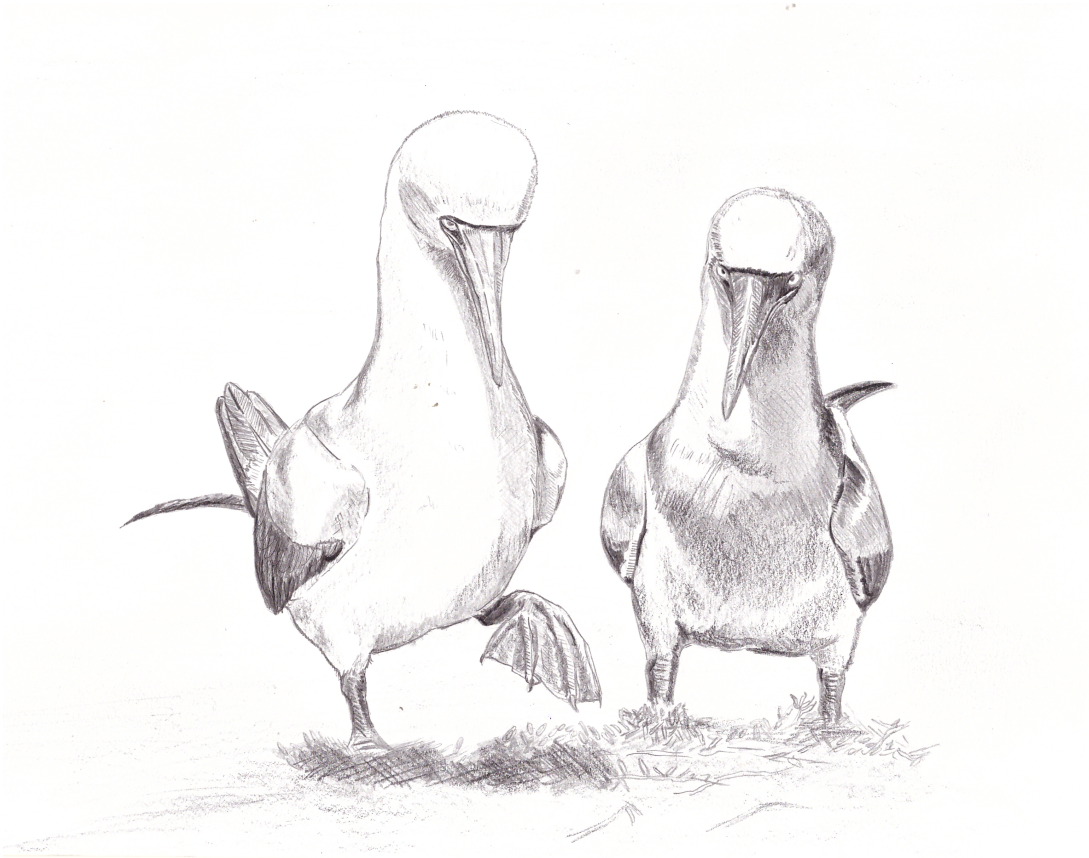
ANA CAROLINA EWBANK

Identification and characterization of antimicrobial resistant genes in the microbiome of seabirds of the Brazilian coast



SÃO PAULO

2021

ANA CAROLINA EWBANK

Identification and characterization of antimicrobial resistant genes in the microbiome of wild seabirds of the Brazilian coast

Thesis submitted to the Postgraduate Program in Patologia Experimental e Comparada of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

Departament:

Patologia

Area:

Patologia Experimental e Comparada

Advisor:

Prof. José Luiz Catão-Dias, Ph.D.

In agreement:_____

Advisor

Co-advisor:

Dr. Fernando Esperón Fajardo, Ph.D

São Paulo

2021

Obs: The final version is available at the FMVZ/USP library.

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

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Ewbank, Ana Carolina
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Título traduzido: Identificação e caracterização de genes de resistências a antimicrobianos no microbioma de aves marinhas da costa brasileira.

Tese (Doutorado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Patologia, São Paulo, 2021.

Programa de Pós-Graduação: Patologia Experimental e Comparada.
Área de concentração: Patologia Experimental e Comparada.
Orientador: Prof. Dr. José Luiz Catão-Dias.
Coorientador: Fernando Esperón Fajardo.

1. Antropização. 2. Antibiótico. 3. *Escherichia coli* resistente a cefalosporinas de espectro estendido. 4. Aves marinhas migratórias. 5. Animais selvagens. I. Título.

Ficha catalográfica elaborada pela bibliotecária Maria Aparecida Laet, CRB 5673-8, da FMVZ/USP.

Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

CERTIFICADO

Certificamos que a proposta intitulada "IDENTIFICAÇÃO E CARACTERIZAÇÃO DOS GENES DE RESISTÊNCIA A ANTIBIÓTICOS DO MICROBIOMA DE AVES MARINHAS DO LITORAL BRASILEIRO", protocolada sob o CEUA nº 1753110716 (ID 002861), sob a responsabilidade de **José Luiz Catão Dias** *e equipe; Ana Carolina Ewbank* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 27/10/2016.

We certify that the proposal "IDENTIFICATION AND CHARACTERIZATION OF ANTIBIOTIC RESISTANCE GENES IN THE MICROBIOME OF SEABIRDS FROM THE BRAZILIAN COAST", utilizing 50 Birds (males and females), protocol number CEUA 1753110716 (ID 002861), under the responsibility of **José Luiz Catão Dias** *and team; Ana Carolina Ewbank* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/27/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 10/2016 a 07/2020 Área: Patologia Experimental E Comparada

Origem:	Não aplicável biotério						
Espécie:	Aves	sexo:	Machos e Fêmeas	idade:	1 a 20 anos	N:	50
Linhagem:	Diversas espécies de aves marinhas de vida livre			Peso:	300 a 5000 g		

Local do experimento: Associação R3 Animal - Centro de Reabilitação

Comentário da CEUA: Serão coletadas amostras de aves marinhas para este projeto, já aprovado pelo Sisbio.

São Paulo, 27 de janeiro de 2021

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

São Paulo, 27 de outubro de 2020
CEUA N 1753110716

Ilmo(a). Sr(a).
Responsável: José Luiz Catão Dias
Área: Patologia Experimental E Comparada

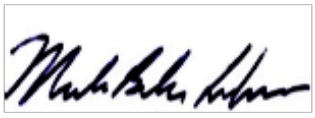
Título da proposta: "IDENTIFICAÇÃO E CARACTERIZAÇÃO DOS GENES DE RESISTÊNCIA A ANTIBIÓTICOS DO MICROBIOMA DE AVES MARINHAS DO LITORAL BRASILEIRO".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ (ID 007101)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 09/setembro/2020) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Fazemos esse pedido devido à extensão do prazo para depósito da tese por parte da Comissão de Pós Graduação da FMVZ - USP em função da pandemia da COVID-19. O mesmo foi postergado até Janeiro de 2021. Não houve e nem haverá qualquer alteração na proposta já aprovada anteriormente pela CEUA, apenas extensão do prazo. Obrigada.".

Comentário da CEUA: "Prorrogação do cronograma para até 01/2021 em função da pandemia. Nada mais será alterado".



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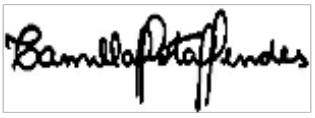

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 59150-5	Data da Emissão: 27/11/2019 12:31:18	Data da Revalidação*: 01/08/2020

De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: ANA CAROLINA EWBANK	CPF: 317.712.058-73
Título do Projeto: IDENTIFICAÇÃO E QUANTIFIÇÃO DOS GENES DE RESISTÊNCIA A ANTIBIÓTICOS NO MICROBIOMA DE AVES MARINHAS DO LITORAL SUL E SUDESTE DO BRASIL.	
Nome da Instituição: Faculdade de Medicina Veterinária e Zootecnia USP	CNPJ: 63.025.530/0019-33

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Captura de Aves	06/2017	07/2020
2	Coleta de Amostras	06/2017	07/2020
3	Transporte de Amostras	06/2017	07/2020

Equipe

#	Nome	Função	CPF	Nacionalidade
1	JULIANA YURI SAVIOLLI	Coleta e transporte de amostras	301.023.498-86	Brasileira
2	RODOLFO PINHO DA SILVA FILHO	Coleta e transporte de amostras	401.790.010-00	Brasileira
3	CRISTIANE KIYOMI MIYAJI KOLESNIKOVAS	Coleta e transporte de amostras, Pesquisador colaborador	176.142.858-67	Brasileira
4	RALPH ERIC THIJL DEL VAL ONORO VANSTREELS	Coleta e transporte de amostras	332.714.958-58	Brasileira
5	RENATA FERREIRA HURTADO	Coleta e transporte de amostras	323.144.298-26	Brasileira
6	LAURA CHRISPIM REISFELD	Coleta e transporte de amostras	338.691.748-89	Brasileira
7	SAMIRA COSTA DA SILVA	Coleta e transporte de amostras, Pesquisador	100.140.327-40	Brasileira
8	Pedro Enrique Navas-Suárez	Coleta e transporte de Amostras, Pesquisador	237.789.788-61	Estrangeira
9	CARLOS SACRISTAN YAGUE	Coleta e Transporte de amostras, pesquisador	236.451.518-18	Estrangeira
10	Gislaine Taimara Dalazen	Coleta e transporte de amostras	032.338.411-08	Brasileira

Ministério do Meio Ambiente - MMA

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Observações e ressalvas

1	A autorização não eximirá o pesquisador da necessidade de obter outras anuências, como: I) do proprietário, arrendatário, posseiro ou morador quando as atividades forem realizadas em área de domínio privado ou dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso; II) da comunidade indígena envolvida, ouvido o órgão indigenista oficial, quando as atividades de pesquisa forem executadas em terra indígena; III) do Conselho de Defesa Nacional, quando as atividades de pesquisa forem executadas em área indispensável à segurança nacional; IV) da autoridade marítima, quando as atividades de pesquisa forem executadas em águas jurisdicionais brasileiras; V) do Departamento Nacional da Produção Mineral, quando a pesquisa visar a exploração de depósitos fossilíferos ou a extração de espécimes fósseis; VI) do órgão gestor da unidade de conservação estadual, distrital ou municipal, dentre outras.
2	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade.
3	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
4	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que específica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
5	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
6	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
7	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
8	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.

