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Isolation and Antimicrobial Resistance Profiles of Enterobacteria from Nestling Grey-Breasted Parakeets (Pyrrhura Griseipectus)

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ABSTRACT

The psittacine *Pyrrhura griseipectus* is a threatened species currently only found in Ceará State, Brazil. A microbiological survey was conducted to determine the composition of the enteric microbiota of this species, as well as the phenotypic profiles of antimicrobial susceptibility presented by the isolates. Cloacal swabs were collected from individual birds and submitted to microbiological processing. Antimicrobial susceptibility profiles were evaluated using the disk diffusion method. Bacteria of the genus *Escherichia, Proteus*, *Citrobacter*, *Pantoea*, *Klebsiella*, *Enterobacter*, *Morganella*, *Hafnia*, *Enterobacter*, and *Serratia* were isolated. The most frequently isolated species were *Escherichia coli*, *Proteus mirabilis* and *Proteus vulgaris*, corresponding to 36.1%, 26.4%, and 8.3%, respectively. Isolates were more frequently resistant to azithromycin and tetracycline, while *Escherichia coli* was the main species presenting multidrug resistance. In conclusion, free-living grey-breasted parakeets may harbor enterobacteria with high antimicrobial resistance rates.

INTRODUCTION

Bacterial resistance to antibiotics has been extensively studied as it is a matter of health concern and of its commercial implications. Studies have shown the increasing role of free-living birds as hosts for bacteria that carry antibiotic-resistance mechanisms (Smith *et al*., 2014; Carroll *et al*., 2015). Therefore, these animals are considered important agents for the emergence of multidrug resistance in several environments around the world, (Hasan *et al*., 2012) as well as for the spreading of resistant bacteria to multiple hosts and places.

It is known that Psittaciformes are capable of harboring numerous emerging zoonotic pathogens, as well as dispersing infected arthropod vectors (Godoy, 2007). Thus, the presence of Gram-negative bacteria, including those belonging to the Enterobacteriaceae family, in their intestinal microbiota has been considered an indication of potential diseases (Bangert *et al*., 1988; Mattes *et al*., 2005). Currently, microbiological studies with psittacine have increasingly isolated enterobacteria in healthy birds (Serafini *et al*., 2015; Lopes *et al*., 2015; Machado *et al*., 2016), which may indicate a more opportunistic role of these agents (Hidasi *et al*., 2013).

Among the enterobacteria relevant to both human and animal health, the role of the bacterium *Escherichia coli* (*E. coli*) as a potential pathogenic agent has been emphasized. *E. coli* is a commensal resident of microbiota of animals and humans (Schremmer *et al*.,1999); however, pathogenic strains are responsible for numerous intestinal and extra-intestinal diseases in both domestic and wild birds (Marietto-Gonçalves *et al*., 2007). The genus *Salmonella* is also considered a major zoonotic

agent, responsible for foodborne infections, especially from poultry products (Tortora *et al*., 2012).

The composition of the enteric microbiota of wild life psittacines is not fully known. In addition, few studies have been conducted with native Brazilian psittacines, emphasizing the importance of research focusing on these species (Alves *et al*., 2013; Vaz *et al*., 2017), and therefore, the *Pyrrhura griseipectus* (Salvadori, 1900), an endemic bird from the Northeast region of Brazil, was chosen as the object of this study. It is found in three areas of the state of Ceará: Ibaretama, Quixadá, and the Baturité Massif (Girão *et al*., 2010). Although this species, which common name is grey-breasted parakeet, has been recently adapted to breed in artificial nests, it builds nests in cavities inside tree trunks in the wild.

The grey-breasted parakeet is currently categorized as an endangered species (Sigrist, 2014, Brasil, 2014) due to its high vulnerability to human activities, particularly those related to illegal wildlife trade and urban expansion. Therefore, research on the composition of their intestinal microbiota, including the presence of bacteria with antimicrobial resistance, are important as the results may be used as tools to assist in the conservation of these birds (Prioste *et al*., 2013). The present study investigated the presence of enterobacteria in wild juvenile grey-breasted parakeets found in artificial nests. Also, the response of these agents to several antibiotics was analyzed.

