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MICROBIOLOGY

Antimicrobial susceptibility of *Staphylococcus* sp. and *Escherichia coli* isolated from captive Amazonian manatee (*Trichechus inunguis*)

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ABSTRACT: The Amazonian manatee (*Trichechus inunguis*) is an aquatic mammal threatened with extinction. However, few studies have investigated the pathogens in this species, which may affect both animal and human health. This study aimed to evaluate the frequency, distribution, and patterns of antimicrobial susceptibility of *Staphylococcus* spp. and *Escherichia coli* colonizing the nasal and rectal cavities of Amazonian manatees kept in captivity at the National Institute for Amazonian Research (INPA) in the state of Amazonas, Brazil. Rectal and nasal swabs from 44 manatees of different ages were used in this study. The genus *Staphylococcus* was isolated from the nasal swabs of 32 (72.7%) animals, with two individuals harboring more than one species of *Staphylococcus*. *S. sciuri* was the most commonly isolated species. Resistance to penicillin was observed in 13 (40.6%) isolates, more frequent than the other antimicrobials tested (P = 0.01). *E. coli* was isolated from the rectal swabs of all animals, with phylogroup B1 being the most frequent among the strains obtained (P = 0.0008). Four isolates (6.8%) were positive for virulence factors, three of which were classified as enterotoxigenic *E. coli* (ETEC) and one as enteropathogenic *E. coli* (EPEC). To our knowledge, this is the first study to evaluate *Staphylococcus* spp. and *E. coli* in Amazonian manatee samples. This study revealed nasal colonization by *Staphylococcus* spp., mainly *S. sciuri*, and diarrheagenic *E. coli* isolates, including antimicrobial-resistant strains.
Key words: Enterobacteriaceae, Trichechidae, Manatins.

Isolamento e sensibilidade antimicrobiana de *Staphylococcus* sp. e *Escherichia coli* de peixe-boi-da-amazônia (*Trichechus inunguis*) em cativeiro

RESUMO: O peixe-boi amazônico (*Trichechus inunguis*) é um mamífero aquático ameaçado de extinção. Apesar disso, poucos estudos investigaram patógenos nessa espécie que podem impactar a saúde animal e humana. O objetivo deste estudo foi avaliar a frequência, distribuição e padrões de suscetibilidade antimicrobiana de *Staphylococcus* sp. e *Escherichia coli* pertencentes à microbiota nasal e retal de peixes-bois da Amazônia mantidos em cativeiro no Instituto Nacional de Pesquisas da Amazônia (INPA) (Amazonas, Brasil). Foram utilizados suabes retais e nasais de 44 peixes-boi de diferentes idades. O gênero *Staphylococcus* foi isolado do swab nasal de 32 (72,7%) animais, sendo que dois indivíduos apresentaram mais de uma espécie de *Staphylococcus*. *S. sciuri* foi a espécie mais comumente isolada. A resistência à penicilina foi observada em 13 (40,6%) isolados, sendo mais frequente do que os outros antimicrobianos testados (P = 0,01). *Escherichia coli* foi isolada dos suabes retais de todos os animais, sendo o filogrupo B1 o mais frequente entre as cepas obtidas (P = 0,0008). Quatro isolados (6,8%) foram positivos para fatores de virulência, três dos quais foram classificados como *E. coli* enterotoxigênica (ETEC) e um isolado como *E. coli* enteropatogênica (EPEC). Este é o primeiro estudo a avaliar *Staphylococcus* spp. e *E. coli* de amostras de peixe-boi da Amazônia. O estudo revelou colonização nasal por *Staphylococcus* sp., principalmente *S. sciuri*, e por isolados diarreiogênicos de *E. coli*, incluindo cepas resistentes a antimicrobianos.
Palavras-chave: Enterobacteriaceae, Trichechidae, peixe-boi.

INTRODUCTION

Manatees (*Trichechus* spp.) are aquatic mammals classified as "vulnerable" by the International Union for Conservation of Nature (IUCN) (MARMONTEL et al., 2016). There are two species of the manatee in Brazil, the American manatee (*T. manatus*) and the Amazonian manatee (*T. inunguis*), both of which are threatened by hunting, accidental capture, and destruction of their natural habitats (BARRETO et al., 2021; CABALLERO et al., 2021; SIDRIM et al., 2015). To reduce the impact of these threats, orphaned American manatee calves, victims of poaching, or those accidentally caught in fishing nets were rescued at various locations in Amazonas State (Brazil) and rehabilitated at the National Institute for Amazonian Research (INPA). These animals remain in captivity for up to 10 years until they meet the requirements for release back into the wild.