Outras ressalvas

1	O pesquisador deverá entrar em contato através do email pesquisaparnamarfn@gmail.com informando a data das expedições ao Arquipélago de Fernando de Noronha e deverá comparecer à sede do ICMBio em Fernando de Noronha para entregar cronograma das atividades antes do início dos trabalhos de campo em FN. Recomendamos que o pesquisador acesse o site https://www.parnanoronha.com.br/pesquisa, se informe e baixe o manual do pesquisador antes de contatar a unidade.	APA Fernando de Noronha

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3	Os métodos podem ser modificados, caso o representante do ICMBio, em campo, julgue pertinente. As regras do Manual de Conduta de Campo da Rebio do Atol das Rocas devem ser cumpridas.	REBIO Atol das Rocas
4	Os pesquisadores estrangeiros PEDRO ENRIQUE NAVAS-SUÁREZ e CARLOS SACRISTAN YAGUE, que são membros da equipe da pesquisa, possuem o vínculo junto a Programa de Bolsas ou Auxílio à Pesquisa patrocinado pela CAPES e a Programa de Bolsas ou Auxílio à Pesquisa patrocinado pela FAPESP, respectivamente. Portanto, estão dispensados de autorização do Ministério da Ciência, Tecnologia e Inovação.	COINF
5	O volume de sangue coletado não deve ultrapassar o equivalente a 1% da massa corporal da ave. Em coletas consecutivas, não deve ultrapassar 2% a cada 14 dias. Não deve ser utilizada punção cardíaca para obtenção da amostra.	CEMAVE Cabedelo-PB
6	Para as UCs do ICMBio Alcatrazes: Ressalva 1) O coordenador do projeto deverá entrar em contato com a gestão da UC antes do início de qualquer atividade, a fim de serem discutidos os pontos de coleta. As coletas deverão, obrigatoriamente, ser sempre acompanhadas por um integrante da unidade de conservação em questão. No caso de desembarque e permanência nas ilhas do arquipélago, a Marinha do Brasil também deverá ser informada. Ressalva 2) É obrigatório o cumprimento do Protocolo de Acesso e Permanência em Unidades de Conservação Insulares - ARIE Ilhas da Queimada Pequena e da Queimada Grande ESEC Tupinambás ? proposto pelo Plano de Ação de Conservação Plano de Ação Nacional para Conservação da Herpetofauna Insular Ameaçada de Extinção ? PAN Herpetofauna Insular	REVIS Arquipélago de Alcatrazes

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Parque Nacional Marinho de Fernando de Noronha	PE	Marinho	Não	Dentro de UC Federal
2	Reserva Biológica do Atol das Rocas	RN	Marinho	Não	Dentro de UC Federal
3	Refúgio de Vida Silvestre do Arquipélago de Alcatrazes		Marinho	Não	Dentro de UC Federal
4	Área de Proteção Ambiental de Fernando de Noronha - Rocas - São Pedro e São Paulo	PE	Marinho	Não	Dentro de UC Federal
5	Florianópolis	SC	Marinho	Não	Fora de UC Federal
6	Litoral do Estado de São Paulo	SP	Marinho	Não	Fora de UC Federal

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Nome da Instituição: Faculdade de Medicina Veterinária e Zootecnia USP	CNPJ: 63.025.530/0019-33

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de amostras biológicas in situ	Outras atividades
2	Coleta/transporte de amostras biológicas ex situ	Atividades ex-situ (fora da natureza)
3	Captura de animais silvestres in situ	Outras atividades

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Captura de animais silvestres in situ	Aves	-
2	Coleta/transporte de amostras biológicas ex situ	Aves	-
3	Coleta/transporte de amostras biológicas in situ	Aves	-

Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Aves)	Fezes, Sangue, Animal encontrado morto ou partes (carcaça)/osso/pele, Fragmento de tecido/órgão, Outras amostras biológicas(Swab retal e de cavidade oral)
2	Método de captura/coleta (Aves)	Puçá, Rede canhão, Outros métodos de captura/coleta(contencao fisica)

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	Faculdade de Medicina Veterinária e Zootecnia USP	Coleção

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Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de Amostra	Qtde.	Data

* Identificar o espécime do nível taxonômico possível.

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Código de autenticação: 0591500520191127

EVALUATION FORM

Author: EWBANK, Ana Carolina

Title: Identification and characterization of antimicrobial resistant genes in the microbiome of wild seabirds of the Brazilian coast

Thesis submitted to the Postgraduate Program in Patologia Experimental e Comparada of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____Decision:_____

Prof. _____

Institution:_____Decision:_____

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Essa tese é dedicada ao meu eterno companheiro de aventuras e desventuras,

Carlos Sacristán.

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RESUMO

EWBANK, A. C. **Identificação e caracterização de genes de resistências a antimicrobianos no microbioma de aves marinhas da costa brasileira.** 180 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

A resistência a antimicrobianos é quintessencial em Saúde Única. Resistência microbiana resulta da plasticidade das bactérias e interações entre microorganismos, hospedeiros e ambiente, influenciada pela pressão antropogênica. O consequente remodelamento dos microbiomas existentes, associado à sua capacidade de disseminação, conferem aos genes de resistências a antimicrobianos (GRAs) o papel de poluentes ambientais que, assim como bactérias resistentes a antimicrobianos (BRA), são indicadores ambientais de antropização. Aves marinhas são excelentes sentinelas da saúde do ecossistema marinho. Neste estudo foram utilizadas técnicas genotípicas (ex.: PCR a tempo real [rtPCR] gelificado e sequenciamento completo [WGS]) e fenotípicas (cultura bacteriana e antibiograma) para avaliar a presença e diversidade dos GRAs e BRA de prioridade em saúde pública (*Escherichia coli* produtora de beta-lactamase de espectro estendido [ESBL-EC] e AmpC [AmpC-EC]) no microbioma de aves marinhas de vida livre de ambientes costeiros e insulares pristinos do BrasilGRAs mediados por plasmídeos foram detectados e quantificados por rtPCR em enemas de (1) 25 aves marinhas (gaivotão [*Larus dominicanus*, n = 14] e pinguim-de-Magalhães [*Spheniscus magellanicus*, n = 11]) à admissão a centro de reabilitação no sul do Brasil, e (2) 308 indivíduos: 104 do Arquipélago de Fernando de Noronha (FNA), Pernambuco (atobá-mascarado [*Sula dactylatra*, n = 48], atobá-marrom [*Sula leucogaster*, n = 31] e fragata-comum [*Fregata magnificens*, n = 25]), e 204 do Atol das Rocas (ROA), Rio Grande do Norte (atobá-mascarado [n = 33], atobá-marrom [n = 33], fragata-comum [n = 35], atobá-de-pé-vermelho [*Sula sula*, n = 33], trinta-réis-das-rocas [*Onychoprion fuscatus*, n = 36], e viuvinha-marrom [*Anous stolidus*, n = 34]) para comparação entre um ambiente intensamente antropizado (FNA) versus um bioma pristino (ROA), no nordeste brasileiro. Ademais, foram utilizadas técnicas fenotípicas e de WGS pra pesquisar ESBL-/AmpC-EC (i) nos mesmos 204 indivíduos de ROA, e (2) em 20 fragatas-comuns de um local inabitado (Arquipélago de Alcatrazes) inserido na antropizada costa sudeste brasileira. Nossos objetivos foram utilizar aves marinhas como bioindicadores de antropização para acessar a ocorrência e disseminação de GRAs e ESBL-/AmpC-EC na costa brasileira, sua epidemiologia e persistência frente à Saúde Única. Nossos resultados mostraram ampla ocorrência e diversidade nos diferentes cenários, especialmente no

antropizado (FNA), que apresentou resusltados consistentes com pressão antropogência: maior significância estatística na prevalência de GRAs conferindo resistencia a sulfonamidas e quinolonas em comparação a ROA, e maior prevalência de *sul*II. Acreditamos que essa seja a primeira detecção de *mec*A em aves marinhas nas Américas, e a primeira de *mcr*-1 em aves marinhas de vida livre no Brasil e migratórias não-sinantrópicas no mundo. Este estudo descreve o primeiro relato do clone pandêmico e de importância em saúde pública ST131 carreando $bla_{\text{CTX-M-8}}$, e constitui o primeiro registro de ST648 carreando $bla_{\text{CTX-M-2}}$ e $bla_{\text{CMY-2}}$, ST117 carreando $bla_{\text{SHV-12}}$ e do novo ST11350 (complexo clonal ST349) carreando $bla_{\text{CTX-M-55}}$ e *fos*A3 em aves selvagens. Mostramos aqui o papel-chave das características biológicas e ecológicas espécie-específicas (ex.: migração, forrageamento) e a relevância da antropização no estudo de resistências a antimicrobianos. Finalmente, enfatizamos o papel das aves marinhas como sentinelas de antropização e seu envolvimento na cadeia de resistências antimicrobianas em Saúde Única.

Palavras-chave: Antropização. Antibiótico. *Escherichia coli* resistente a cefalosporinas de espectro estendido. Aves marinhas migratórias. Animais Selvagens.