MATERIAL AND METHODS

Study area

The environment of Baturité Massif (4°16'S, 38°56'W), located approximately 100 km from Fortaleza (capital city of Ceará State, Brazil), consists of moist forests in the midst of a dry and warm region, and it is an Environmental Protection Area (EPA). With an altitude of 600 meters in some areas, the EPA of Baturité Massif includes the municipalities of Aratuba, Baturité, Capistrano, Guaramiranga, Mulungu, Pacoti, Caridade, and Redenção.

Sampling

This study was authorized by the Brazilian agency for the environment and natural renewable resources (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, IBAMA) under SISBIO protocol n. 37211334, and approved by the Local Ethics Committee for the Use of Animals of the State University of Ceará (Protocol 4832011/14).

In order to contribute for the conservation of this species, artificial nests made of wood were placed inside private properties nearby the natural habitat of the parakeets. The nests are part of the conservation program of this species promoted by the non-governmental organization Associação de Pesquisa e Presevação de Ecossistemas Aquáticos (AQUASIS). Sampling was conducted during the reproductive period of the species, which occurs in the rainy season in Ceará State, from February to May in 2014 and again in 2015. A total of 79 healthy juvenile grey-breasted parakeets, with approximately 2 to 3 weeks of age were selected, and sampled for intestinal microbiota analysis. The birds were removed from the artificial nests, and carefully physically restrained to reduce stress as much as possible (Godoy, 2007). Birds were individually identified with leg bands and then submitted to the sampling procedure by using individual sterile cloacal swabs to obtain the fecal material. After sampling, individuals were promptly returned to the nests.

The cloacal swab samples were placed in tubes containing buffered peptone water broth, and maintained in a box containing ice in order to preserve their intrinsic properties. The collected material was transported to the Laboratório de Estudos Ornitológicos (LABEO) of the Universidade Estadual do Ceará, for microbiological analysis.

Microbiological analysis

The samples stored in tubes containing buffered peptone water were removed from the ice box and incubated in a bacteriological incubator at 37 °C for 24 h, which were the temperature and duration applied in all analytical procedures in this study. After this step, 1-mL aliquots of the initial samples were transferred to tubes containing selenite cystine broth or brain heart infusion broth, and incubated again (Lopes *et al*., 2015). A loopful of each broth was streaked on MacConkey agar enteric and Hektoen agar plates, and incubated. One to three bacterial colonies from each sample were selected according to the morphological characteristics presented in each selective media (Lopes *et al*., 2015).

The isolates were submitted to the following biochemical tests for the bacterial identification: investigation of classic fermentation characteristics in TSI (Triple-Sugar-Iron) agar, decarboxylation reactions in LIA (Lysine Iron agar), production of sulfide and indole and motility in SIM medium, and biochemical activities in methyl red and Voges-Proskauer tests, ornithine

decarboxylase, mannitol, arabinose, raffinose, dulcitol, adonitol, inositol, urease, rhamnose, citrate, malonate, glucose and lactosere actions (Koneman *et al*., 2010)

The presence of *E. coli* species was confirmed both by the classical biochemical profile shown and by the metallic green sheen that indicates this bacterial growth in Eosin Methylene Blue (EMB) agar (Koneman *et al*., 2010). Samples with biochemical profile of the genus *Salmonella* were further investigated using the rapid agglutination test with sera anti-somatic "O" (Lopes *et al*., 2015).

Antimicrobial resistance

The antimicrobial susceptibility test was performed using the disk diffusion method, using the standards provided by Clinical and Laboratory Standards Institute (CLSI, 2012). Antibiotics of the following classes were selected: aminoglycosides, cephalosporins, chloramphenicol, macrolides, polymyxins, quinolones, sulfonamides, tetracycline and trimethoprim. Therefore, discs containing the following antibiotics with respective concentrations were used: azithromycin (15 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), streptomycin (10 µg), gentamycin (10 µg), tetracycline (30 µg), ceftiofur (30 µg), polymyxin B (300 µg), sulfamethoxazole and trimethoprim (25 µg) and sulfonamide (300 µg).

