird (*Fregata magnificens*) from the coast of the State of São Paulo. Vilela (2012), investigated this microorganism in fecal samples of House Sparrows (*Passer domesticus*) that lived around farms in the State of Pernambuco and found lower percentages (13.2%). Callaway (2014) analyzed cloacal swab samples from 376 migratory birds, which included Brown-Headed Cowbird (*Molothrus ater*), Common Grackle (*Quiscalus quiscula*) and Cattle Egret (*Bubulcus ibis*), and found even lower rates, 3.7% (14/376).

Serratia rubidea was the third most isolated species of bacteria. It is considered an important human pathogen, since it is a common agent of nosocomial infections, mostly in the urinary tract (Menezes *et al.*, 2004). Diseases caused by *Serratia* in birds are uncommon but can occur, mostly in an opportunistic manner in immunocompromised birds due to stress in captivity, inappropriate weather conditions, parasitic diseases, among other causes (Fudge *et al.*, 2001; Guimarães *et al.*, 2006) Free-living birds can acquire this microorganism from the contaminated environment in which they live and may act as disseminators. Spena (2020) isolated *Serratia rubidea* from oral swab samples of Eurasian Thick-knee (*Burhinus oedicnemus*) and associated this finding with a diet composed of invertebrates found in the feces of ruminants. This bacterial species has also been reported to be isolated from lakes in Poland that were occupied by Great Cormorants (*Phalacrocorax carbo*). Researchers have associated this finding with the leaching of feces and excreta by rain leading this and other species of enterobacteria into the lake (Wiśniewska *et al.*, 2007).

The other enterobacteria that occurred less frequently in the analyzed samples can also occasionally cause health damages, with previous reports in scientific literature involving free-range or domestic birds. In addition to sharing virulence factors with other enteropathogens, such as *Escherichia coli*, *Hafnia alvei* has been reported to cause serious infections in laying hens (Albert *et al.*, 1992; Real *et al.*, 1997). Miniero Davies (2018) described an outbreak of mortality associated with *E. tarda* affecting fish, domestic ducks, and a wild heron that shared a lake located on a farm in the state of São Paulo, Brazil. Davies (2016) described *Klebsiella pneumoniae* expressing virulence and antibiotic resistance genes in psittacine and passerine birds from illegal trade. *Cronobacter sakazakii* has been reported in broilers with clinical signs, causing high mortality and decreased egg production (Amer & Mekky, 2019). *Proteus* sp. are also potentially pathogenic for birds, causing foot injuries and affecting the respiratory system, causing air sacculitis and caseous pneumonia in cases of immunosuppression (Godoy *et al.*, 2009). Bacteria from the *Arizona* group have often been isolated from feces of adult chickens and turkeys, but have also been reported to occur in wild birds, such as the Canadian crane (Williams *et al.*, 1968; Windingstad *et al.*, 1977). However, more severe cases have been reported in industrial birds, such as mortality in turkeys, as well as clinical signs of salmonellosis and omphalitis in broiler chickens (Sato *et al.*, 1966).

Antimicrobial-resistant strains are detected more frequently in birds raised in captivity than those that live in the wild. In addition to the possibility of inappropriate use of antibiotics, this may occur when birds have greater contact with other animals that possess and disseminate resistant strains (Machado *et al.*, 2016; Gaio *et al.*, 2019). However, our research isolated strains with relevant antimicrobial resistance rates from free-living birds, mainly involving ampicillin, nalidixic acid, and amoxicillin associated with clavulanic acid. Some studies involving free-living birds have also reported varying rates of resistance to these three antibiotics in isolates of enterobacteria with. Carreira (2019) researched samples of cloacal swabs from free-living birds captured in the Metropolitan region of Fortaleza, Brazil, and observed that the acquired resistance rates to amoxicillin associated with clavulanic acid, as well as to nalidixic acid, were lower than the results found in this study. Tsubokura (1995) analyzed *Escherichia coli* isolates from the feces of several migratory bird species collected in the coastal region of Japan and found that less than 10% of the samples were resistant to ampicillin. These same researchers used the feces of 54-day-old Hyline chicks and found the resistance to ampicillin to be of approximately 39.0%. These variations can often be attributed to the conditions found in different habitats (Carter *et al.*, 2018), as demonstrated by several studies that measure resistance levels in isolates from birds under different conditions or captured in different environments (Shobrak *et al.*, 2014; Atterby *et al.*, 2016; Giacopello *et al.*, 2016; Ramey *et al.*, 2018; Tormoehlen *et al.*, 2019).

