

1 Wild birds as reservoirs of multidrug-resistant enterobacteria in 2 Mulungu, Brazil

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26 ABSTRACT

27 Caatinga is a biome unique to Brazil that is degraded by anthropogenic actions, which
28 lead to the loss of biodiversity putting many species at risk of extinction. The Ceará
29 State is located in the Caatinga and has a rich avifauna comprised of 433 species
30 including 13 species that are threatened with extinction, which are found in the Baturité
31 Massif. The aim of this study was to investigate the frequency and diversity of
32 enterobacteria in wild birds and to determine their susceptibility to antimicrobials.
33 Cloacal swab samples were collected from 50 individuals of 28 different species,
34 including the Ceara Gnatheter (*Conopophaga cearae*) and Red-necked Tanager
35 (*Tangara cyanocephala cearensis*), which are classified as vulnerable (VU) by the
36 Brazilian Ministry of the Environment. A total of 55 isolates belonging to 14 different
37 species of Enterobacteriaceae were identified. Among these, *Pantoea agglomerans* and
38 *Escherichia coli* were the most prevalent species with isolation rates of 36% and 26%,
39 respectively. The highest rate of antimicrobial resistance found was to ampicillin
40 (41.8%), followed by nalidixic Acid (36.3%) and amoxicillin associated with clavulanic

41 acid (32.7%). The drugs with the best efficacy were tobramycin (96.4%), ciprofloxacin
42 (92.6%) and tetracycline (90.9%). Multidrug resistance was observed in 23.5% of the
43 analyzed strains. This research provides important information about the composition of
44 the cloacal microbiota of wild birds in Mulungu, Brazil, as well as their health status. In
45 addition, these results demonstrate that they harbor multidrug-resistant strains of
46 Enterobacteriaceae.

47 **Introduction**

48 It is estimated that the Caatinga biome is home to 548 species of birds, which are
49 distributed in 74 families and represent 28.6% of the total number of species recorded in
50 Brazil [1]. The Ceará State, Brazil, is located in this biome, which has dry landscapes as
51 its main geographic feature. Nonetheless, it presents other striking phytophysiognomies,
52 such as coastal forests, often associated with extensive mangroves, Cerrado fragments,
53 in addition to remnants of Atlantic Forest and Forest Amazon embedded in the semiarid
54 zone [2, 3, 4, 5], as occurs in the enclaves of humid forest occurring in the Baturité
55 Massif [6]. The Baturité Massif has been under strong anthropogenic pressure and since
56 its original occupation it has suffered severe environmental degradation caused by
57 deforestation, fires, introduction of exotic species, dismemberment of sites, predatory
58 hunting and growth of urban centers, which have been important factors for the
59 alteration of the local biota [7]. In addition, it is home to 13 bird species that are
60 classified as threatened in the Red List of the Brazilian Ministry of the Environment [8].
61 Therefore, the Baturité Massif is a priority area for avian conservation in Northeastern
62 Brazil [9].

63 The environmental degradation can promote notable negative consequences on
64 wildlife [10]. Environmental pollution is one of the anthropogenic actions that puts the
65 conservation of avifauna at risk, mainly on aspects related to the dissemination of
66 pathogens that are important for animal and public health, such as Salmonella and other
67 enteropathogens [11, 12]. Furthermore, free-ranging birds may come into contact with
68 residues of antibiotics or resistant microorganisms when exposed to contaminants in the
69 environment in which they live [13, 14, 15]. This may affect the health of birds,
70 considering that factors such as ingestion of antibiotics and infection by pathogenic
71 organisms may alter the microbiota of birds [16, 17, 18].

72 Several studies with free-living birds have shown that they may carry strains of
73 bacteria from the Enterobacteriaceae family with resistance to multiple antimicrobials

74 [19, 20, 21]. Over several decades, antimicrobial resistance has become a global clinical
75 and public health threat against the effective treatment of common infections caused by
76 resistant pathogens, resulting in treatment failure and increased mortality [22]. The
77 development of bacterial resistance can be explained by the natural evolution of
78 microorganisms. However, the widespread and misuse of antibacterial agents in humans
79 and animals has accelerated this process [22]. In recent years, substantial evidence has
80 been provided linking the high presence of antimicrobial-resistant bacteria in the
81 environment with anthropogenic sources [23, 24]. In this context, there is a growing
82 interest in researches involving the environment, including wildlife, in order to better
83 understand the effects of pollution and antimicrobial resistance derived from
84 anthropogenic impacts in ecosystems [25, 26, 27].

85 Anthropogenic effects on wildlife are poorly investigated, and the extent to
86 which animal populations contribute to the spread of antibiotic resistance is still
87 unknown. Therefore, considering that there are few studies investigating the contact that
88 free-living birds have with multidrug-resistant enterobacteria in the Ceará State, which
89 are generally limited to a few species, greater elucidation is needed [28, 29, 15, 30].