ABSTRACT

EWBANK, A. C. **Identification and characterization of antimicrobial resistant genes in the microbiome of seabirds of the Brazilian coast.** 180 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

Antimicrobial resistance is a quintessential One Health issue. Microbial resistance results from bacteria genetic plasticity and interactions among microbials, hosts and the environment, enhanced by anthropogenic pressure. The consequent remodeling of the existing microbiomes, associated with their dissemination capacity, confer antimicrobial resistance genes (ARGs) the role of environmental pollutants and, alongside antimicrobial resistant bacteria (ARB), indicators of environmental anthropization. Seabirds are excellent sentinels of the marine ecosystem health. We used genotypic (i.e., gelled real-time PCR [rtPCR] and whole genome sequencing [WGS]) and phenotypic techniques (bacterial culture and antimicrobial sensitivity tests) to evaluate the presence and diversity of ARGs and ARB of critical priority (extended-spectrum beta-lactamase [ESBL]-producing *Escherichia coli* [ESBL-EC] and AmpC-producing *E.coli* [AmpC-EC]) in the microbiome of wild seabirds inhabiting coastal and insular environments in Brazil. Gelled rtPCR reactions detected and quantified selected plasmid-mediated ARGs in enemas of (1) 25 seabirds (kelp gull [*Larus dominicanus*, n = 14] and Magellanic penguin [*Spheniscus magellanicus*, n = 11]) upon admission to a rehabilitation center in southern Brazil, and (2) 308 individuals: 104 from the Fernando de Noronha Archipelago (FNA), Pernambuco (masked boobies [*Sula dactylatra*, n = 48], brown boobies [*Sula leucogaster*, n = 31] and magnificent frigatebirds [*Fregata magnificens*, n = 25]), and 204 from Rocas Atoll (ROA), Rio Grande do Norte (masked boobies [n = 33], brown boobies [n = 33], magnificent frigatebirds [n = 35], red-footed boobies [*Sula sula*, n = 33], sooty terns [*Onychoprion fuscatus*, n = 36], and brown noddies [*Anous stolidus*, n = 34]) to compare the highly anthropized (FNA) versus the pristine biome (ROA), northeastern Brazil. Additionally, we used phenotypic techniques and WGS to survey ESBL-/AmpC-EC in cloacal swabs of (1) the same 204 ROA individuals, and (2) 20 magnificent frigatebirds from an uninhabited site (Alcatrazes Archipelago, São Paulo) Brazil), inserted in the anthropized southeastern Brazilian coast. Our goals were to use seabirds as environmental bioindicators of anthropization to assess the occurrence and dissemination of ARGs and ESBL-/AmpC-EC in the Brazilian coast, and their epidemiology and persistence through a One Health approach. Our findings showed their wide occurrence and diversity throughout the evaluated scenarios, especially in the anthropized (FNA), which presented results consistent with anthropogenic pressure: statistically significant higher prevalence of

sulfonamide- and quinolone-encoding ARGs in comparison with ROA, and higher *sul*II gene prevalence. To our knowledge, this is the first detection of *mec*A in seabirds in the Americas, and of *mcr*-1 gene in wild free-ranging seabirds in Brazil and in free-ranging migratory non-synanthropic seabirds worldwide. This is the first description of the pandemic and public health relevant ST131-O25b harboring $bla_{\text{CTX-M-8}}$, and the first report of ST648 harboring $bla_{\text{CTX-M-2}}$ and $bla_{\text{CMY-2}}$, ST117 harboring $bla_{\text{SHV-12}}$, and of a novel ST11350 (ST349 clonal complex) harboring $bla_{\text{CTX-M-55}}$ and *fos*A3 in wild birds. We showed the key role of species-specific biological and ecological characteristics (e.g., migration, foraging strategies) and the relevance of anthropization in the study of antimicrobial resistance. Finally, we highlight the role of seabirds as anthropization sentinels and their involvement in the One Health chain of antimicrobial resistance.

Keywords: Anthropization. Antibiotic. Extended-spectrum cephalosporin-resistant *Escherichia coli*. Migratory seabird. Wildlife.

SUMMARY

1. LITERATURE REVIEW

1.1 A BRIEF HISTORY ON ANTIBIOTICS: FROM ANCIENT TIMES TO THE CURRENT ISSUE OF ANTIMICROBIAL RESISTANCE

Contrary to our common anthropocentric belief that antibiotics have entered our lives by our own doing and desire at the dawn of the modern "antibiotic era", studies show that antimicrobials have accompanied us from a very early start. Many ancient cultures in Ancient Serbia, China and Greece, used molds, soil, and plants to treat bacterial infections, believing that they would influence the spirits or the gods responsible for inflicting illnesses and suffering (DURAND et al., 2019). Traces of tetracycline were detected in human skeletal remains from ancient Sudanese Nubia dating back to 350–550 CE (BASSETT et al., 1980; NELSON et al., 2010) and the late Roman period sampled from the Dakhleh Oasis, Egypt. The postulated intake of tetracycline in the diet of these populations is believed to have had a protective effect, based on the low rate of infectious diseases documented in the Sudanese Nubian population and the lack of signs of bone infection in the samples from the Dakhleh Oasis (ARMELAGOS, 1969; COOK et al., 1989).

The beginning of the modern "antibiotic era" is usually associated with the names of Paul Ehrlich (1854-1915) and Alexander Fleming (1881-1955). Ehrlich elaborated a theory of a "magic bullet" that would be capable to selectively target solely the disease-causing microbes and not the host. His research was focused on finding a therapeutic drug to a then endemic and almost incurable sexually transmitted disease, caused by the spirochete *Treponema pallidium*, known as syphilis. Finally, he found an organoarsenic derivative, commercially named Salvarsan, that along with the more soluble and less toxic Neosalvarsan, was the most frequently prescribed drug in Europe, until 1940s, when it was replaced by penicillin (MAHONEY et al., 1943).

Penicillin is probably the most well known "character" in the long history of humanity and antimicrobial interactions. Although Alexander Fleming is generally known as the discoverer of penicillin, in 1928, it was Ernest Duchesne (1874–1912), a French medical student, who in 1896, originally discovered the antimicrobial properties of *Penicillium*. He observed that Arab stable boys kept their saddles in a dark and damp room to encourage mold growth on its surface, which they said helped heal saddle sores. Duchesne then prepared a suspension from the mold and injected it into diseased guinea pigs along with a lethal dose of virulent typhoid bacilli and observed that all animals remained healthy. Unfortunately, at the

time of his discovery, his work was ignored because of his young age and unknown status (POUILLARD, 2002).

Alexander Fleming then “rediscovered” the penicillin, and this time such discovery was not ignored; instead, it triggered a revolution and consolidated the **“**modern era of antibiotics” (PIDDOCK, 2012; SENGUPTA et al., 2013), first prescribed to treat serious infections in the 1940s (CENTER FOR DISEASE CONTROL AND PREVENTION, 2013). The discovery of antibiotics was a turning point in human history (DAVIES; DAVIES, 2010). Over the first several following decades (referred to as the “golden age” of antibiotic use) they revolutionized medicine in many ways and quickly became extremely important corner stones of modern healthcare, eased patients’ suffering and saved countless lives (DAVIES; DAVIES, 2010; CANTAS et al., 2013). The first half of the 20th century unrolled on the success of the natural antibiotic penicillin and the synthetic antimicrobial sulfonamides (CANTAS et al., 2013).

Unfortunately, the concern about over-use and misuse caught up fast with this healthcare marvelous. Such finding was not a surprise to Sir Alexander Fleming, who during his 1945 Nobel Prize acceptance speech voiced his worries that the time might come when penicillin “can be bought by anyone in the shops” and that the inevitable exposure of microbes to non-lethal doses of the drug would “make them resistant” (WORLD HEALTH ORGANIZATION, 2014). His concern proved soon to be true after penicillin started being sold without medical prescription, with the first report of penicillin-resistant bacterial strains soon after, in 1946 (BARBER; ROZWADOWSKA-DOWZENKOAND, 1948). Since then, the “cat and mouse” game of antimicrobials and resistance has never stopped.

Currently, we are faced with an even more worrying fact; the emergence of new bacterial strains that are simultaneously resistant to several antimicrobials (called multidrug-resistant bacteria [“MDR”]), that may eventually become resistant to all available antimicrobials (pandrug-resistance [“PDR”]), leading us back to a “pre-antibiotic era”, when procedures currently considered routine represented potential health challenges (e.g., surgeries, cesarean sections) (WORLD HEALTH ORGANIZATION, 2020; EUROPEAN CENTER FOR DISEASE PREVENTION AND CONTROL, 2020).

Over 75 years after Flemming’s Nobel Prize speech, we are still threatened by antimicrobial resistance, an issue that is continuously becoming more and more complex in a global scale (POUILLARD, 2002; LEVY; MARSHALL, 2004; ALANIS, 2005; PALLETT; HAND, 2010). The difference between now and then, are the social, economic and political

contexts in which we find ourselves facing this issue, associated with a globalized society and trade.

1.2 BRIEF DEFINITION OF ANTIMICROBIAL RESISTANCE

Antimicrobial drugs are medicines that are active against a spectrum of infections, caused by viruses (antivirals), bacteria (antibiotics), fungi (antifungals) and parasites (including antimalarials) (WORLD HEALTH ORGANIZATION, 2014). Antimicrobial resistance refers to an antimicrobial drug that is no longer (as) effective against the organism it is targeting, either if it is an antiviral, antibacterial, antiparasitic or antifungal drug, thus resulting in difficult, costly, or even impossible therapy (SMITH; COAST 2012; WORLD HEALTH ORGANIZATION, 2014).

For the purposes of this study we will focus and discuss bacterial antimicrobial resistance. There are two types of drugs against bacteria: antibiotics and antimicrobials. Antibiotics are microorganism-produced molecular substances capable of, at low concentrations, inhibit or kill other microorganisms. On the other hand, antimicrobial is any substance – either natural, semisynthetic or synthetic in origin – that inhibits the growth of or kills microorganisms, causing little to no damage to the host. In summary: all antibiotics are antimicrobials, but not all antimicrobials are antibiotics (WORLD HEALTH ORGANIZATION, 2015).

1.3 ANTIMICROBIAL RESISTANCE: A COMPLEX AND MULTIFACETED ONE HEALTH ISSUE

Antimicrobial resistance (AMR) is a quintessential One Health issue, one of the most serious global clinical and public health threats of the 21st century, posing economic, social and political burden and implications, and greatly impacting the human-animal-environment interface (WORLD HEALTH ORGANIZATION, 2000; 2014; DA COSTA et al., 2013; SMITH et al., 2014; JOBBINS; ALEXANDER, 2015; LIU et al., 2016; QUEENAN et al., 2016).