The rate of resistance to meropenem detected in free-living birds in this research should also be highlighted (14.5%). Several studies involving wild birds, free-living or not, as well as domestic birds, present lower rates of resistance to this drug or no resistance at all (Iroha *et al.*, 2015; Sousa *et al.*, 2019; Foti *et al.*, 2020; Ong *et al.*, 2020; Beleza *et al.*, 2021). Nevertheless, it is a more important to point out that this is a high-cost carbapenemic drug with use restricted to hospitals in Brazil. Moreover, it is a last resort for the treatment of infections and is widely prescribed to human patients with septic conditions in intensive care for severe infections caused by Gram-negative hospital pathogens, including Enterobacteriaceae (Khan *et al.*, 2014; Blumentrath *et al.*, 2019; De Carmargo *et al.*, 2021). Although the recommendations for the use of this drug are restrictive, reservoirs of microorganisms resistant to it are increasing, not only in hospitals, but also in the community and the environment. An important new source of this type of resistance development is being observed in livestock, companion animals, and wildlife (Guerra *et al.*, 2014).

Concerning the total number of isolated enterobacterial strains (23.6%) and specifically *Escherichia coli* (15.4%), worrisome rates of multidrug resistance were observed, considering that these are Gram-negative bacteria from free-living animals. Other studies have also demonstrated the occurrence of multidrug resistance in bacteria isolated from cloacal swabs in free-living birds (Foti *et al.*, 2020; Nascimento *et al.*, 2003). However, it is not so simple to obtain a proper comparison of data from other studies, since there are few published articles specifically involving free-living birds and isolates of enterobacteria in general from cloacal swabs. One of these studies involves Gray-breasted Parakeets (*Pyrrhura griseipectus*), in which total bacterial isolates presented a lower multidrug resistance rate (11.1%) (Nascimento *et al.*, 2003). Concerning *Escherichia coli*, it is possible to observe that the results in relation to multidrug resistance are the most varied. However, it is possible to find similar rates (Machado *et al.*, 2018), as well as percentages lower than 5.4% or higher than 23.1% (Carrol *et al.*, 2015; Ong *et al.*, 2020).

Densely populated urban areas are historically seen as hotspots for antibiotic resistant bacteria (Ledeberg *et al.*, 1997; Singer *et al.*, 2007), but microorganisms with these characteristics associated with humans have been described in non-clinical environments, such as in remote areas of the planet, far from direct anthropogenic pressure, apparently free from exposure to antibiotics, as in regions of the Amazon, Bolivia and Antarctica. It is suspected that this resistance may have been caused by the existence of military bases, domestic animals, water, fishing boats, scientific expeditions and/or on-board tourism in these regions (Gottdenker *et al.*, 2005; Pallecchi *et al.*, 2007; Bartoloni *et al.*, 2009; Portugal *et al.*, 2015; Hernández *et al.*, 2016). It is important to emphasize that the cause of antibiotic resistance may not always be related to environmental pressures caused by man, as is the case of those that are naturally induced by microorganisms that produce natural antibiotics (Salyers *et al.*, 1997).

The considerable resistance rates detected in isolates from birds captured in Mulungu, particularly those tested with ampicillin, amoxicilin+clavulunate, meropenem, and nalidixic acid, may indicate that some contact with anthropogenic residues has occurred. Thus, we can consider that wild birds included in our study may be working as indicators of environmental contamination. In this context, we found that free-living birds may be affected by the environment they live in, acquiring multidrug-resistant bacteria. At some point, this condition can harm the conservation of species, or may cause these animals to act as reservoirs of resistant bacteria (Brinkmeyer *et al.*, 2003; Benskin *et al.*, 2009; Bonnedahl *et al.*, 2014; Hernández *et al.*, 2016). Thus, the emergence and evolution of antibiotic resistance among pathogenic bacteria represents a serious public health issue on a global scale (Allen *et al.*, 2010; Martinez *et al.*, 2009).

CONCLUSION

This study revealed that the investigated wild free-living birds harbor a diverse cloacal microbiota regarding the Enterobaceriaceae family. The phenotypic analysis of the isolates revealed the occurrence of bacterial resistance to several of the tested antimicrobials. Among these, the resistance rates to ampicillin and nalidixic acid may be considered high, since these isolates originated from free-living animals, which naturally suffer low selective pressure by antibiotics. The percentage of resistance found to meropenem (14.5%) was also higher than normally expected, since it is a drug with use restricted to hospitals. A relevant multidrug resistance rate was also detected in this study (23.5%), and this shows that birds associated with local extinction risk, such as Ceara Gnateater (*Conopophaga cearae*), are also being affected.

Although this research did not investigate the direct or indirect relationship of wild birds in the Region of Mulungu-CE with sources of contamination, such as sewage water, dumps, crops, soil, and domestic or wild animals, it is possible to assume that they could have some contact with contaminating agents, which explains the multidrug resistance rates detected in the cloacal microbiota isolates. Furthermore, birds that have been infected by these microorganisms may also be carrying resistant bacteria to other wild birds or to domestic animals.

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