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

93 **Material and methods**

94 **Characterization of the Study Area**

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

99 The study was carried out in the city of Mulungu, Ceará, which is located in the
100 Baturité Massif that contains 16 cities: Aratuba, Baturité, Canindé, Capistrano, Charity,
101 Guaramiranga, Mulungu, Redenção, Pacoti, Palmacia, Acarape, Barreira, Aracoiaba,
102 Guaiúba, Maranguape and Itapiúna. Within the region known as the Baturité Massif, an
103 Environmental Protection Area (APA) is located at an approximate distance of 120
104 kilometers from the state capital, Fortaleza. This APA presents its highest peak at 1115
105 meters in altitude and is composed by tropical pluvial subdeciduous forest and pluvio-
106 nebular subevergreen forest (average annual temperatures of 24° to 26° with average

107 annual rainfall of 1,737.5 millimeters and with hot sub-humid and humid tropical
108 conditions) with trees up to 30 meters high, river springs and waterfalls. This region
109 show a marked contrast to the surrounding semiarid backwoods (sertão) in the middle of
110 a hot dry region. It has high anthropogenic activity, such as agriculture, livestock and
111 urban growth, and presents mostly altered vegetation [31, 32, 33].

112 Sample collection

113 The capture of birds was conducted for 3 months (october, november and
114 december 2019) with the aid of 4 mist nets (Ecotone Mist nets - 1030/12-nailon; length:
115 12 cm; height: 3.2m; mesh: 30x30cm; denier: 110/2; 4 bags, fixed with rods at the
116 ends).

117 The nets were placed 20 cm above the ground in linear transects in the forest.
118 These were opened at dawn and closed at dusk (7:00 am to 5:00 pm) and were checked
119 every 20 minutes to remove the captured birds.

120 The species were identified according to the *Avis Brasilis* field guide [34] and
121 by consulting the list of birds in Brazil made available by the Brazilian Committee for
122 Ornithological Records - CBRO [35].

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

128 Microbiological procedure

129 Once at the Ornithological Studies Laboratory (LABEO), samples were
130 transferred from Stuart media to 5 mL of 1% Peptone Water (Kasvi®) and were
131 cultured. The incubation conditions were standardized at 37°C/24h for all the steps of
132 the microbiological procedure. Aliquots of 0.5mL were collected from the peptone
133 water samples and transferred to tubes containing Brain-Heart Infusion (Kasvi®) (BHI)
134 and Selenite-Cystine (Kasvi®) (SC) enrichment broths. In addition, aliquots of 0.05mL
135 were collected and transferred to Rappaport-Vassiliadis broth (Kasvi®) (RP). After
136 incubation, a loopful was collected from each broth and streaked on plates containing
137 Brilliant Green agar (Himedia®), Salmonella-Shigella agar (Himedia®) and
138 MacConkey agar (Kasvi®), following incubation. Different colonies were collected
139 from each plate and were inoculated into tubes containing Triple Sugar Iron Agar

140 (Kasvi®). To identify the enterobacteria, biochemical tests were used, including SIM
141 Medium (Himedia®), lysine-decarboxylase (LIA) (Kasvi®), ornithine-decarboxylase
142 (Himedia®), methyl red (VM), Voges-Proskauer (VP) (Himedia®), Urea (Dynamic
143 Formula®), Simmons Citrate Agar (Himedia®), Arginine Decarboxylase (Exodus
144 Cientifica®), Malonate Broth (Himedia®), Lactose (Merck®), Sucrose (Dinâmica®),
145 Mannitol (Dinâmica®), Arabinose (Dinâmica®), Raffinose (Dinâmica®), Rhamnose
146 (Dinâmica®), Sucrose (Dinâmica®), Dulcitol (Dinâmica® ®), Adonitol (Dinâmica®),
147 Inositol (Sigma®) and Sorbitol (Sigma®) [36].