It is hard to imagine an issue that epitomizes the principles of One Health more than AMR does (ROBINSON et al., 2016). In a straightforward description, the One Health approach is defined as “the collaborative effort of multiple disciplines – working locally, nationally, and globally – to attain optimal health for people, animals and our environment” (AMERICAN VETERINARY MEDICAL ASSOCIATION, 2008). AMR has clear links to

each of these three pillars, and most importantly, relies heavily on historical, cultural, socio-economical and political factors aside from "health" *per se*.

There are four main genetic reactors in which antimicrobial resistance evolves: (1) human and animal microbiota; (2) places in which susceptible individuals are crowded and exposed to bacterial exchange (e.g., hospitals, farms, rehabilitation centers); (3) biological residues originated in the secondary reactor (e.g., wastewater, lagoons); and (4) the soil and the surface or ground water environments (BAQUERO et al., 2008). Antimicrobial resistance "circulates" within the One Health chain through direct contact, food supply (e.g., meat, fish, eggs and dairy products), or environmental pathways (DA COSTA et al., 2013).

1.4 ANTIMICROBIAL RESISTANCE: A NATURAL PHENOMENON

The development of bacterial resistance results from a naturally occurring phenomenon that predates the existence of humans (HALL; BARLOW 2004; D'COSTA et al., 2006; BHULLAR et al., 2012) and is ubiquitous in the environment (e.g., air [SAPKOTA et al., 2006; LI et al., 2018], water [POIREL et al., 2005], soil [ALLEN et al., 2009; FORSBERG et al., 2012], and glaciers [USHIDA et al., 2010; SEGAWA et al., 2013]). Such phenomenon is promoted by bacteria genetic plasticity and interactions among microbial agents, host organisms and the environment (DA COSTA et al., 2013; FINLEY et al., 2013).

The study of antimicrobial resistance must have a broader approach, aside just from clinical microbiology, focusing on evolutionary and ecological contexts (AMINOV; MACKIE, 2007; AMINOV, 2009; 2010), because of two important factors: (1) the extensive diversity of ARGs in the environmental microbiota, accumulated throughout billions of years of evolution and (2) the absence of barriers among the ecological compartments in the microbial world and the possibility of horizontal gene transfer (HGT) mediated by mobile genetic elements (MGE) within them (AMINOV, 2011).

MGEs allow bacteria to rapidly adapt to new antimicrobials, as well as transfer ARGs, especially when exposed to subdoses (ALLEN; STANTON, 2014). Among them, plasmid-related antimicrobial resistance genes have increased epidemiological relevance - both medical and practical – by encompassing most antibiotics currently in clinical use (especially the commonly used cephalosporins, fluoroquinolones and aminoglycosides) (BENNETT, 2008), and for being capable of HGT between bacterial communities (RAMÍREZ-CASTILLO et al., 2014). Moreover, plasmids may carry one or more antibiotic resistance, metabolic and virulence genes (BENNETT, 2008).

HGT drives the evolution of bacteria (HARRISON; BROCKHURST, 2012). The uptake of genes or operons from the 'mobile gene pool' promotes rapid adaptation to novel environments without the need of relying in rare beneficial mutations arising spontaneously within a population (JAIN et al., 2003). Thus, HGT confers new phenotypic traits (or suites of traits) that allow access to novel ecological niches, posing as evolutionary and ecological innovations (OCHMAN, et al., 2000; WIEDENBECK; COHAN, 2011). Transfer of antimicrobial resistance genes (ARGs) plays an important role in the development of MDR in bacteria (FORSBERG et al., 2012) and is demonstrated through the rapid global spread of antimicrobial resistance throughout bacterial populations (BENNETT, 2008). HGT is mediated by three different mechanisms: transformation, transduction and conjugation (HARRISON; BROCKHURST, 2012; VON WINTERSDORFF et al., 2016). Natural transformation is a process by which cells take up naked DNA from the environment. In transduction, DNA is transferred between cells via a phage vector (bacteriophages), which are also important mechanisms for the horizontal transfer of virulence genes between bacteria, and facilitate transfer of MGE (HARRISON; BROCKHURST, 2012). Conjugation is the transfer of DNA, often mediated by conjugative plasmids, through direct cell-to-cell contact (HARRISON; BROCKHURST 2012). HGT can occur between taxonomically distinct bacterial lineages, and even between kingdoms (HEINEMANN; SPRAGUE, 1989), aside from being considered an important driver of bacterial speciation (OCHMAN, et al., 2000; WIEDENBECK; COHAN, 2011).

Plasmids are platforms of assemble and reassortment of gene arrays not involved in basic cell growth and multiplication; instead, they contain genes that may be useful periodically to enable the cell to exploit some particular environmental challenge (e.g. antibiotic resistance, heavy metal resistance, virulence determinants) (BENNETT, 2008). Plasmid genomes are modular in structure (NORMAN et al., 2009), and can be subdivided into a core "backbone" of genes encoding functions (replication, segregation and conjugation), and "accessory" genes encoding traits beneficial to the bacterial host. Replication is the defining function of a plasmid. The replication region generally consists of an origin of replication and proteins that recruit the host's own DNA replication machinery (i.e. polymerase molecules, tRNAs and ribosomes) to carry out replication. Genes regulating plasmid replication are also common and ensure a stable number of plasmid copies in the host. Segregation systems act to minimize the loss of the plasmid during cell division. Conjugation genes allow the plasmid to transmit horizontally through cell-to-cell transfer, either through "mate pair formation" (e.g., pilus) or by moving and establishing one strand of

the plasmid DNA into the recipient cell ("mobilizable" plasmids) (NORMAN et al., 2009; HARRISON; BROCKHURST, 2012).

1.5 ANTIMICROBIAL RESISTANCE AND ANTHROPOGENIC ACTIVITIES: WHY, HOW AND WHERE

Despite having ancient origins, the "resistome" - the genetic elements that encode the bioactive molecules synthesized by environmental bacteria to either cooperate with or antagonize other members of the community (GAZE et al., 2013), have been severely impacted by anthropogenic activities, which are considered one of the primary leading forces towards antimicrobial resistance (WRIGHT, 2007; WORLD HEALTH ORGANIZATION, 2012). The use, misuse and overuse of antimicrobials in human and veterinary medicine, aquaculture and agriculture (CHENG et al., 2013; YANG et al., 2013; BENGTSSON; GREKO, 2014), in addition to waste disposal and spillover of antimicrobials and their metabolites (e.g., pharmaceutical manufacturing waste [GRAHAM et al., 2011; GUO et al., 2018], and domestic and agricultural waste releases into the environment [WEST et al., 2011; CUMMINGS et al., 2011]), have altered bacterial ecosystem dynamics, leading to a significantly increased selective pressure (WRIGHT, 2007; WORLD HEALTH ORGANIZATION, 2014).

Worryingly, such scenario unravels in a time of stunted discovery and research on new antimicrobials. From the late 1960s through the early 1980s, the pharmaceutical industry introduced many new antibiotics to solve the resistance problem (SPELLBERG; GILBERT, 2014), but this rate of discovery has fallen dramatically (O'Neill, 2016). Between 1968 and 2000, no new antimicrobial classes were discovered (they originated from breakthroughs of previous decades) and although two were discovered in 2000 and 2003, it is important to highlight that these drugs targeted only Gram-positive bacteria; there is still no new class candidates for Gram-negative bacteria (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL AND EUROPEAN MEDICAL AGENCY, 2009; SMITH; COAST 2012; O'NEILL, 2016). This scientific hurdle is exacerbated by the complex regulatory requirements involved and the decline in investment by the pharmaceutical industry and public funders, with the former opting to benefit from drugs more easily manufactured and with higher commercial return, such as chemotherapic drugs (VENTOLA, 2015; O'NEILL, 2016). In spite of that, due to the worldwide increasing importance of infections caused by multidrug resistant Gram-negative bacteria, especially to carbapenems,

new antimicrobial combinations such as meropenem-varbobactam and ceftazidime-avibactan have recently broadened therapeutic options in nosocomial infections (HAYDEN et al., 2020).

Nowadays, the AMR problem is also a One World issue (ROBINSON et al., 2016). The globalization of the food system, with increasing movement of livestock and agricultural produce, combined with increasing human travel, facilitates the rapid spread and mixing of emerging genes confering AMR (ROBINSON et al., 2016). Good examples are the emergence of a plasmid-mediated resistance gene to colistin (*mcr*-1), a last-resort antibiotic, identified in people and pigs in China that rapidly spread to other continents (LIU et al., 2016, MCGANN et al., 2016), and the spread of the New Delhi metallobeta-lactamase 1 (NDM-1), a transmissible genetic element encoding resistance genes against most known betalactam antibiotics, that emerged in New Delhi, India, in 2008 (MOELLERING, 2010). These are just two recent examples that clearly demonstrate that AMR is a global problem, thus requiring global solutions, that will not necessarily be the same in every country (ROBINSON et al., 2016).

1.6. ANTIMICROBIAL RESISTANCE ACROSS THE THREE ONE HEALTH PILLARS: AN INTRICATE CHAIN

1.6.1 HEALTH, SOCIAL AND ECONOMICAL BURDENS TO SOCIETY

The emergence and transmission of resistance threatens to compromise and undermine many of the current medical advances (CARS et al., 2008). Treatment failure due to AMR increases the costs of care associated with: complementary exams (e.g., laboratory tests) and additional or alternative treatments, often much more expensive and with additional side-effects, lengthier stays in hospitals, delayed antimicrobial treatment (until the correct drug is administered), prolonged illness, disability, and even death. Additionally, treatments for antimicrobial-resistant organisms may lead to longer time off work, reduced quality of life and productivity losses, increased family burden, increase in private insurance coverage and patient out-of-pocket expenses, among others (SMITH; COAST, 2012). AMR may also create burden through secondary effects on healthcare systems and/or society, when they compromise the ability to prevent or cure infections that may result from such treatments (e.g., surgery, radiotherapy and chemotherapy) (SMITH; COAST, 2012; NAYLOR et al., 2018).