Staphylococci are gram-positive, coccoid bacteria that are commensals of several species, but

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they can also be the cause of a wide variety of diseases in animals and humans (LISOWSKA-ŁYSIAK et al., 2021; SANTANA et al., 2022a ; WALTHER et al., 2017; WIELER et al., 2011). There is little knowledge about the species of *Staphylococcus* that colonize animals of wild species, with most studies being case reports of infection (GARCÊS et al., 2019; LUZZAGO et al., 2014; SAHIN-TÓTH et al., 2022).

Escherichia coli is also a commensal bacterial agent in humans and animals, but it can cause a range of conditions, from mild enteric conditions to infections in various organs and potentially fatal sepsis (ALFURAIJI et al., 2022; KOLENDA et al., 2015; SONG et al., 2022). Some animals also act as carriers of *E. coli* potentially pathogenic to humans (ABREHAM et al., 2019; KOLODZIEJEK et al., 2022; PIÉRARD et al., 2012); however, the role of wild animals as reservoirs of *E. coli* and its relevance to public health is still not well understood (MERKER BREYER et al., 2022; TORRES et al., 2022).

In addition to the pathogenic potential of *E. coli* and *Staphylococcus* bacteria, there is increasing concern regarding their potential for antimicrobial resistance in humans and animals (AWORH et al., 2021; RASMI et al., 2022; RAUBER et al., 2016; VIEGAS et al., 2022). Evidence shows that wild animals can act as reservoirs and disseminators of resistant microorganisms (BESSALAH et al., 2021; ROTH et al., 2019; SUAY-GARCÍA et al., 2019).

Therefore, this study aimed to evaluate the frequency, distribution, and patterns of antimicrobial susceptibility of *E. coli* and *Staphylococcus* spp. found in the nasal and rectal cavities of Amazonian manatees held in captivity at the INPA, Amazonas, Brazil.

MATERIALS AND METHODS

Samples

A one-year study was conducted using non-probability sampling, where all available animals were sampled. A total of 44 Amazonian manatees, of different age groups and sexes, from the INPA were included in this study (Table 1). A total of 76 nasal and rectal swabs were collected, of which 21 were from calves (47.7%), 14 were from juveniles (31.8%), and nine were from adults (20.4%), with the majority being female. The time spent in captivity by the sampled calves varied from 3 days to 40 months, with an average of 15 months. The juveniles in captivity were from 29 to 70 months of age, with a mean age of 56. All sampled adults were in captivity for over five years, with a maximum of 36 years (mean = 19 years).

Calves were housed in circular fiberglass tanks with 636 m^3 freshwater, whereas juveniles were kept in oval fiberglass tanks filled with 2,268 m^3 freshwater. Calves were fed exclusively on an artificial milk formula (water, whole milk powder, canola oil, Aminomix (Vetnil, Brazil), soybean meal, rolled oats, and corn flour) four times per day (MADURO et al., 2020). A combination of milk formula with mixed cultivated vegetables and grasses (carrots, lettuce, pumpkin, cabbage, *Brachiaria mutica*, and *Eichhornia crassipes*) was offered to the juveniles thrice daily. The adults were fed once daily, exclusively on a mixture of cultivated vegetables and grass.

For sampling, the animals were physically restrained and placed over a sponge mattress outside the water. All individuals were clinically monitored and considered healthy when samples were collected. Nasal and rectal swabs were collected in Stuart medium (Olen®, China) and were refrigerated and sent to the Laboratory of Bacterioses and Anaerobe Research at the Veterinary School of the Federal University of Minas Gerais (Belo Horizonte, Brazil) to be processed within four days.

Staphylococcus sp. and detection of mecA

Swabs were seeded onto saline mannitol agar (MSA; Difco, USA) and incubated at 37 °C for 24 h to isolate *Staphylococcus* spp. Presumptive colonies from each sample were plated on Müller Hinton agar (MH; Difco, USA) and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, as previously described

Table 1 - Collected samples, animal age category, and culture results for *Escherichia coli* and *Staphylococcus* spp. in captive Amazonian manatee (*Trichechus inunguis*).