148 Antimicrobial susceptibility profile

149 The isolates were submitted to antimicrobial susceptibility test using the Kirby-
150 Bauer disk diffusion technique [37], and the inhibition zones were compared to the
151 standards established by the Clinical and Laboratory Standards Institute-CLSI [38].
152 Eleven antimicrobials of 7 pharmacological classes were tested: Quinolones (Nalidixic
153 Acid, 30µg); Fluroquinolones (Ciprofloxacin, 5µg); Aminoglycosides (Gentamicin,
154 10µg and Tobramycin, 10µg); Tetracyclines (Tetracycline, 30µg); Macrolides
155 (Azithromycin, 15µg); Sulfonamides (Sulfamethoxazole + Trimethoprim, 25 µg); Beta-
156 lactams (Penicillin: Ampicillin, 10 µg and Amoxicillin + Clavulanic Acid 10 µg,
157 Cephalosporins: Ceftriaxone, 30 µg and Carbapenems: Meropenem 10µg); (All
158 antimicrobials from Oxoid Ltd., Cambridge, UK confirm brand*). Isolates that
159 expressed resistance or intermediate phenotypes were interpreted as resistant. Bacteria
160 were considered resistant to multiple drugs (RMD) when resistance occurred to at least
161 three classes of antibiotics [39]. The *Escherichia coli* ATCC 25922 strain was used as a
162 control sample. To perform the test, isolates were cultured in tubes containing 5 mL of
163 Brain-heart Infusion broth (BHI), and placed in bacteriological incubator for 24 hours at
164 37°C. Subsequently, aliquots of the broth were seeded onto MacConkey agar plates and
165 again incubated. Afterwards, two to three colonies were selected and placed in 5mL
166 tubes of saline solution. Then, a swab was moistened in the turbid saline solution
167 (which contained a turbidity of 0.5 according to the Mcfarland Nephelometric scale)
168 and streaked on the surface of a plate containing Mueller-Hinton agar (Kasvi®), to
169 which antimicrobial discs were placed. After the plates were incubated at 37°C for 24h,
170 the inhibition zones were measured and results were interpreted as sensitive or resistant.

171 Results

172 During the study, a total of 50 birds of 28 different species distributed in 13
 173 families were captured (Table 1). The most frequent species was Pectoral Sparrow
 174 (*Arremon taciturnus*), in a total of 5 individuals, followed by the occurrence of Yellow-
 175 bellied Elaenia (*Elaenia flavogaster*) and Ruddy Ground-Dove (*Columbina talpacoti*),
 176 both with 4 individuals. Two rare species classified as vulnerable (VU) were also
 177 collected, which were the Ceara Gnateater (*Conopophaga cearae*) and the Red-necked
 178 Tanager (*Tangara cyanocephala cearensis*) [8].
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Table 1. List of bird species captured in the city of Mulungu, Ceará, Brazil

Family	Common and scientific name	Captured animals
Thraupidae	Red-necked Tanager (<i>Tangara cyanocephala cearensis</i>)	2
	Burnished-buff Tanager (<i>Stilpnia cayana</i>)	1
	Bananaquit (<i>Coereba flaveola</i>)	2
	Palm Tanager (<i>Thraupis palmarum</i>)	3
	Guira Tanager (<i>Hemithraupis guira</i>)	1
	Orange-headed Tanager (<i>Thlypopsis sordida</i>)	1
Tyrannidae	Gray Elaenia (<i>Myiopagis caniceps</i>)	2
	Mouse-colored Tyrannulet (<i>Phaeomyias murina</i>)	1
	Short-crested Flycatcher (<i>Myiarchus ferox</i>)	1
	Yellow-bellied Elaenia (<i>Elaenia flavogaster</i>)	4
Columbidae	Gray-fronted Dove (<i>Leptotila rufaxilla</i>)	2
	Ruddy Ground-Dove (<i>Columbina talpacoti</i>)	4
Trochilidae	Ruby-topaz Hummingbird (<i>Chrysolampis mosquitus</i>)	1
	Fork-tailed Woodnymph (<i>Thalurania furcata</i>)	2
	Rufous-breasted Hermit (<i>Glaucis hirsutus</i>)	3
Passerellidae	Pectoral Sparrow (<i>Arremon taciturnus</i>)	5
Dendrocolaptidae	Straight-billed Woodcreeper (<i>Dendroplex picus</i>)	2
	Planalto Woodcreeper (<i>Dendrocolaptes platyrostris</i>)	1
	Lafresnaye's Woodcreeper (<i>Xiphorhynchus guttatoides eytoni</i>)	1
	Red-billed Scythebill (<i>Campylorhamphus trochilirostris</i>)	1
Turdidae	Pale-breasted Thrush (<i>Turdus leucomelas</i>)	1
	Rufous-bellied Thrush (<i>Turdus rufiventris</i>)	2
Furnariidae	Pale-legged Hornero (<i>Furnarius leucopus</i>)	2
Picidae	Green-barred Woodpecker (<i>Colaptes melanochloros</i>)	1

Conopophagidae	Ceara Gnateater (<i>Conopophaga cearae</i>)	1
Icteridae	Variable Oriole (<i>Icterus pyrrhopterus</i>)	1
Thamnophilidae	Great Antshrike (<i>Taraba major</i>)	1
Hirundinidae	Southern Rough-winged Swallow (<i>Stelgidopteryx ruficollis</i>)	1
Total	28	50

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181 A total of 55 strains distributed in 14 different bacterial species were detected in
182 the analyzed samples. The prevalence of positive birds for at least one bacterial species
183 was 52.0%. *Pantoea agglomerans* and *Escherichia coli* were the most prevalent ones,
184 occurring in 36.0% (18/50) and 26% (13/50) of the investigated birds. *Serratia rubidaea*
185 was the third most isolated bacterial species, followed by *Hafnia alvei*, which
186 presented isolation rates of 14.0% (7/50) and 10.0% (5/50), respectively (Table 2).