1.6.2 HEALTH, SOCIAL AND ECONOMICAL BURDENS TO VETERINARY MEDICINE

The antimicrobials used in veterinary medicine were introduced in the mid-1940s, right after they became available for treating human diseases (GUSTAFSON; BOWEN, 1997; MCEWEN, 2006). Although some of them are exclusively designed for veterinary use, the majority belongs to the same antimicrobial classes as those used in humans, largely comprising identical or very similar molecules (SWANN, 1969; HEUER et al., 2009).

The administration of antimicrobials to livestock is not limited to therapy; it also includes growth promotion, prophylaxis, and metaphylaxis (ANTHONY et al., 2001; ANDERSON et al., 2003; CASEWELL et al., 2003; CABELLO, 2006). In order to increase and accelerate the production process, antimicrobials are routinely added to animal feed to get them faster to slaughter weight using less feed, as well as to offset the infection risks of raising animals in modern, intensive systems often under inadequate conditions (crowding, stress, poor hygiene and nutrition) (WALLINGA et al., 2015). Stress lowers the immune system function of these animals; thus antimicrobials end up being especially useful in intensive confinements (HARDY, 2002). This widespread use of antimicrobials in animal production (livestock and aquaculture), especially at sub-therapeutic doses and during long exposure periods, contributes to the emergence of antimicrobial-resistant bacteria (ARB) and has significant public health implications. ARB of animal origin can be transmitted to humans by direct contact (SMITH et al., 2013), and through food products (GESER et al., 2012) and the environment (GRAHAM et al., 2009) (VAN BOECKEL et al., 2015; ROBINSON et al., 2016). This situation also provides ideal conditions for the amplification of ARGs that may have arisen in humans or the environment (ROBINSON et al., 2016).

On the other hand, from a microbiological perspective, increased and improved biosecurity measures in livestock production, such as poultry, as well as decreased or zero use of antimicrobial drugs do not necessarily mean that resistance will be controlled. Instead, resistant pathogenic and commensal bacteria can persist and spread within and between premises due to complex and varied reasons, including: bacterial adaptations to improve fitness costs related to survival and replication of resistance genes and associated proteins, horizontal transmission of genetic resistance factors between bacteria, transfer of bacteria (by moving animals, workers, and equipment), ineffective cleaning and disinfection strategies, and co-selection of resistance to especific drugs upon use of other antimicrobials, heavy metals, or biocides (DAVIES; WALES, 2019).

Furthermore, in the last decades, companion animals have increased in numbers and acquired a new social role in modern society, leading to an increased attention to their welfare and closer contact between humans and their pets. Such changes have contributed to the transmission and sharing of ARGs and ARB, including MDR, between humans and companion animals, constituting another potential risk to public health (WEESE; DUIJKEREN 2010; WIELER et al., 2011; EWERS et al., 2014; POMBA et al., 2017).

1.6.3 THE ROLE OF THE ENVIRONMENT: A STILL POORLY UNDERSTOOD PLAYER WITH (LIKELY) LIMITLESS POTENTIAL

The environment constitutes a vast and still poorly understood reservoir of antibiotic resistance genes (ALLEN et al., 2010; MONIER et al., 2011). Environmental bacteria are quantitatively the most prevalent organisms, thus serving as sources for AMR genes that can become incorporated, over time, into pathogens of people and animals (ROBINSON et al., 2016). For instance, the gene encoding for CTX-M enzyme, a rapidly growing family of extended-spectrum beta-lactamase (ESBLs) with significant clinical impact, are currently considered the most prevalent beta-lactamases found in *Escherichia coli* isolates worldwide and often located in clinical pathogens, is very similar with chromosomally encoded beta-lactamases from the typically environmental bacteria *Kluyvera* spp. (BONNET, 2004; CANTÓN; COQUE, 2006; D'ANDREA et al., 2013; HUMENIUK et al., 2002; POIREL et al., 2002; RODRÍGUEZ et al., 2004; ZHAO et al., 2013; ZHANG et al., 2014). Additionally, genes encoding quinolone resistance may be present in environmental bacteria. The origin of the *qnr*A gene, able to confer low level quinolone resistance, was identified in the chromosome of *Shewanella algae*, a Gram-negative species widely distributed in marine and freshwater environments (POIREL et al., 2005). Furthermore, studies suggest that *qnrS* likely has origins in chromosomal genes from *Vibrio splendidus* (CATTOIR et al. 2007), while the chromosome of *Citrobacter* is the potential source of plasmid-mediated *qnrB* (POIREL et al., 2005; JACOBY et al. 2011).

Not only most antibiotics are produced by environmental microorganisms (NEWMAN; CRAGG, 2016), they may also be enriched by human-related activities and persist in soil and aquatic environments (ALLEN et al. 2010; ZHANG et al., 2015). Consequently, the environment turns into a reactor where bacteria from different origins, antimicrobials, disinfectants, and heavy metals are mixed, contributing to the evolution and dissemination of AMR (BAQUERO et al., 2008). Additionally, physical forces (e.g., wind

and watershed) are important drivers of the spread of ARGs (ALLEN et al., 2010; KELLOGG; GRIFFIN, 2006). Soil and water microbiome play complex and critical roles in ecosystem functions such as the recycling of carbon and nutrients. Disrupting these vital processes by creating an imbalance may threaten planetary health (WHITMEE et al., 2015), potentially pushing ecosystems beyond critical environmental thresholds (SCHEFFER et al., 2009). Thus, understanding the ecology of ARGs and natural microbial communities in various environmental compartments, the pressures and circumstances that lead to their evolution and dissemination, including their reservoirs, mechanisms, diversity, prevalence, ecological significance, and transfer from unmanaged ecosystems to the human milieu is of utter relevance in the advancement of therapies against antimicrobial-resistant pathogens (AMINOV; MACKIE, 2007; ALLEN et al., 2009; ALLEN et al., 2010; MONIER et al., 2011).

1.6.3.1 THE AQUATIC ENVIRONMENT

Water environments are an important factor in the dispersal and evolution of AMR by acting as a media where bacteria from the human-animal-environment interface and their genes, genetic platforms, and genetic vectors interact, many a times, under the influence of antimicrobials, disinfectants, and heavy metals released therein (BAQUERO et al., 2008).

Antimicrobial resistance contaminants can be directly released by human (e.g., treated and untreated sewage, hospital, clinical, industrial waste, fecal contamination of surface waters) and veterinary sources (e.g., aquaculture and animal farm, discharges, and agricultural runoff) to the primary reception system (e.g., via wastewater treatment plants [WWTPs]) (SZCZEPANOWSKI et al., 2004; SCHLÜTER et al., 2007; MARTI et al., 2014; SINGH et al., 2019). Wastewater Treatment Plants (WWTPs) effluents are the main route through which antibiotics are released into the environments, therefore, promoting ARGs dissemination in this media (MARTI et al., 2014; GAO et al., 2018). Common technologies applied in WWTPs such as biological treatment provide an ideal environment for horizontal gene transfer due to the high bacterial densities, and high oxygen and nutrient concentrations they promote, allowing bacteria to be in continuous direct contact with antibiotics and resistant bacteria (RIZZO et al., 2013; MARTI et al., 2014). Over 90 different ARGs have been detected in WWTP influents, including 12 types of widely used antibiotics (GAO et al., 2018). Among them, *sul* and *tet* genes have been reported to be more abundant than other ARGs in studies focusing on different types of WWTPs (XU et al., 2015; CHEN; ZHANG,

2013a; GAO et al., 2018).

Furthermore, antibiotic resistance contaminants can leach to groundwater or be carried by runoff and erosion to the secondary reception system (groundwater and surface water) (TENNSTEDT et al., 2005; CHEN et al., 2007; AUERBACH et al., 2007), and finally converge to the tertiary reception system (estuaries and nearby coastal and ocean systems) under the influence of hydrological dynamics. The effects of anthropogenic activities gradually weaken, whereas the effect of environmental changes (pollutants and the physical and chemical factors) increases from river to ocean systems. Moreover, a mixture of other pollutants (e.g., disinfectants and heavy metals), their metabolites, resistant bacteria, and physical and chemical factors (e.g., pH, salinity) contribute to the evolution and dissemination of antibiotic resistance in the aquatic environment (BAQUERO et al., 2008; LAXMINARAYAN; CHAUDHURY, 2016; GAO et al., 2018). Consequently, aquatic compartments such as water and sediment may have a significant role in driving ARG transfer, ecology, and evolution (TAYLOR et al., 2011).

1.7 WILDLIFE: THE INTERSECTION OF THE ONE HEALTH PILLARS

Wildlife exists across multiple trophic levels, therefore capable of accumulating and dispersing resistance determinants within ecosystems (HASSELL et al., 2009). Although not naturally exposed to antimicrobial therapy in the wild (CARROLL et al., 2015), the increasing pressure from expanding human populations and reduced availability of natural habitats due to changes in land-use, force wildlife species to seek alternative sources of food and shelter (HASSELL et al., 2009; ARNOLD et al., 2016). Consequently, wildlife gets closer to humans, agricultural facilities (e.g., manure and slurry), and associated contaminated environments (e.g., refuse dumps, landfills, abattoir viscera ponds, sewage treatment plants) (CARROLL et al., 2015), ultimately increasing the potential for antimicrobial resistance transfer between these compartments (DOLEJSKA et al., 2007; ALLEN et al., 2010; HASSELL et al., 2009; 2017; AHLSTROM et al., 2018). This process of landscape transformation by human impact – know as “anthropization” (SAMOILENKO et al., 2018), has been suggested as a driving factor in the epidemiology of ARGs and ARB in wildlife (ATTERBY et al., 2017; AHLSTROM et al. 2018; NIETO-CLAUDIN et al., 2019; SACRISTÁN et al., 2020).