Age group	--Nasal swab (%)--	--Rectal swab (%)--	----TOTAL (%)----	---------------------Culture---------------------	
				E. coli	*Staphylococcus* spp.
Adult	9 (20.4)	5 (15.6)	14 (18.4)	5 (20)	7 (21.8)
Juvenile	14 (31.8)	13 (40.6)	27 (35.5)	8 (32)	7 (21.8)
Calf	21 (47.7)	14 (43.7)	35 (46)	12 (48)	18 (56.2)
TOTAL (%)	44 (100)	32 (100)	76 (100)	25 (100)	32 (100)

(ASSIS et al., 2017) using a FlexControl MicroFlex LT mass spectrometer (Bruker Daltonics, USA) with a 60-Hz nitrogen laser, in which up to 240 laser shots were used. Parameters for mass range detection were defined to allow the identification from 1,960 to 20,137 m/z, where Ion source 1 was 19.99 kV, Ion source 2 was 18.24 kV, and the lens voltage was 6.0 kV for data acquisition. Prior to measurement, calibration was performed using a standard control (*Escherichia coli* DH5 alpha; Bruker Daltonics, USA). The real-time (RT) identification score criteria were those recommended by the manufacturer: a score ≥ 2.3 indicated a species-level identification. Isolates with MALDI-TOF, scores under 2.300 were submitted to sequencing the *rpoB* gene (MELLMANN et al., 2006).

Antimicrobial susceptibility tests were performed using the disk diffusion method on agar according to the Institute of Clinical and Laboratory Standards Institute (CLSI) guidelines M100-S30 and VET01S (CLSI, 2020a; CLSI, 2020b). The antimicrobials tested were based on the recommendations of CLSI (CLSI, 2020a; CLSI, 2020b) and considered one representative compound from each antimicrobial class commonly used in human and veterinary medicine: cefoxitin (CFO:30 µg), penicillin G (PEN:10 IU), tetracycline (TET:30 µg), trimethoprim/sulfamethoxazole (SUT:25 µg), chloramphenicol (CLO:30 µg), erythromycin (ERI:5 µg), clindamycin (CLI:2 µg), gentamicin (GEN:10 µg), rifampicin (RIF:5 µg), and ciprofloxacin (CIP:5 µg) (DME, BRA). *Staphylococcus aureus* ATCC 25923 was used as the control. Isolates were considered multidrug resistant (MDR) if they were resistant to three or more classes of antimicrobials (SWEENEY et al., 2018). In addition, *Staphylococcus* spp. isolates were tested by polymerase chain reaction (PCR) to determine whether they carried the *mecA* gene (MURAKAMI et al., 1991).

Escherichia coli

For *E. coli* isolation, nasal and fecal swabs were plated on MacConkey agar (MC; Difco, USA) and incubated for 24 h at 37 ºC. Up to three selected lactose-producing colonies from each sample were subjected to species-specific PCR, as previously described (MCDANIEL et al., 1996) and using the following primers: 5′-ACCTGCGTTGCGTAAATA-3′ forward and 5′-GGGCGGGAGAAGTTGATG-3′ reverse. The *E. coli* isolates were then classified into one of the following phylogroups: A, B1, B2, C, D, E, F, or Clade I (CLERMONT et al., 2013). The presence of virulence genes associated with enterotoxigenic *E. coli* (ETEC: *sta, stb, lt, f5, f18, f41, f4,* and *987p*), enteropathogenic *E. coli* (EPEC: *eae, bfpA, iha, toxB,* and *efa1*), Shiga toxin-producing *E. coli* (STEC: *stx1, stx2, ehxA,* and *saa*), enterohemorrhagic *E. coli* (EHEC: *eae, iha, toxB, efa1, stx1, stx2, ehxA,* and *saa*), *E. coli* necrotoxic (NTEC: *cnf1, cnf2,* and *f17*), enteroaggregative *E. coli* (EAEC: *astA, aggR, aaf,* and *pet*) and enteroinvasive *E. coli* (EIEC: *ipaH*) were screened using PCR (RAMOS et al., 2019).