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Table 2- 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)
<i>Pantoea agglomerans</i>	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%)
<i>Escherichia coli</i>	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%)
<i>Serratia rubidaea</i>	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%)
<i>Hafnia alvei</i>	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%)
<i>Enterobacter gergoviae</i>	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%)
<i>Edwardsiella tarda</i>	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%)
<i>Klebsiella pneumoniae</i>	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%)
<i>Proteus vulgaris</i>	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%)
<i>Proteus mirabilis</i>	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%)
<i>Cronobacter sakazakii</i>	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%)
<i>Enterobacter cloacae</i>	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%)
<i>Arizona spp</i>	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%)
<i>Yersinia enterocolitica</i>	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%)
<i>Shigella spp</i>	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

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214 Negative samples were obtained from birds of the Hirundinidae family. On the
 215 other hand, all species of the Dendrocolaptidae, Turdidae, Picidae, Conopophagidae,
 216 Icteridae and Thamnophilidae family had at least one bacterial isolate. In the
 217 Trochilidae family, there was only one (1/6 species) bird that presented bacterial
 218 growth, which was a Rufous-breasted Hermit (*Glaucis hirsutus*) that was positive for
 219 *Pantoea agglomerans*. Another family with a low number of positive birds was
 220 Thraupidae that presented only two birds positive for enterobacteria (2/10 species). An
 221 Orange-headed Tanager (*Thlypopsis sordida*) was positive for *Hafnia alvei* and *Pantoea*
 222 *agglomerans*, whereas a Bananaquit (*Coereba flaveola*) was positive for *Pantoea*
 223 *agglomerans* and *Escherichia coli*. The Tyrannidae family had the same number of
 224 positive birds (2/8 species), two individuals of Yellow-bellied Elaenia (*Elaenia*
 225 *flavogaster*) from which *Pantoea agglomerans* and *Serratia rubidaea* were isolated
 226 from one sample and *Escherichia coli* was isolated from the other. Lafresnaye's
 227 Woodcreeper (*Xiphorhynchus guttatoides eytoni*) was the species with the highest
 228 number of isolated enterobacteria (*Enterobacter cloacae*, *Serratia rubidaea*,
 229 *Escherichia coli*, *Edwardsiella tarda*, *Hafnia alvei* and *Arizona* spp). The Red-necked
 230 Tanager species (*Tangara cyanocephala* subsp. *cearensis*) classified as vulnerable (VU)
 231 had no isolates, while the species Ceara Gnateater (*Conopophaga cearae*) classified as
 232 vulnerable (VU) was positive for *Proteus mirabilis* and *Pantoea agglomerans* (Table 3).

Table 3. 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	Red-necked Tanager (<i>Tangara cyanocephala cearensis</i>)	-
	Burnished-buff Tanager (<i>Stilpnia cayana</i>)	-
	Bananaquit (<i>Coereba flaveola</i>)	-
	Palm Tanager (<i>Thraupis palmarum</i>)	-
	Red-necked Tanager (<i>Tangara cyanocephala cearensis</i>)	-
	Palm Tanager (<i>Thraupis palmarum</i>)	-
	Palm Tanager (<i>Thraupis palmarum</i>)	-
	Guira Tanager (<i>Hemithraupis guira</i>)	-
	Orange-headed Tanager (<i>Thlypopsis sordida</i>)	<i>Hafnia alvei</i> / <i>Pantoea agglomerans</i>
Bananaquit (<i>Coereba flaveola</i>)	<i>Pantoea agglomerans</i> / <i>Escherichia coli</i>	
Tyrannidae	Gray Elaenia (<i>Myiopagis caniceps</i>)	-
	Gray Elaenia (<i>Myiopagis caniceps</i>)	-
	Mouse-colored Tyrannulet (<i>Phaeomyias murina</i>)	-