A suspension of the isolated bacteria was prepared by inoculating the pure colonies into sterile saline solution to reach inoculum turbidity equal a 0.5 McFarland turbidity standard. The inocula were streaked on Mueller-Hinton agar plates, and the antibiotic discs containing the antimicrobial were placed on the plates, and incubated. After incubation, the results were interpreted by measuring the inhibition zones around the discs (Lopes *et al*., 2015). The strains that showed phenotypic resistance to three or more antibiotics were considered multidrug resistant. The *E. coli* strain ATCC 25922 was used as control sample.

RESULTS

A total of 144 strains were isolated, and belonged to the genera *Escherichia*, *Proteus*, *Citrobacter*, *Pantoea, Klebsiella*, *Enterobacter*, *Morganella*, *Hafnia*, *Enterobacter*, and *Serratia*. All birds were positive for at least one of the isolated bacteria. In addition, more than one species per fecal sample were detected in some cases.

The most frequently isolated species were: *Escherichia coli*, with 52 isolates (36.1%), followed by *Proteus mirabilis,* with 38 isolates (26.4%), *Proteus vulgaris* with 12 isolates (8.3%), and *Citrobacter freundii,* with 10 isolates (6.9%). The frequency of the other species ranged between 1 and 7 isolates, and no *Salmonella* sp. was isolated. The results are shown in Table 1.

Table 1 – Absolute numbers (n)and frequencies (%) of bacterial isolates, and frequency of positive birds per isolate obtained in the 79 individual cloacal swab samples collected from *Pyrrhura griseipectus* nestlings in Ceará State, Brazil

Bacterial isolates	Isolates	Positive birds	Frequency
	n	(%)	(%)
Escherichia coli	52	65.8	36.1
Proteus mirabilis	38	48.1	26.4
Proteus vulgaris	12	15.2	8.3
Citrobacter freundii	10	12.7	6.9
Pantoea agglomerans	7	8.9	4.8
Klebsiella pneumoniae	6	7.6	4.2
Enterobacter aerogenes	5	6.3	3.5
Morganella morganii	3	3.8	2.1
Hafnia alvei	2	2.5	1.4
Enterobacter cloacae	2	2.5	1.4
Citrobacter amalonaticus	2	2.5	1.4
Citrobacter diversus	2	2.5	1.4
Enterobacter sakazakii	1	1.3	0.7
Serratia liquefaciens	1	1.3	0.7
Serratia rubidae	1	1.3	0.7
Total	144	-	100.0

Out of the 144 bacterial isolates obtained, 141 were submitted to the antimicrobial susceptibility test, and three samples were not used due to conservation issues. Three of the antibiotic compounds tested showed low efficacy against the isolates: 38.3%,18.4%, and 13.5% of the isolates were resistant to azithromycin, tetracycline, and sulfonamide, respectively (Table 2).

The results in Table 2 show that, other than the species *Serratia liquefaciens*, *Serratia rubidae* and *Enterobacter cloacae*, the isolates presented variable resistance levels to 9 out of the 11tested antibiotics; only ciprofloxacin and ceftiofur fully inhibited all of the tested strains. In this context, 21 (14.9%) isolates presented multidrug resistance, with *E. coli* isolates as the most frequent, accounting for 33% of the isolates (7/21) and presenting resistance to azithromycin, chloramphenicol,sulfametoxazole+trimetropim, sulfonamide, and tetracycline, with a variation ranging from 8.2% to 28.6%.

Table 2 – Antibiotic resistance of enterobacteria isolated from *Pyrrhura griseipectus* nestlings of Ceará State, Brazil.