One *E. coli* isolate per animal was subjected to antimicrobial susceptibility testing. Two isolates were tested for animals that were positive for more than one *E. coli* phylogroup. Antimicrobial susceptibility tests were performed using disk diffusion on agar according to the Institute of Clinical and Laboratory Standards guideline VET01S (CLSI, 2020a). The following antimicrobials were tested: trimethoprim/sulfamethoxazole (SUT:25 µg), enrofloxacin (ENO:5 µg), gentamicin (GEN:10 µg), neomycin (NEO:30 µg), ceftiofur (CFT:30 µg), amoxicillin/clavulanic acid (AMC:30 µg), ampicillin (AMP:10 µg), florfenicol (FLF:30 µg), doxycycline (DOX:30 µg), oxytetracycline (OT:30 µg) (zones of inhibition were interpreted according to CLSI, 2020), and ciprofloxacin (CIP:5 µg) (zones of inhibition were interpreted according to EUCAST, 2022) (DME, BRA). *E. coli* ATCC 25922 was used as the control.

RESULTS AND DISCUSSION

Bacteria of the genus *Staphylococcus* were isolated from nasal swabs of 32 (72.7%) individuals. *S. sciuri* was the most commonly isolated species in this study (Table 2). There was no association between the isolation of *Staphylococcus* species and animal categories ($P > 0.05$).

Resistance to penicillin and tetracycline was observed in 13 (40.6%) and three (9.3%) isolates, respectively (Figure 1). Two isolates (6.2%) exhibited simultaneous resistance to both antimicrobials. The highest resistance rate among the tested strains ($P = 0.0111$) was for penicillin, whereas all isolates were sensitive to cefoxitin, rifampicin, chloramphenicol, ciprofloxacin, and gentamicin.

Twenty-five (25/44, 56.8%) animals tested positive for *E. coli*, and a total of 59 isolates were obtained from rectal swabs. Of the total number of isolates, 39 (66.1%) were classified as phylogroup B1 (Table 3), which had more isolates than the others ($P = 0.0008$). Four isolates (6.8%) were positive for virulence factors, three of which were classified as ETEC (positive for LT and 937p virulence factors), and one isolate was classified as EPEC (positive for the *eae* gene, which codes for intimin) (Table 4).

Table 2 - Distribution of *Staphylococcus* spp. isolates in nasal swabs of captive Amazonian manatees (*Trichechus inunguis*) (n= 44).

Species	--Total (%)--
S. sciuri	17 (38.6)
S. kloosii	4 (9)
S. haemolyticus	3 (6.8)
S. warneri	2 (4.5)
S. gallinarum	2 (4.5)
S. xylosus	1 (2.3)
S. pseudoxylosus	1 (2.3)
S. pasteuri	1 (2.3)
S. hominis	1 (2.3)
Negatives	12 (27.3)
Total	44 (100)

Almost one-third (11/37 = 29.7%) of the isolates were resistant to at least one antimicrobial class, and two isolates (2/37 = 5.4%) were multidrug-resistant (Figure 1). Resistance to ampicillin and trimethoprim/sulfamethoxazole was most frequent among *E. coli* isolates, whereas resistance to ceftiofur and gentamicin was not detected in the present study.

Few studies have been conducted on commensal microorganisms and bacterial pathogens