	Short-crested Flycatcher (<i>Myiarchus ferox</i>)	-
	Yellow-bellied Elaenia (<i>Elaenia flavogaster</i>)	<i>Pantoea agglomerans</i> / <i>Serratia rubidaea</i>
	Yellow-bellied Elaenia (<i>Elaenia flavogaster</i>)	-
	Yellow-bellied Elaenia (<i>Elaenia flavogaster</i>)	<i>Escherichia coli</i>
	Yellow-bellied Elaenia (<i>Elaenia flavogaster</i>)	-
Columbidae	Gray-fronted Dove (<i>Leptotila rufaxilla</i>)	<i>Pantoea agglomerans</i> / <i>Serratia rubidaea</i>
	Gray-fronted Dove (<i>Leptotila rufaxilla</i>)	-
	Ruddy Ground-Dove (<i>Columbina talpacoti</i>)	<i>Escherichia coli</i>
	Ruddy Ground-Dove (<i>Columbina talpacoti</i>)	<i>Pantoea agglomerans</i> / <i>Escherichia coli</i>
	Ruddy Ground-Dove (<i>Columbina talpacoti</i>)	-
	Ruddy Ground-Dove (<i>Columbina talpacoti</i>)	<i>Escherichia coli</i> / <i>Cronobacter sakazakii</i> / <i>Serratia rubidaea</i>
Trochilidae	Ruby-topaz Hummingbird (<i>Chrysolampis mosquitus</i>)	-
	Woodnymph (<i>Thalurania furcata</i>)	-
	Rufous-breasted Hermit (<i>Glaucis hirsutus</i>)	-
	Rufous-breasted Hermit (<i>Glaucis hirsutus</i>)	-
	Rufous-breasted Hermit (<i>Glaucis hirsutus</i>)	<i>Pantoea agglomerans</i>
	Woodnymph (<i>Thalurania furcata</i>)	-
Passerellidae	Pectoral Sparrow (<i>Arremon taciturnus</i>)	<i>Escherichia coli</i> / <i>Pantoea agglomerans</i>
	Pectoral Sparrow (<i>Arremon taciturnus</i>)	<i>Escherichia coli</i>
	Pectoral Sparrow (<i>Arremon taciturnus</i>)	<i>Pantoea agglomerans</i> / <i>Hafnia alvei</i> / <i>Serratia rubidaea</i> / <i>Klebsiella pneumoniae</i>
	Pectoral Sparrow (<i>Arremon taciturnus</i>)	<i>Enterobacter gergoviae</i> / <i>Pantoea agglomerans</i>
	Pectoral Sparrow (<i>Arremon taciturnus</i>)	-
Dendrocolaptidae	Straight-billed Woodcreeper (<i>Dendroplex picus</i>)	<i>Escherichia coli</i>
	Straight-billed Woodcreeper (<i>Dendroplex picus</i>)	<i>Pantoea agglomerans</i>
	Planalto Woodcreeper (<i>Dendrocolaptes platyrostris</i>)	<i>Hafnia alvei</i> / <i>Pantoea agglomerans</i>
	Lafresnaye's Woodcreeper (<i>Xiphorhynchus guttatoides eytoni</i>)	<i>Enterobacter cloacae</i> / <i>Serratia rubidaea</i> / <i>Escherichia coli</i> / <i>Edwardsiella tarda</i> / <i>Hafnia alvei</i> / <i>Arizona</i> spp
	Red-billed Scythebill (<i>Campylorhamphus trochilirostris</i>)	<i>Pantoea agglomerans</i> / <i>Yersinia enterocolitica</i> / <i>Escherichia coli</i>
Turdidae	Pale-breasted Thrush (<i>Turdus leucomelas</i>)	<i>Proteus vulgaris</i>
	Rufous-bellied Thrush (<i>Turdus rufiventris</i>)	<i>Pantoea agglomerans</i> / <i>Serratia rubidaea</i> / <i>Escherichia coli</i> / <i>Enterobacter gergoviae</i>
	Rufous-bellied Thrush (<i>Turdus rufiventris</i>)	<i>Hafnia alvei</i> / <i>Escherichia coli</i>
Furnariidae	Pale-legged Hornero (<i>Furnarius leucopus</i>)	<i>Pantoea agglomerans</i> / <i>Shigella</i> spp./ <i>Escherichia coli</i>
	Pale-legged Hornero (<i>Furnarius leucopus</i>)	-
Picidae	Green-barred Woodpecker (<i>Colaptes melanochloros</i>)	<i>Pantoea agglomerans</i> / <i>Serratia rubidaea</i>
Conopophagidae	Ceara Gnateater (<i>Conopophaga cearae</i>)	<i>Proteus mirabilis</i> / <i>Pantoea</i>

	<i>agglomerans</i>	
Icteridae	Variable Oriole (<i>Icterus pyrrhopterus</i>)	<i>Pantoea agglomerans</i>
Thamnophilidae	Great Antshrike (<i>Taraba major</i>)	<i>Pantoea agglomerans/Edwardsiella tarda/Serratia rubidaea</i>
Hirundinidae	Southern Rough-winged Swallow (<i>Stelgidopteryx ruficollis</i>)	-

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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*), nalidixic acid with a rate of 36.3% (20/55) and amoxicillin associated with Clavulanic Acid with 32.7% (18/55), were the second and third antimicrobials with the highest resistance rates. Tobramycin, ciprofloxacin and tetracycline were the drugs that showed the best efficacy with 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 the *Cronobacter sakazakii* and *Enterobacter cloacae* strains, both showed resistance only to Nalidixic Acid (Table 4).