Additionally, once directed to rehabilitation centers, the presence of ARGs and ARB in their microbiome may interfere, and sometimes, even hamper successful therapy. As seen in nosocomial settings, rehabilitation centers may be highly contaminated by antimicrobials and their metabolites, as well as by ARGs and ARB, and exert intense selective pressure over the local resistome (BLYTON et al. 2015; HAENNI et al. 2020). Thus, rehabilitation centers are a very important and informative setting for the study of resistance within the One Health interface by being potential hot spots for the acquisition, interaction, and development of resistance, facilitating their exchange among wildlife, humans (e.g., staff) and the environment, both while in-care and upon release (HAENNI et al. 2020). Further studies are necessary to clarify if and how ARGs and ARB can be transmitted from wildlife to humans or domestic animals, which is the main concern of clinicians and policymakers (ARNOLD et al., 2016).

1.7.1 SEABIRDS: SENTINELS OF THE MARINE ECOSYSTEM

Seabirds present unique physiological and morphological adaptations that allow them to feel equally at home on land, in the air, and in the water, and be able to rapidly switch from one to the other. The definition of seabird may vary, but the bird groups traditionally considered as seabirds are: Sphenisciformes (penguins), Procellariiformes (albatrosses and petrels), Pelecaniformes (cormorants, boobies and pelicans), and Charadriiformes (gulls, terns, skuas, skimmers, and auks). While all species among the Sphenisciformes and Procellariiforme are seabirds, among the Pelecaniformes, various species of cormorant, anhinga, and pelican can be strict seabirds, or freshwater birds, or are able to thrive in both environments. Charadriiformes comprises five groups considered to be primarily seabirds; while auks and skuas are strict seabirds, different species of gulls, terns, and skimmers are associated with the sea, freshwater, or estuaries (BROOKE, 2000).

Seabirds are long-lived, wide-ranging, and upper trophic level marine predators present in all marine ecosystems and oceans of the world, from coastline to pelagic and open seas (ORO; MARTÍNEZ-ABRAÍN, 2009). This avian group faces many threats, including entanglement in fishing gear, overfishing of food sources, climate change, pollution, hunting, trapping, disturbance, direct exploitation, development, energy production, and introduced species (predators [i.e., rats and cats] introduced to breeding islands that were historically free of land-based predators) (CROXALL et al., 2012; CRAWFORD et al., 2017; DIAS et al., 2019).

By acting as predators, scavengers and cross-ecosystem nutrient ancillaries, seabirds play important roles in the processes, function and resilience of island and marine ecosystems (PALECZNY et al., 2015). Essentially, seabirds respond rapidly to environmental changes, and due to their behavior and population dynamics, are excellent sentinels of the marine ecosystem health, capable of reflecting natural and anthropogenic changes to the environment (RABINOWITZ et al., 2010), including ARGs and ARB (GUENTHER et al., 2012; HERNANDEZ et al., 2013; BONNEDAHL et al., 2015; STEDT et al., 2015; ATTERBY et al., 2016; ATTERBY et al., 2017; AHLSTROM et al., 2018). Several studies have used microbiologic culture-dependent methods to evaluate the prevalence of ARGs in free-ranging seabirds in Europe (gulls [DOLEJSKA et al., 2007; POETA et al., 2008; LITERAK et al., 2010; RADHOUANI et al., 2009, 2011; ANTILLES et al., 2015; MERKEVICIENE et al., 2017]), the Americas (gulls [MARTINY et al., 2011; BÁEZ et al., 2014; BONNEDAHL et al., 2014; 2015; HASAN et al., 2014; ANTILLES et al., 2015; CARROLL et al., 2015; ATTERBY et al., 2016; LIAKOPOULOS et al., 2016; RUZAUSKAS; VASKEVICIUTE et al., 2016; TORO et al., 2016] and penguins [PRICHULA et al., 2016]), Asia (gulls [HASAN et al., 2014), Eurasia (gulls [HERNANDEZ et al., 2010]), Oceania (gulls [MUKERJI et al., 2019]), and Antarctica (penguins [RAHMAN et al., 2015]).

1.7.2 ARGS AND ARB AS INDICATORS OF ANTHROPOGENIC ACTIVITY

ARGs are environmental pollutants that not only behave like typical chemical pollutants (e.g., leaching to groundwater or being carried by runoff or erosion), but that can also be vertically transmitted via proliferation dynamics or passed among bacteria via horizontal transfer (PRUDEN et al., 2006; GAO et al., 2018). Moreover, ARGs are considered “easy to get, hard to lose” pollutants that may be detected in antibiotic-free environments and even in the absence of selective pressure (MARTI et al., 2014). Similarly, ARB have been used as markers of anthropization (GUENTHER et al., 2012; HERNANDEZ et al., 2013; BONNEDAHL et al., 2015; STEDT et al., 2015; AHLSTROM et al., 2018), including in remote locations (SJÖLUND et al., 2008; HERNANDEZ et al., 2010; ARDILES-VILLEGAS et al., 2011; ATTERBY et al., 2016; HERNANDÉZ; GONZÁLEZ-ACUÑA 2016; GUENTHER et al., 2017).

In this study, we will focus in one particular interesting and broadly studied representative: *Escherichia coli*. Member of the Enterobacterales family, *E. coli* are Gram-negative, non-sporulating facultative anaerobe that inhabits the intestines and faeces of warm-

blooded animals and reptiles (BERG 1996; GORDON; COWLING 2003). *E. coli* can be easily disseminated into different ecosystems through water, soil, food, and other media (SKURNIK et al., 2006; RADHOUANI et al., 2009); therefore, its presence is largely used as an indicator of environmental contamination and anthropogenic activity (BONNEDAHL et al., 2009; TENAILLON et al., 2010; PESAPANE et al., 2013).

Extended Spectrum Beta-Lactamase (ESBL) or plasmid-mediated AmpC beta-lactamase producing *E. coli* are resistant to a very important antimicrobial class: betalactams (i.e., penicillins, cephalosporins, carbapenems and monobactams). Betalactams are the most commonly used class of antimicrobials, especially in the therapy of common infections (e.g., pneumonia and urinary tract infections), but also in severe and life threatening infections (e.g., bacteremia and sepsis), and pre-surgical prophylactic treatment (BROLUND et al., 2014; BROLUND; SANDEGREN, 2016). ESBL enzymes are capable of hydrolyzing third and forth generation cephalosporins, as well as monobactams, and are inhibited by clavulanic acid and tazobactam. Genes encoding ESBLs belong most commonly to the TEM, SHV and CTX-M families, especially to the latter, which is rapidly emerging worldwide (EWERS et al., 2012). AmpC beta-lactamases, on the hand, hydrolyze third (but not fourth) generation cephalosporins and cephamycins, and are not inhibited by clavulanic acid. The most relevant families of genes encoding AmpC beta-lactamases are CMY, ACC, DHA and FOX - the most prevalent being CMY (especially represented by the $bla_{\text{CMY-2}}$ gene) (JACOBY, 2009). ESBL- and AmpC-producing *E. coli* are a rapidly emerging public health issue (WORLD HEALTH ORGANIZATION, 2014), described in several epidemiological settings within the human-animal-environmental interface (MESA et al., 2006; EWERS et al., 2012; EGERVÄRN et al., 2017; MUGHINI-GRAS et al., 2019; DE CARVALHO et al., 2020). Genes for beta-lactamase enzymes can be encoded within an organism's chromosome or transmitted on plasmids (LISTER, 2000; JACOBY; MUNOZ-PRICE 2005). In the later case, these genes often encode for diverse resistance mechanisms, allowing them to express resistance to multiple and unrelated antimicrobials (MEDEIROS, 1997).

1.8 HOW TO APPROACH AND UNDERSTAND AMR IN BRAZIL

Antimicrobial usage is an important driver for increasing AMR levels; however, the spread of resistant bacteria and/or the genes encoding resistance are probably much more relevant in AMR dissemination and prevalence (COLLIGNON et al., 2018). Thus, in order to understand a country's scenario and eventually elaborate mitigation measures, one must take

both factors into consideration. Comprehensive and detailed guidelines setting solid concepts and goals to tackle AMR are currently available (O'NEILL, 2016; WORLD HEALTH ORGANIZATION, 2015; 2017); however, each country has its particular characteristics – from cultural values to political engagement and enforcement. This myriad of characteristics ultimately makes up for the biggest challenge when it comes to acting on the issue of AMR: what works for a particular country may not work for another one. Therefore, it is crucial to consider a country's social, economic and political characteristics. Considering this line of thought, what is the Brazilian scenario?

Brazil is a country of continental proportions and estimated population of over 212 million people, the sixth largest in the world (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2021). It is largely known for its social inequality, which resulted from historical factors regarding its economic, political, and sociological development (GRIESSE, 2007). According to the World Bank parameters, despite being classified as an upper middle-income country, Brazil has 25.3% (53.38 million people) of its population living below the poverty line and 6.5% (13.7 million people) below the extreme poverty line (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2019). The country's Human Development Index (HDI) - potential human development in a national average of achievements in three basic dimensions: health, education and income – is 0.765, the 84th rank among the 189 evaluated countries. Conversely, its Inequality-Adjusted Human Development Index (IHDI) - interpreted as the level of human development when inequality is accounted for, is 0.570, a significant overall loss of 25.5% (UNITED NATIONS HUMAN DEVELOPMENT REPORT, 2020). Additionally, to further analize the issue of national inequality, one may also consider the Gini index (that ranges from 0 [perfect equality] to 1 [maximum inequality, situation in which one single individual receives all the income of an economy]), one of the best known inequality indicators, broadly used for comparisons between countries and their ranking. According to the World Bank, Brazil remains one of the most unequal countries in the world regarding citizen income; in 2019, the Gini index related to house income *per capita* was 0,543 (ranking in 156th place), an increase in comparison with 2012 (0,540), when it first started being measured, and 2015 (0,524), the lowest value in the series (World Bank, 2020; World Development Indicators: Distribution of income or consumption, in: http://wdi.worldbank.org/table/1.3; INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2020).