Species	AZI	POL	GEN	NAL	SUL	TET	CFT	SUT	CLO	STR	CIP
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Citrobacter amalonaticus	1 (50)	1 (50)	0	1 (50)	0	1 (50)	0	0	0	0	0
Citrobacter diversus	0	1 (50)	0	1 (50)	0	0	0	0	0	0	0
Citrobacter freundii	5 (50)	7 (70)	0	1 (10)	1 (10)	5 (50)	0	1 (10)	0	0	0
Enterobacter aerogenes	2 (40)	1 (20)	0	1 (20)	1 (20)	2 (40)	0	1 (20)	1 (20)	0	0
Enterobacter cloacae	0	0	0	0	0	0	0	0	0	0	0
Enterobacter sakazakii	0	0	0	1 (100)	0	0	0	0	0	0	0
Escherichia coli	4 (8.2)	1 (2.0)	1 (2.0)	3 (6.1)	10 (20.4)	14 (28.6)	0	7 (14.3)	5 (10.2)	1 (2.0)	0
Hafnia alvei	1 (50)	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	5 (83.3)	0	0	1 (16.7)	0	0	0	0	0	1 (16.7)	0
Morganella morganii	2 (66.7)	NR	0	33,3	33,3	3 (100)	0	0	0	0	0
Pantoeae agglomerans	2 (28.6)	1 (14.3)	0	1 (14.3)	1 (14.3)	1 (14.3)	0	2 (28.6)	0	0	0
Proteus mirabilis	25 (65.8)	NR	1 (2.6)	7 (18.4)	4 (10.5)	NR	0	5 (13.1)	2 (5.2)	4 (10.5)	0
Proteus vulgaris	7 (58.3)	NR	0	1 (8.3)	2 (16.7)	NR	0	0	0	0	0
Serratia liquefaciens	0	0	0	0	0	0	0	0	0	0	0
Serratia rubidae	0	0	0	0	0	0	0	0	0	0	0
Total (n=141)	54 (38.2)	12 (8.5)	2 (1.4)	17 (12.0)	19 (13.5)	26 (18.4)	0	16 (11.3)	8 (5.8)	6 (4.2)	0

AZI: Azithromycin, POL B: Polymycin B, GEN: Gentamycin, NAL: Nalidix acid, SUL: Sulfonamide, TET: Tetracycline, CTF: Ceftiofur, SUT: Sulfametoxazole + Trimetropim, CLO: Chloramphenicol, EST: Streptomycin, CIP: Ciprofloxacin, NR: Natural Resistance

The table 3 shows the resistance profiles of *E. coli* strains, the major representative of multidrug resistance. One strain was resistant to 9 antibiotics, while the other six presented resistance patterns from three to five antibiotics.

Table 3 – Multidrug resistant profiles of *Escherichia coli* strains isolated

Number of isolates	Multidrug Resistance Profiles
1	AZI, POL,GEN,NAL,SUL,TET,SUT,CLO,STR
1	NAL,SUL,TET,SUT,CLO
2	SUL,TET,SUT
3	SUL,TET,SUT,CLO
Total: 7	

SUL: Sulfonamide, TET: Tetracycline, AZI: Azithromycin, POL: Polymycin B, GEN: Gentamycin, NAL: Nalidixic acid, CLO: Chloramphenicol, STR: Streptomycin, SUT: Sulfametoxazole-trimetropim

DISCUSSION

There are few studies on the enteric bacterial microbiota of free-living birds, especially those associated with its development altricial birds. Therefore, to the best of the authors' knowledge, this is the first study developed to evaluate the enteric microbiota of the endangered species *Pyrrhura griseipectus*. This study provides new information that will help to fill knowledge gaps on the health of these birds in the wild.

The results show that *E. coli* as the most prevalent bacterium in the microbiota, in agreement with the literature reporting the presence of this bacterium in the microbiota of young and adult wild birds (Dongen *et al*., 2013). Hidasi *et al*. (2013) studied the intestinal microbiota of parrots belonging to different species (Psittaciformes) seized from illegal trade and also found that *E. coli* was the most prevalent species (33.8%). Vaz *et al.* (2017) detected that 72.7% of wild red-tailed Amazon parrot (*Amazona brasilensis*) nestlings sampled from artificial wooden nests harbored *E. coli*; however, the authors considered this result within the normal microbiological profile of such birds.

In the present study, *E. coli* and other enterobacteria may have had a commensal behavior at the time of sampling, because they were isolated from apparently healthy birds with no specific signs of bacterial disease (Szmolka & Nagy, 2013). However, while *E. coli* strains may be part of the normal enteric microbiota, some pathogenic strains still may potentially induce virulence mechanisms, resulting colibacillosis, salpingitis, respiratory diseases, omphalitis, or septicemia (Godoy, 2007). In addition, *E. coli* populations in the bird's intestines are likely to trigger a virulence response when subjected to environmental changes or to oscillations of the host's immune system (Ewers *et al*., 2009).