Table 4. 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 (%)
<i>Pantoea agglomerans</i> , 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%)
<i>Escherichia coli</i> , 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%)
<i>Serratia rubidae</i> , 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%)
<i>Hafnia alvei</i> , n=5	-	2 (40.0%)*	-	2 (40%)*	1 (20%)	2 (40%)*	-	1 (20%)	2 (40%)	2 (40%)	-
<i>Enterobacter gergoviae</i> , n=2	1 (50%)	-	-	1 (50%)	-	1 (50%)	-	-	-	-	-
<i>Edwardsiella tarda</i> , n=2	-	2 (100%)*	-	1 (50%)	-	2 (100%)	1 (50%)	-	-	-	-
<i>Klebsiella pneumonia</i> , n=1	1 (100%)	-	-	1 (100%)*	1 (100%)	1 (100%)	1 (100%)	-	-	1 (100%)	1 (100%)
<i>Proteus vulgaris</i> , n=1	-	1 (100%)*	-	-	1 (100%)	1 (100%)	-	1 (100%)*	-	1 (100%)	1 (100%)
<i>Proteus mirabilis</i> , n=1	-	1 (100%)*	-	-	1 (100%)	-	-	-	-	1 (100%)	-
<i>Cronobacter sakazakii</i> , n=1	-	-	-	-	-	-	-	-	-	1 (100%)	-
<i>Enterobacter cloacae</i> , n=1	-	-	-	-	-	-	-	-	-	1 (100%)	-
<i>Arizona</i> spp, n=1	-	-	-	1 (100%)	-	1 (100%)	-	-	-	-	-
<i>Yersinia enterocolitica</i> , n=1	-	1 (100%)*	-	1 (100%)	-	-	-	-	-	-	-
<i>Shigella</i> 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

267 Among the birds classified as vulnerable (VU) by the list of the Official National
 268 List of Fauna Species Endangered with Extinction [8], it was not possible to detect
 269 resistance in samples collected from the Red-necked Tanager (*Tangara cyanocephala*
 270 *cearensis*), since there was no isolation of any bacteria. However, the *Proteus mirabilis*
 271 strain that was isolated from a Ceara Gnateater (*Conopophaga cearae*) was resistant to
 272 azithromycin, ceftriaxone and nalidixic acid. In addition, the *Pantoea agglomerans*
 273 strain that was isolated from the same individual was resistant to eight out of twelve
 274 tested antibiotics (gentamicin, nalidixic acid, ceftriaxone, amoxicillin + clavulanic acid,
 275 ciprofloxacin, ampicillin, ciprofloxacin and meropenem), which correspond to five of
 276 the seven antimicrobial classes.

277 As expected, when considering the bacterial species that have intrinsic resistance
 278 mechanisms, resistance to at least one of the tested antimicrobials was observed in all of
 279 the strains. However, when considering only acquired resistance, 10 isolates (18.2%)
 280 were sensitive to all of the investigated drugs. Multidrug resistance (acquired cases) was
 281 observed in 13 isolates (23.5%), and three strains were resistant to seven antibiotics.
 282 From the total of 13 *Escherichia coli* strains, 2/13 (7.7%) presented multidrug resistance
 283 and 4/13 (30.8%) of the strains were sensitive to all of the studied antimicrobials (Table
 284 5).

Table 5. 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 <i>Escherichia coli</i> (%)	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%)

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289 **Discussion**

290 In this study, more than half of the samples were positive to some of the
291 investigated enterobacteria. Despite the isolation of fourteen different species of
292 bacteria, birds were not necessarily suffering from any pathological condition. In
293 addition to the low frequency of isolation, some of these microorganisms may occur
294 naturally in these birds, considering that these strains have been isolated previously
295 from healthy birds either in the wild or in cages [40, 41, 42, 43, 44, 45, 30]. Species of
296 the Enterobacteriaceae family, *Escherichia coli* in particular, do not belong to the
297 intestinal microbiota of granivorous pet birds, because feed composed exclusively of
298 seeds has been shown to provoke an inhibitory effect of this bacterial species [46]. In
299 this sense, the detection of Enterobacteriaceae in cloacal samples of granivorous birds
300 should be observed with caution, as it suggests favorable conditions for the
301 development of potential pathologies [47]. However, it is important to highlight that the
302 bird species that were captured in this study have an omnivorous diet. This may explain
303 the natural presence of enterobacteria, since the occurrence of these microorganisms in
304 the digestive tract is influenced by the composition of their nutrition [46]. Other factors
305 may also have influenced the isolation of these bacteria, such as direct or indirect
306 contact with domestic animals, as well as environmental contamination by human
307 action. In the natural environment in which they live, several species of enterobacteria
308 also occur, as they are ubiquitous, in soil or water. Thus, Enterobacteriaceae have been
309 isolated mainly from omnivorous, piscivorous and healthy carnivore birds [46, 48, 49,
310 50, 51].