Poverty and public spending on healthcare are able to affect health outcomes; therefore, they should be considered when discussing AMR and elaborating efficient

mitigating measures (COLLIGNON et al., 2018). First, it is crucial to understand the dynamics between poverty ("the pronounced deprivation of well-being") and public health. Poverty can refer to income (i.e., low individual or household income) and non-income parameters (e.g., limited education, unemployment or precarious employment, inadequate housing conditions). It is strongly linked with access to basic sanitation (clean water supply, sewage collection, and garbage removal systems). In 2019, 90,6% of the general brazilian population lived in housing with direct or indirect garbage removal services, 84,7% with clean water supply, and 65,8% with sewage collection and removal by a collection or pluvial system) (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2020). Moreover, poverty greatly increases the risk of contracting infectious diseases. Poor waste and sanitation, poor water quality, housing overcrowding and inadequate nutrition are all linked to inadequate practices to prevent infection and risk of not recovering from infectious diseases, which may be further aggravated by the lack of access to healthcare (ALIVIDZA et al., 2018; COLLIGNON et al., 2018).

The Brazilian public healthcare system (Sistema Único de Saúde – SUS / Universal Health System), was established in the 1988 Brazilian Federal Constitution, based on the principles of universalization, equity and integralization, with the goal of providing free healthcare access to all Brazilian citizens (CONSTITUIÇÃO DA REPÚBLICA FEDERATIVA DO BRASIL, 1988). The SUS incorporates a network of teaching and research institutions (e.g., universities, public health institutes), which interact with state and municipal secretariats, the Ministry of Health, agencies and foundations, creating a healthcare training network. Hence SUS's legacy regarding advances in the health surveillance system, sanitary control, pharmaceutical assistance, transplants, SAMU, smoking control, and HIV/AIDS (PAIM, 2018).

In spite of the 150 million Brazilian citizens that depend on SUS for healthcare and the Brazilian Constitution proclaim that health is a right of all and the duty of the state, the Brazilian executive, legislative and judicial sectors have not ensured the basic conditions for the economic and scientific-technological sustainability of SUS (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2020; PAIM, 2018). Unfortunately, as a result, SUS has been largely neglected, suffering from structurally deteriorated medical centers and lack of resources (e.g., understaffed, with limited and outdated materials and equipment) (PAIM, 2018). Additionally, self-medication and over-the-counter acquisition is a serious issue in Brazil, with deep cultural roots, aggravated by misinformation and limited to no access to public healthcare, associated with inadequate antimicrobial selection and dosing, and with

shorter treatment courses. Additionally, non-prescription use has been speculated to play an important role - potentially as important as over-use, in selecting and maintaining high levels of community antimicrobial resistance (MORGAN et al., 2011; ROSSI 2011; SANTA-ANA-TELLEZ et al., 2013).

In terms of economy, Brazil relies heavily on agriculture and livestock activities; corresponding to 18.5% and 8.1%, respectively, of the 2020 total GDP (gross domestic product) of 26.6% (CENTRO DE ESTUDOS AVANÇADOS EM ECONOMIA APLICADA/ ESCOLA SUPERIOR DE AGRICULTURA "LUIZ DE QUEIROZ"/ UNIVERSIDADE DE SÃO PAULO - CEPEA/ESALQ/USP; In: https://www.cepea.esalq.usp.br/br/pib-do-agronegocio-brasileiro.aspx). Both activities are known to employ wide amounts of antimicrobials to increase productivity (CAPITA; ALONSO-CALLEJA, 2013). According to the only official data source available in Brazil - the National Union of Animal Health Products Industries (Sindicato Nacional da Indústria de Produtos para Saúde Animal - SINDAN), antimicrobials were the third most sold drugs in 2017, accounting for 15.2% (approximately BRL 0.8 billion) of the profits that the industry of animal health products (both animal production and small animals) earned that year (BRL 5.3 billion). Moreover, the recent globally rising demand for animal protein for human consumption, has forced countries such as Brazil to adopt livestock production systems that rely heavily on antimicrobial consumption (VAN BOECKEL et al., 2015). In the same study, Brazil was estimated to consume approximatelly 9% (around 5,683 tonnes) of the total global consumption of antimicrobials in food animal production (estimated at 63,151; ±1,560) in 2010. In spite of its relevance to the AMR issue, Brazil lacks public transparency regarding the annual volume of antimicrobials used in livestock and agriculture, as well as its use purpose (therapeutic, prophylactic or zootechnical feed additives to enhance animal development) (CARDOSO et al., 2019).

In an attempt to address the issue of AMR, the Brazilian Ministry of Health, the Ministry of Agriculture and the Health Regulatory Agency (Agência Nacional de Vigilância Sanitária - ANVISA), among other governmental institutions, have issued regulations in the effort of further understanding, monitoring and controlling AMR in Brazil. Among them, there are two that must be mentioned: Resolution Nr. 20 and the National Action Plan for Prevention and Control of Antimicrobial Resistance (PAN-BR). Resolution Nr. 20, issued by ANVISA in 2011, listed 119 types of antimicrobials that must only be sold upon a duplicated prescription by a physician - in an attempt to control inappropriate over-the-counter purchase in the country (ANVISA, 2011). PAN-BR is a five-year plan (2018-2022) with a One Health

approach, elaborated in accordance with World Health Organization, the Food and Agriculture Organization of the United Nations (FAO) and World Organization for Animal Health (OIE) guidelines, with the goals of: (1) promoting public awareness and expanding related scientific data; (2) decreasing the incidence of infectious diseases (e.g., sanitation and access to potable water); (3) promoting conscious antimicrobial use in human and animal medicine; and (4) promoting sustainable use of antimicrobials and the research/development of new drugs and diagnostic methods (MINISTÉRIO DA SAÚDE, 2018).

1.9 GENERAL OBJECTIVE

The general objective of the present work was to investigate the presence and diversity of ARGs and ARB, respectively, in the microbiome and microbiota of wild seabirds from the Brazilian coast, in order to assess the impacts of anthropization on the marine environment and the complex epidemiological chain of antimicrobial resistance through a One Health approach.

1.10 SPECIFIC OBJECTIVES

• To investigate the microbiome of wild seabird species through highly sensitive real time polymerase chain reaction (rtPCR) methods in order to detect and quantify selected plasmid-mediated ARGs encoding resistance to eight different antimicrobial classes (tetracyclines [*tet*(A), *tet*(B), *tet*(Y), *tet*(K), *tet*(M), *tet*(Q), *tet*(S), *tet*(W)], aminoglycosides [*aad*A and *str*], sulfonamides [*sul*I and *sul*II], phenicols [*cat*I and *cat*II], macrolides [*erm*(B) and *erm*(F)], quinolones [*qnr*B and *qnr*S], betalactams [bla_{TEM}, $bla_{\text{CTX-M}}$ and *mec*A], and polymyxins [*mcr*-1]) in different scenarios: (1) upon admission into a rehabilitation center (Associação R3 Animal, Santa Catarina state, southern Brazil), and (2) to compare an anthropized environment (Fernando de Noronha Archipelago, Pernambuco) and a pristine biotope (Rocas Atoll, Rio Grande do Norte state), in northern Brazil.

• To use microbiological techniques and whole genome sequencing (WGS) to identify and characterize ESBL-producing *E. coli* bacterial lineages, serotypes, resistome, plasmidome and virulome present in the microbiota of wild seabird species in uninhabited islands in northern (Rocas Atoll, Rio Grande do Norte state) and southeastern Brazil (Alcatrazes Archipelago, São Paulo state).

2 OCCURRENCE AND QUANTIFICATION OF ANTIMICROBIAL RESISTANCE GENES IN THE GASTROINTESTINAL MICROBIOME OF TWO WILD SEABIRD SPECIES WITH CONTRASTING BEHAVIORS

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ABSTRACT

Antimicrobial resistance genes (ARGs) are environmental pollutants and anthropization indicators. We evaluated human interference in the marine ecosystem through the ocurrence and quantification (real-time PCRs) of 21 plasmid-mediated ARGs in enema samples of 25 wild seabirds, upon admission into rehabilitation: kelp gull (*Larus dominicanus*, n = 14) and Magellanic penguin (*Spheniscus magellanicus*, n = 11). Overall, higher resistance values were observed in kelp gulls (nonmigratory coastal synanthropic) in comparison with Magellanic penguins (migratory pelagic non-synanthropic). There were significant differences between species (respectively, kelp gull and Magellanic penguin): ARGs occurrence (bla_{TEM} [p = .032]; *tet*M [p = .015]; *tet*A [p = .003]; and *sul*II [p = .007]), mean number of ARGs per sample (p = .031), ARGs mean load percentage (*aad*A [p = .045], *tet*A [p = .031], *tet*M [p = .016], bla_{TEM} [p = .032], *sul*II [p = .008]), percentage of genes conferring resistance to an antimicrobial class (betalactams [p = .036] and sulfonamides [p = .033]), mean number of genes conferring resistance to one or more antimicrobial classes (p = .024]), percentage of multiresistant microbiomes (p = .032), and clustering (p = .006). These differences are likely due to these species' contrasting biology and ecology - key factors in the epidemiology of ARGs in seabirds. Additionally, this is the first report of *mec*A in seabirds in the Americas. Further studies are necessary to clarify the occurrence and diversity of ARGs in seabirds, and their role as potential sources of infection and dispersal within the One Health chain of ARGs.

Keywords: anthropization; marine pollution; antibiotic resistance; wildlife; gull; penguin; One Health.

2.1 INTRODUCTION

Antimicrobial resistance is an issue of serious public health concern with global economic, social and political implications affecting human and animal populations, as well as the environment (DA COSTA et al., 2013; JOBBINS; ALEXANDER, 2015; SMITH et al., 2014). This worldwide phenomenon is compromising our ability to treat infectious diseases, and undermining or preventing advances in health and medicine (WORLD HEALTH ORGANIZATION WEBSITE, 2019). Microbial resistance is the result of natural bacteria genetic plasticity and interactions between microbial agents, host organisms and the environment (DA COSTA et al., 2013; HIDASI et al., 2013), enhanced by the selective pressure exerted by antimicrobial usage and over-prescription in human and veterinary medicine treatments, animal and fish production (i.e., zootechnical feed additives to enhance animal development, and prophylaxis), agriculture and food technologies (DA COSTA et al., 2013; HIDASI et al., 2013; ROCA et al., 2015). The consequent remodeling of the existing microbiomes (group of all the genomic elements of a specific microbiota), associated with their dissemination capacity, confer antimicrobial resistance genes (ARGs) the role of environmental pollutants (BLASER; FALKOW, 2009; D'ARGENIO; SALVATORE, 2015) and indicators of environmental anthropization (JOBBINS; ALEXANDER, 2015; RADHOUANI et al., 2011; SACRISTÁN et al., 2020).