The possibility of psittacines to harbor potentially pathogenic *E. coli* strains, while showing no overt clinical signs, is a relevant health concern as these birds may serve as asymptomatic reservoirs. The studies of Saidenberg *et al*. (2012) showed the presence of *E. coli* with virulence genes reported in cases of severe illness in clinically asymptomatic parrots. This indicates that these birds may suffer disease in a near future or may

excrete this pathogen in the environment, potentially contaminating other animals.

The second most frequently isolated microorganisms in this study belong to the genus *Proteus* sp., and were represented by the species *Proteus mirabilis* and *Proteus vulgaris*. These bacilli were previously isolated in young birds, and it was identified that their population tends to decrease as the birds grow older (Naldo *et al*., 1998). It is considered an opportunistic microorganism, and upper respiratory system disorders and pododermatitis have been associated with infections by *Proteus* sp. in wild birds (Olinda *et al*., 2012).

Citrobacter freundii, considered as an opportunistic and ubiquitous bacteria (Fernandez *et al*. 2011), has been detected as the primary agent in cases of sudden death in captive parrots (Churria *et al*., 2014). *Pantoea agglomerans* is a potential human pathogen (Cruz *et al*., 2007) and it is commonly found in nature, especially in plants, water, soil, and animals (Delétoile *et al*., 2009). This suggests that free-living birds may acquire these bacteria from direct contact with leaves, water, or food particles found in their habitats (Gibbs *et al*., 2007).

Enterobacteria of the genera *Klebsiella, Enterobacter, Morganella, Hafnia* and *Serratia* were less frequently isolated in this study; however, their isolation in wild birds was previously reported in other studies (Gibbs *et al*., 2007; Santos *et al*., 2010). Those bacteria are responsible for occasional infections and, in some cases, can function as primary pathogens (Gerlach, 1994, Hidasi *et al*., 2013, Davies *et al*., 2016)

The absence of *Salmonella* was also observed in other Brazilian studies, with low detection rates in apparently healthy wild birds, whether maintained in captivity or in the wild (Allgayer *et al*., 2009; Santos *et al*., 2010; Lopes *et al*., 2015, Machado *et al*., 2016). Because psittacines are very sensitive to avian salmonellosis, particularly at young ages (Marietto-Gonçalves *et al*., 2010), the absence of *Salmonella* spp in the present study suggests that the evaluated birds were not exposed to any pathogen of this genus (Godoy, 2007). In addition, no dead parakeets were found in the present study to allow their evaluation.

Interestingly, *Salmonella* shedding is usually intermittent (Gerlach, 1994) and it is possible that an infected bird may not shed it at the time of sampling (Butron & Brightsmith, 2010). Another possible explanation for the absence of *Salmonella* spp. in the present study may be the young age of the sampled parakeets, since enteric microbiota of birds gradually changes as they grow up (Kohl, 2012). Moreover, Hidasi *et al*. (2013) state that *Salmonella* spp. are uncommon habitants of the microbiota of captive and wild life psittacines.

The enterobacteria detected in this research study is explained by the fact that young birds are colonized by microbes immediately after hatch, and acquire a microbial biomass by contact with environmental microorganisms, as well as by parental feeding, both of which considered the main sources of microbial transmission to developing birds (Mills *et al*., 1999). Moreover, the young birds may have acquired this harmful microbial load by direct contact with other animals that might have visited the artificial nests, such as mice, bats, and other avian species, or by indirect contact with their secretions (Allgayer *et al*., 2009; Serafini *et al*., 2015).

The recent interest in the study of the free-living birds stems from the perception that these birds may be carriers of antimicrobial resistance mechanisms, as the host species may acquire dangerous pathogens as well as disseminate them (Beskin *et al*., 2009). Although free-living birds have infrequent direct contact with antimicrobial agents, they are still capable to host resistant bacteria or to be infected by these microbes (Radhouani *et al*., 2012). Interestingly, the studied parakeets had direct contact with their parents in a reduced space in the artificial nests, which could facilitate a process of bacterial transmission between the birds and through the environment of the nests.

Greater resistance was primarily observed to azithromycin, followed by sulfonamide and nalidixic acid. The first is an important antibiotic of the macrolides class, and used in human and veterinary medicine for the treatment of infections caused by Gram-positive and Gram-negative bacteria (Mitchell, 2005). A significant resistance to this antibiotic has been detected in bacterial samples from psittacines seized from illegal trade, which may be explained by its inadequate use, as well as by the transmission of resistant clones (Lopes *et al*., 2015).