311 The most prevalent bacterial species isolated from birds in this study was
312 *Pantoea agglomerans*. This microorganism can rarely cause infections, while it
313 normally acts as a commensal species that colonizes the normal intestinal microbiota.
314 Biodiversity studies report the isolation of *Pantoea agglomerans* in the microbiota of
315 several plants and insects [52, 53], which serve as a food source for several of the bird
316 species that were captured. *Escherichia coli* was also among the most isolated
317 microorganisms (23.2%) and is likewise an ubiquitous organism, which can be found in
318 soil, water and vegetation [54, 55]. Although its presence does not necessarily mean a
319 sign of illness, on some occasions, such as when they acquire virulence genes, this
320 bacterium can cause pathological problems in humans and animals, including birds [41].

321 The prevalence of *E. coli* in studies involving free-ranging birds is quite varied. Saviolli
322 [56] describes the presence of the microorganism in 60.0% of samples from
323 Magnificent Frigatebird (*Fregata magnificens*) from the coast of the State of São Paulo.
324 Vilela et al. [57] investigated this microorganism in fecal samples of House Sparrows
325 (*Passer domesticus*) that lived around farms in the State of Pernambuco and found
326 lower percentages (13.2%). Callaway et al. [58] analyzed cloacal swab samples from
327 376 migratory birds, which included Brown-Headed Cowbird (*Molothrus ater*),
328 Common Grackle (*Quiscalus quiscula*) and Cattle Egret (*Bubulcus ibis*), and found
329 even lower rates, 3.7% (14/376).

330 *Serratia rubidea* was the third most isolated species of bacteria. It is considered
331 an important human pathogen as a common agent of nosocomial infections mainly of
332 the urinary tract [59]. Diseases caused by *Serratia* in birds are uncommon but can occur
333 mostly in an opportunistic manner in immunocompromised birds due to stress in
334 captivity, inappropriate weather conditions, parasitic diseases, among other causes [60,
335 61]. Free-living birds can either acquire this microorganism from the contaminated
336 environment in which they live and may act as disseminators. Spena et al. [62] isolated
337 *Serratia rubidea* from oral swab samples of Eurasian Thick-knee (*Burhinus*
338 *oedicnemus*) and associated this finding with a diet composed of invertebrates found in
339 the feces of ruminants. This bacterial species has also been reported to be isolated from
340 lake waters in Poland, which was occupied by Great Cormorant (*Phalacrocorax carbo*).
341 Researchers have associated this finding with the leaching of feces and excreta during
342 rains leading this and other species of enterobacteria into the lake [63].

343 The other enterobacteria that occurred less frequently in the analyzed samples
344 can also occasionally cause damage to health and reports have already been described in
345 the scientific literature involving free-range or domestic birds. In addition to sharing
346 virulence factors with other enteropathogens, such as *Escherichia coli*, *Hafnia alvei* has
347 been reported to cause serious infections in laying hens [64, 65]. Miniero Davies et al.
348 [66] described an outbreak of mortality associated with *E. tarda* affecting fish, domestic
349 ducks and a wild heron that shared a lake located on a farm in the state of São Paulo,
350 Brazil. Davies et al. [67] described *Klebsiella pneumoniae* expressing virulence and
351 antibiotic resistance genes in psittacine and passerine birds from illegal trade.
352 *Cronobacter sakazakii* has been reported in broilers with clinical signs causing high
353 mortality and decreased egg production [68]. *Proteus* sp. are also potentially pathogenic
354 for birds as the cause of foot injuries and involvement of the respiratory system causing

355 air sacculitis and caseous pneumonia in cases of immunosuppression [69]. Bacteria
356 from the *Arizona* group have often been isolated from feces of adult chickens and
357 turkeys but have also been reported to occur in wild birds, such as the Canadian crane
358 [70, 71]. However, more serious occurrences have been reported in industrial birds,
359 such as mortality in turkeys, as well as clinical signs of Salmonellosis and omphalitis in
360 broiler chickens [72, 1].