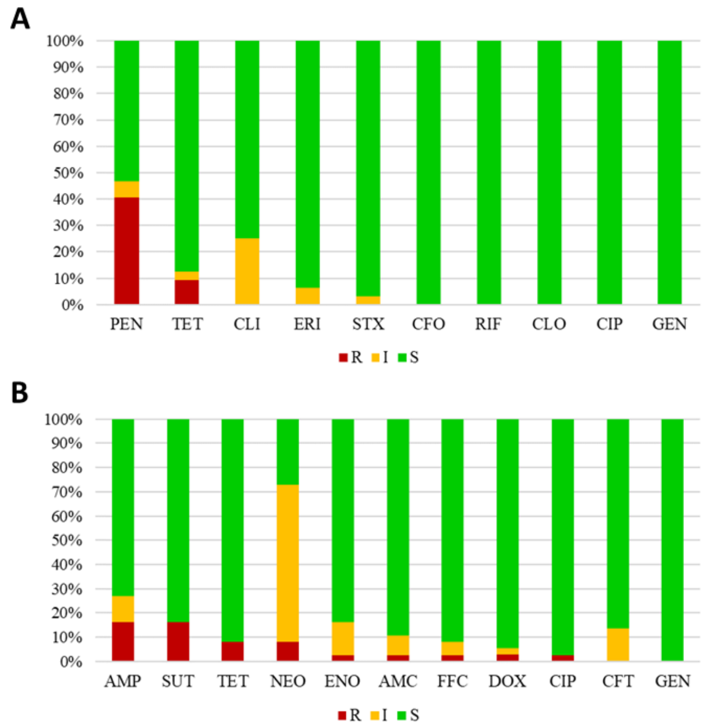

Figure 1 - Overall frequency of antimicrobial resistance among *Staphylococcus* spp. (A) and *Escherichia coli* isolates (B) from captive Amazonian manatee (*Trichechus inunguis*), Brazil. Legend: R – Resistant; I – Intermediate Resistance; S –Susceptible; cefoxitin (CFO), penicillin (PEN), tetracycline (TET), trimethoprim/sulfamethoxazole (SUT), chloramphenicol (CLO), erythromycin (ERI), clindamycin (CLI), gentamicin (GEN), rifampicin (RIF), and ciprofloxacin (CIP), enrofloxacin (ENO), neomycin (NEO), ceftiofur (CFT), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), florfenicol (FLF), doxycycline (DOX).

Table 3 - Number of isolates and frequency of Clermont phylogroups of *Escherichia coli* identified from rectal swabs of 25 captive Amazonian manatees (*Trichechus inunguis*).

Phylogroup	--Total positive isolates (%)--
B1	39 (66.1)
D	9 (15.2)
A	7 (11.8)
F	3 (5.1)
E	1 (1.7)
Total	59 (100)

in wild animals, particularly endangered species (DE OLIVEIRA et al., 2021; LONCARIC et al., 2019). Among animals belonging to the order Sirenia, the manatee (genus *Trichechus*, specifically the species *Trichechus inunguis*) stands out. Studying this species in the wild is difficult due to its habitat and behavior, as it can camouflage itself in the dark waters of the Amazon rivers, avoiding contact with humans as much as possible (ARÉVALO-SANDI et al., 2016). To date, studies on *Staphylococcus* and *E. coli* in animals of the order Sirenia have been limited to case reports, and few studies have evaluated the colonization and antimicrobial sensitivity of the pathogens affecting these animals (SUZUKI et al., 2019; SILVA et al., 2017; VERGARA-PARENTE et al., 2003).

The *Staphylococcus* genus has recently become a major concern due to increasing antimicrobial resistance and frequent reports of infection in a variety of hosts, including wild animals (FEBLER et al., 2018; GARCÍA et al., 2020; MATIAS et al., 2018; WENDLANDT et al., 2013). Recent studies have suggested an interconnection between humans, animals, and the environment, with non-domestic animals potentially serving as carriers of staphylococci and antimicrobial resistance genes located in mobile genetic elements (ABDULLAHI et al., 2021; ROSSI et al., 2020). In the present study, *S. sciuri* was the most common species in the three animal categories studied and was found in almost half of the animals (40.9%). *S. sciuri* is known to colonize a wide range of hosts (GARCÍA et al., 2020; HAUSCHILD et al., 2007; SARAIVA et al., 2021) and is frequently described as an opportunistic pathogen in several animals species and humans (CARVALHO et al., 2022; NEMEGHAIRE et al., 2014; SANTANA et al., 2022a).

Other species of the genus *Staphylococcus* identified in this study have also been reported. *S. kloosii*, *S. haemolyticus*, and *S. warneri* have been reported to infect humans and colonize various animals, such as birds of prey, bats, and trout, as well as some domestic species (FOUNTAIN et al., 2019; DOS SANTOS et al., 2018; SIGLER; HENSLEY, 2013; SOUSA et al., 2016). *S. haemolyticus* is a notable pathogen frequently associated with human infections, particularly that of neonates (CZEKAJ et al., 2015; ELTWISY et al., 2022; PEREIRA et al., 2014; WESTBERG; et al., 2022).

Thirteen (40.6%) *Staphylococcus* isolates showed resistance to at least one antimicrobial agent, a frequency similar to that observed in previous studies on some wild animals (SANTANA et al., 2022a, 2022b; SOUSA et al., 2014). This higher frequency of penicillin resistance is consistent with the results of previous studies on various animals, such as tortoises and wild birds (RUIZ-RIPA et al., 2019; SANTANA et

Table 4 - Frequency of *E. coli* virulence genes in strains isolated from rectal swabs of captive Amazonian manatees (*Trichechus inunguis*) and their respective phylogroups and antimicrobial resistance.