In addition, the detected resistance to tetracycline and sulfonamide, used both in human and veterinary medicine, may indicate a wide spread of resistant bacteria in different environments, as shown in previous studies with bacteria of animal origin (Hirsh & Zee, 2009; Hu *et al.*, 2013).

The isolation of antibiotic-resistant *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pantoea agglomerans*, *Proteus* sp., and *E. coli* in the present study is a significant finding, as they may transmit resistance genes to different bacterial genera (Levinson, 2010),

allowing the exchange of these genes between commensal bacteria and those with elevated pathogenic potential, which may persist for longer periods in the host's enteric microbiota.

Regarding the resistance results obtained from *E. coli* strains, previous studies have shown similar profiles in strains isolated from migratory birds. The research of Bonnedahl *et al*. (2009), for example, showed that *E. coli* isolated from juvenile seagulls presented significant rates of resistance to tetracycline. In addition, a study with wild birds in rehabilitation environments before their reintroduction in the wild performed by Hidasi *et al*. (2013) found *E. coli* strains with high levels of resistance to chloramphenicol (33.1%), tetracycline (69.1%) and sulfonamide (71.5%). Both studies demonstrate that bacterial resistance may be related to direct or indirect exposure to human activity, and it is usually associated with selective pressure by antibiotics.

In this study, seven *E. coli* strains showed multidrug resistance, out of which one presented resistance to 9 of the 11 antibiotics tested. This phenomenon was previously demonstrated in other studies with free-living birds, even in harsh environments with little human presence, as reported by Sjølund *et al*. (2008), who isolated multidrug resistant *E. coli* strains from the enteric microbiota of birds in the Arctic. This complex bacterial capacity to develop resistance mechanisms has been the focus of numerous studies, and frequently, one of the main challenges is to determine the origin of such resistant strains in wild animals, especially birds, whose ability of vast mobilization makes them capable of disseminating numerous microbes in various regions.

The resistant bacteria isolated in this study may also have been horizontally transmitted by the parents, which may have had contact with other animals or even with products of human origin (i.e., food leftovers and adverse secretions) due to the strong presence of private estates and tourist activities in the region where the studied birds were found. According to Nascimento *et al*. (2010), this region has experienced a significant human population growth in recent years, especially in urban areas, and are associated with the development of the construction industry and the tourism activities.

In this context, the increasing manipulation of birds and artificial boxes to monitor the birth and growth of fledglings may be a contamination source. Therefore, the use of adequate personal protection equipment is essential to ensure safety in the bird management, as well as to avoid prolonged contact, which may cause animal stress and direct contamination. The probability of free-living birds to harbor resistant bacteria increases according to their proximity to anthropogenic centers (Cole *et al*., 2005), reflecting the use of antibiotics in the region where they are found (Bonnedahl *et al*., 2009).

Another possibility is the contamination of the artificial nests by secretions of several invasive animals. In addition, the contact with migratory birds, whose ability to reach multiple territories makes them relevant foci of microbial spread is a hypothesis widely advocated in previous studies to explain the primary source of multidrug resistance bacteria in regions far from urban centers (Dolejska *et al*., 2007). In the Baturité Massif, a survey of the avifauna showed a wide number of avian species, including migratory birds, which supports this hypothesis (Albano & Girão, 2008). Moreover, it is also possible that the resistance mechanisms of these bacteria were triggered by natural environmental pressure, which promoted the occurrence of strains with such characteristics even in the absence of antimicrobial use (Nascimento *et al*., 2003).

Whatever the origin of these bacteria in the examined birds, their presence may indicate that grey-breasted parakeets may could act as reservoirs of these enteric microorganisms, highlighting the need of further microbiological studies to understand their pathogenicity in these wild birds. Therefore, a health monitoring program of young and adult grey-breasted parakeets would bring a deeper understanding of the epidemiological evolution of their colonization by enterobacteria. In addition, the current study may serve as a first step for further research on the grey-breasted parakeet, contributing for its conservation program as well as to emphasize the importance of Brazilian biodiversity.

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