361 It is always more expected to detect cases of antimicrobial resistance in birds
362 raised in captivity than those that live in the wild. In addition to the possibility of
363 inappropriate use of antibiotics, this may occur when birds have greater contact with
364 other animals that possess and disseminate resistant strains [73, 74]. However, our
365 research showed cases of free-living bird strains with relevant antimicrobial resistance
366 rates, mainly involving ampicillin, nalidixic acid and amoxicillin associated with
367 clavulanic acid. Some studies involving free-living birds has also reported resistance to
368 these three antibiotics in isolates of enterobacteria with varying rates. Carreira [75]
369 researched samples of cloacal swabs from free-living birds captured in the Metropolitan
370 region of Fortaleza, Brazil and observed that the acquired resistance rates of amoxicillin
371 associated with clavulanic acid, as well as nalidixic acid, were lower than the results
372 found in this study. Tsubokura et al. [76] analyzed *Escherichia coli* isolates from the
373 feces of several migratory bird species collected in the coastal region of Japan found
374 that less than 10% of the samples were resistant to ampicillin. These same researchers
375 used the feces of 54-day-old Hyline chicks and found the resistance to ampicillin to be
376 approximately 39.0%. These variations can often be attributed to the conditions found
377 in different habitats [77], as demonstrated by several studies that measure resistance
378 levels in isolates from birds under different conditions or captured in different
379 environments [78, 24, 79, 80, 81].

380 The rate of resistance to meropenem detected in free-living birds in this research
381 should also be highlighted (14.5%). Several studies involving wild birds, free-living or
382 not, as well as domestic birds, present lower rates of resistance to this drug or none at all
383 [82, 83, 30, 84, 85]. However, a more relevant point is the fact that this drug is a high
384 cost carbapenemic with restricted use to hospitals in Brazil. In addition, it is a last resort
385 for the treatment of infections and is widely prescribed to human patients with septic
386 conditions in intensive care of severe infections by Gram-negative hospital pathogens,
387 including Enterobacteriaceae [86, 87, 88]. Although the recommendations for the use of
388 this drug are restricted, the reservoirs of these organisms are increasing, not only in

389 hospitals, but also in the community and the environment. An important new source of
390 resistance development of such organisms is observed in livestock, companion animals
391 and wildlife [89].

392 Concerning the total number of isolated enterobacterial strains (23.6%) and
393 specifically *Escherichia coli* (15.4%), worrisome rates of multidrug resistance were
394 observed, considering that these are Gram-negative bacteria from free-living animals.
395 Other studies have also demonstrated the occurrence of multidrug resistance of bacteria
396 isolated from cloacal swabs in free-living birds [90, 85]. However, it is not so simple to
397 obtain a proper comparison of data from other studies, since there are few published
398 articles specifically involving free-living birds and isolates of enterobacteria in general
399 from cloacal swabs. One of these studies involves Gray-breasted Parakeets (*Pyrrhura*
400 *griseipectus*), whose total bacterial isolates presented a lower multidrug resistance rate
401 (11.1%) [30]. Concerning *Escherichia coli*, it is possible to observe that the results in
402 relation to multidrug resistance are the most varied. However, it is possible to find
403 similar rates [15], percentages lower than 5.4% or higher than 23.1% [26, 84].

404 Densely populated urban areas are historically seen as hotspots for antibiotic
405 resistant bacteria [91, 92] but microorganisms with these characteristics associated with
406 humans have been described in non-clinical environments, such as in remote areas of
407 the planet, far from direct anthropogenic pressure, apparently free from exposure to
408 antibiotics, as in regions of the Amazon, Bolivia and Antarctica. It is suspected that this
409 resistance may have been caused by the existence in these regions of military bases,
410 domestic animals, water, fishing boats, scientific expeditions and/or on-board tourism
411 [93, 94, 95, 96, 97]. Although it is important to emphasize that the cause of antibiotic
412 resistance may not always be related to environmental pressures caused by man, as is
413 the case of those that are naturally induced by microorganisms that produce natural
414 antibiotics [98].

415 The considerable resistance rates detected in isolates from birds captured in
416 Mulungu, more particularly those tested with ampicillin, amoxicilin+clavulunate,
417 meropenem and nalidixic acid, may indicate that some contact with anthropogenic
418 residues has occurred. Thus, we can consider that wild birds included in our study may
419 be working as indicators of environmental contamination. In this context, we found that
420 free-living birds can be considered victims of the environment in which they live,
421 acquiring multidrug resistant bacteria. At some point, this condition can harm the
422 conservation of species, or they may act as reservoirs of resistant bacteria [97, 99, 100,

423 101]. Thus, the emergence and evolution of antibiotic resistance among pathogenic
424 bacteria represent a serious public health issue on a global scale [102, 103].

425

426 **Conclusion**

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

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

445

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