Animal identification	------Category------	-----Phylogroup-----	Pathotype (virulence factors)	--Antimicrobial resistance--
233	Juvenile	A	ETEC (K88 and LT)	-
242	Juvenile	B1	EPEC (*eae*)	SUT
278	Calf	B1	ETEC (937p)	AMP
281	Calf	A	ETEC (K99)	AMC; SUT

SUT - sulfamethoxazole/trimethoprim; AMP – ampicillin; AMC - amoxicillin/clavulanic acid.

al., 2022a). Some authors have hypothesized that this frequency of resistant isolates is due to the widespread use of beta-lactams, particularly penicillin, in both human and veterinary medicine (COLLIGNON et al., 2016; MCEWEN & COLLIGNON, 2018). It is worth noting that the use of antimicrobials in these animals is rare, which suggests indirect dissemination of resistance mechanisms and/or resistance (REBELO et al., 2021; VERSTRAETE et al., 2022).

It is not surprising that most animals tested positive for *E. coli*, as this microorganism is commensal in animals (BLOUNT, 2015; LAGERSTROM & HADLY, 2021; LEIMBACH et al., 2013; MURPHY et al., 2021; TENAILLON et al., 2010). Among the isolates, phylogroup B1 was the most prevalent, corroborating studies on several animal species that showed a high prevalence of this phylogroup in their gastrointestinal microbiota (LAGERSTROM & HADLY, 2021; RAMOS et al., 2019; TENAILLON et al., 2010; XAVIER et al., 2022). Phylogroups A and D were detected in approximately 27% of the animals. These two phylogroups are more common in humans (BAILEY et al., 2010) and have been reported at lower frequencies in some animal species than those found in this study (KILANI et al., 2017; XAVIER et al., 2022). This detection raises two hypotheses: either isolates from phylogroups A and D are part of the microbiota of these animals, unlike some wild species already studied (CRISTÓVÃO et al., 2017; ZURFLUH et al., 2019), or there is external influence, such as contamination of riverbeds by human and domestic animal waste or transmission by contaminated feed (AMARSY et al., 2019; GUENTHER et al., 2011; KARAKAYA et al., 2022).

Four *E. coli* isolates were positive for virulence factors and were classified as ETEC or EPEC. These virulence factors are commonly associated with enteric colibacillosis in cattle and pigs (AWAD et al., 2020; DUBREUIL et al., 2016). These findings suggest that Amazonian manatees may host diarrheagenic strains of *E. coli* and, combined with the detection of strains of phylogroups A and D, further supports the hypothesis that colonization is secondary to indirect contact with domestic animal waste.

Interestingly, almost one-third of the tested *E. coli* isolates were resistant to at least one antimicrobial, and resistance to ampicillin, sulfamethoxazole/trimethoprim, and tetracycline was the most common. Notably, these are the antimicrobials most commonly used in human and veterinary medicine (BOECKEL et al., 2019; VITTECOQ et al., 2016).

Similar to that of *Staphylococcus* spp., few studies on *E. coli* in animals belonging to the order *Sirenia* make comparisons difficult. These results suggest that manatees may spread resistance genes through the water in which they live, which could constitute environmental pollution (GUENTHER et al., 2011).

In conclusion, this study suggests that Amazonian manatees in captivity are commonly colonized by *Staphylococcus* spp., mainly *S. sciuri*, and diarrheagenic *E. coli* isolates, including multidrug resistant strains. Future studies may clarify the possible influence of riverbed contamination on microbial colonization. In addition, sampling of free-living animals is necessary to better understand the influence of captivity on the microbiota of Amazonian manatees.

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DECLARATION OF CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHORS' CONTRIBUTIONS

All the authors contributed equally to the conception and writing of this manuscript. All the authors critically revised and approved the final version of the manuscript.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was conducted with permission from the Instituto Chico Mendes de Conservação da Biodiversidade, (ICMBio-SISBIO) permit 72503-2. In addition, the study satisfied the ethics and animal welfare criteria of the Comitê de Ética em Experimentação Animal da União Federal Instituto do Amazonas.

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