Seabirds are long-lived, wide-ranging, and upper trophic level marine predators present in all marine ecosystems and oceans of the world, from coastline to pelagic and open seas (ORO; MARTÍNEZ-ABRAÍN, 2004). By acting as predators, scavengers and cross-ecosystem nutrient ancillaries, seabirds play important roles in the processes, function and resilience of island and marine ecosystems (PALECZNY et al., 2015). Essentially, seabirds respond rapidly to environmental changes, and due to their behavior and population dynamics, are excellent sentinels of the marine ecosystem health, reflecting natural and anthropogenic changes to the environment (RABINOWITZ et al., 2010), including pollution by ARGs (ATTERBY et al., 2016; 2017; EWBANK et al., 2020). In seabirds, most ARGs studies have focused on synanthropic species, due to their proximity to anthropized areas and feeding habits, and relied on classic microbiological techniques (bacterial culture and sensitivity testing) (RADHOUANI et al., 2011; AHLSTROM et al., 2018; MUKERJI et al., 2019). Nevertheless, recent studies have shown that biological and ecological factors (e.g., migration and feeding niche) are also relevant to the issue of ARGs in wild birds (DOLEJSKA et al., 2019; MARCELINO et al., 2019; EWBANK et al., 2020). Additionally,

most bacteria are not cultivable (HAMADY; KNIGHT, 2009; MONIER et al., 2011), and culture methods do not favor mobile genetic elements (e.g., plasmids), which encode most ARGs (NIETO-CLAUDIN et al., 2019; CAO et al., 2020). Thus, in order to promote a more comprehensive approach, we employed highly sensitive real time polymerase chain reaction (rtPCR) methods (SACRISTÁN et al., 2020; CEVIDANES et al., 2020) to detect and quantify 21 selected plasmid-mediated ARGs in the gastrointestinal microbiome of two wild seabirds species (kelp gulls [*Larus dominicanus*] and Magellanic penguins [*Spheniscus magellanicus*]) upon admission to a rehabilitation center. The goals of this study were to (i) assess the presence and load of ARGs in these individuals and (ii) evaluate our findings in light of selected biological and ecological parameters (i.e., dispersal [migratory and non-migratory], feeding niche [coastal and pelagic], and interaction with human-impacted areas [synanthropic and non-synanthropic]). We hypothesized that due to their non-migratory coastal synanthropic behavior (BIRDLIFE INTERNATIONAL, 2020), kelp gull would present higher occurrence and load of ARGs than the migratory pelagic non-synanthropic Magellanic penguin (RUOPPOLO et al., 2012; BOERSMA et al., 2013).

2.2 MATERIALS AND METHODS

2.2.1 Sample collection

Fresh fecal samples were immediately obtained by enema (EWBANK et al., 2020) in 25 physically restrained birds (14 kelp gulls and 11 Magellanic penguins) upon admission at the wildlife rehabilitation center (Associação R3 Animal, Florianópolis, Santa Catarina state, southern Brazil). All birds included in the study came directly from their rescue sites (beach), and did not receive previous veterinary care prior to their arrival at the center. Total DNA extraction was carried out by a pressure filtration technique (QuickGene DNA tissue kit S, Fujifilm, Tokyo, Japan), according with the manufacturer's instructions. The 16S rRNA gene was amplified by real time PCR (rtPCR) in ten-fold dilutions of each extracted sample (PEAK et al., 2007; JIANG et al., 2013, Supplementary materials) to verify adequate concentration of bacterial DNA. A sample was considered validated when its ten-fold dilution showed a cycle threshold (Ct) less than 25 (ESPERÓN et al., 2018). To normalize the study, ct was obtained based on the fluorescence variation value ($(\Delta F/\Delta C) = 0.02$) (NIETO-CLAUDIN et al., 2019). Once validated, samples were analyzed by rtPCR for 21 selected ARGs encoding resistance to

eight antimicrobial classes: tetracyclines (*tet*(A), *tet*(B), *tet*(Y), *tet*(K), *tet*(M), *tet*(Q), *tet*(S) and *tet*(W) [JIANG et al., 2013], aminoglycosides (*aad*A [DEVARAJAN et al., 2016] and *str* [WANG et al., 2014]), sulfonamides (*sul*I, *sul*II), chloramphenicols (*cat*I and *cat*II [Jiang et al., 2013]), macrolides (*erm*(B), *erm*(F) [Chen et al., 2007]), quinolones (*qnr*B [CUMMINGS et al., 2011] and *qnr*S [MARTI; BALCÁZAR, 2013]); betalactams (bla_{TEM} [DEVARAJAN et al., 2016] and *mec*A [TSURU et al., 2015]), and polymyxins (*mcr*-1 [NIETO-CLAUDIN et al., 2019]) (Supplementary materials). The estimation of the percentage of bacteria harboring ARGs (mean load percentage of each ARG), was based on the formula % gene $X = 10^{[2 + 0.33 (ct16S - ct\ gene\ X)]}$, with ct as the cycle threshold (16S rRNA regarding bacterial determination and X for each evaluated gene), and 0.33 as the mean slope for all the evaluated genes. Results were expressed in $\log_{10}$ scale of the hypothetical percentage of bacteria presenting each gene, ranging from -8 (sample considered negative) to +2 (when 100% of the bacteria in the sample presented the ARG) (NIETO-CLAUDIN et al., 2019). The same thermal cycle was used for all rtPCR reactions (6' 95°C, 40x (10" 95°C, 30" 60°C)), with alignment and extension in the same step, at constant 60ºC. A melting curve step was performed at the end of the rtPCR reaction (NIETO-CLAUDIN et al., 2019). As per SACRISTÁN et al., 2014, we applied the term "multiresistant microbiome" when a fecal sample presented at least three ARGs encoding resistance to different classes of antimicrobials (ESPERÓN et al., 2018; NIETO-CLAUDIN et al., 2019; SACRISTÁN et al., 2020). All samples used in this study were collected as part of the Santos Basin Beach Monitoring Project (Projeto de monitoramento de Praias da Bacia de Santos - PMP-BS), licensed by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) of the Brazilian Ministry of Environment (ABIO Nº 640/2015), and in full compliance with the Biodiversity Information and Authorization System (SISBIO 59150-4). All procedures were performed according to the Ethical Committee in Animal Research of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (process number 1753110716).

2.2.2 Statistical analysis

The k-means clustering method was used to investigate the resistance patterns (GENESIS software v. 1.7.7, Graz University of Technology, Graz, Austria), by assigning each sample to one cluster. Two clusters were selected, corresponding to low (value = 0) and high (value = 1) levels of ARGs. The Mann-Whitney U non-parametric test was used to establish the differences between species regarding: ARGs occurrence, mean number of

ARGs per sample, mean load percentage of each ARG, the mean number of genes conferring resistance to one or more antimicrobial classes in each sample, percentage of multiresistant microbiomes, resistance patterns, and k-means clustering. All statistical analyses were performed in R software (R Development Core Team 3.0.1., 2013), with a significance level of $p < 0.05$.

2.3 RESULTS

All the tested samples validated for the 16S rRNA gene. All animals, with the exception of one individual (96 %, 24/25), were positive to at least one ARG (Table 1). ARGs results and clusters (Figure 1), according with the species, are described below.

2.3.1 Kelp gull

The *bla*$_{TEM}$ gene presented the highest occurrence (79%, 11/14), followed by *qnr*B (64%, 9/14), *tet*(Q) (57%, 8/14), *sul*II (50%, 7/14), *tet*(B), *tet*(M) and *aad*A (43%, 6/14), *tet*(A), *erm*(B) and *erm*(F) (36%, 5/14), *tet*(W) and *qnr*S (29%, 4/29), *str* (21%, 3/21), *tet*(S), *sul*I, *cat*I, *cat*II and *mec*A (14%, 2/14), and *tet*(K) (7%, 1/14). The *tet*(Y) and *mcr*-1 genes were not detected in this group. The mean number of ARGs per sample was 6.4 (with min=1 and max=15). The *bla*$_{TEM}$ gene presented the highest mean load percentage (-2.2) (considering ≥ -3 as the median value, with -8 [min] and +2 [max]).

When clustered by antimicrobial class, kelp gulls were positive to one or more genes encoding resistance to tetracycline, fluorquinolone and betalactams (79%, 11/14), sulfonamides and macrolides (50%, 7/14), aminoglycosides (43%, 6/14), and phenicols (21%, 3/14). No gulls presented ARGs encoding polymyxin resistance (*mcr-1*). The mean number of genes conferring resistance to one or more antimicrobial classes presented in each gull sample was four. Additionally, 71% (10/14) of the gulls presented multiresistant microbiomes (Table 1), of these, five presented two similar patterns: a tetracycline, sulfonamide, fluorquinolone, betalactam, aminoglycoside, phenicol and macrolide combination (30%; 3/10), and a tetracycline, sulfonamide, fluorquinolone and betalactam combination (20%; 2/10).

2.3.2 Magellanic penguin

The *tet*(Q) gene presented the highest occurrence (55%, 6/11), followed by *qnr*B (45%, 5/11), bla_{TEM} and *tet*(W) (36%, 4/11), *erm*(F) (27%, 3/11), *tet*(B), *tet*(Y) and *erm*(B) (18%, 2/11), *sul*I and *aad*A (9%, 1/11). Genes *tet*(A), *tet*(K), *tet*(M), *tet*(S), *sul*II, *str*, *cat*I, *cat*II, *qnr*S, *mec*A and *mcr*-1 were not detected. The mean number of ARGs per sample was 2.7 (with a maximum of 8 genes per individual). Only one penguin did not present any of the tested ARGs. None of the genes presented mean load percentage ≥ -3.

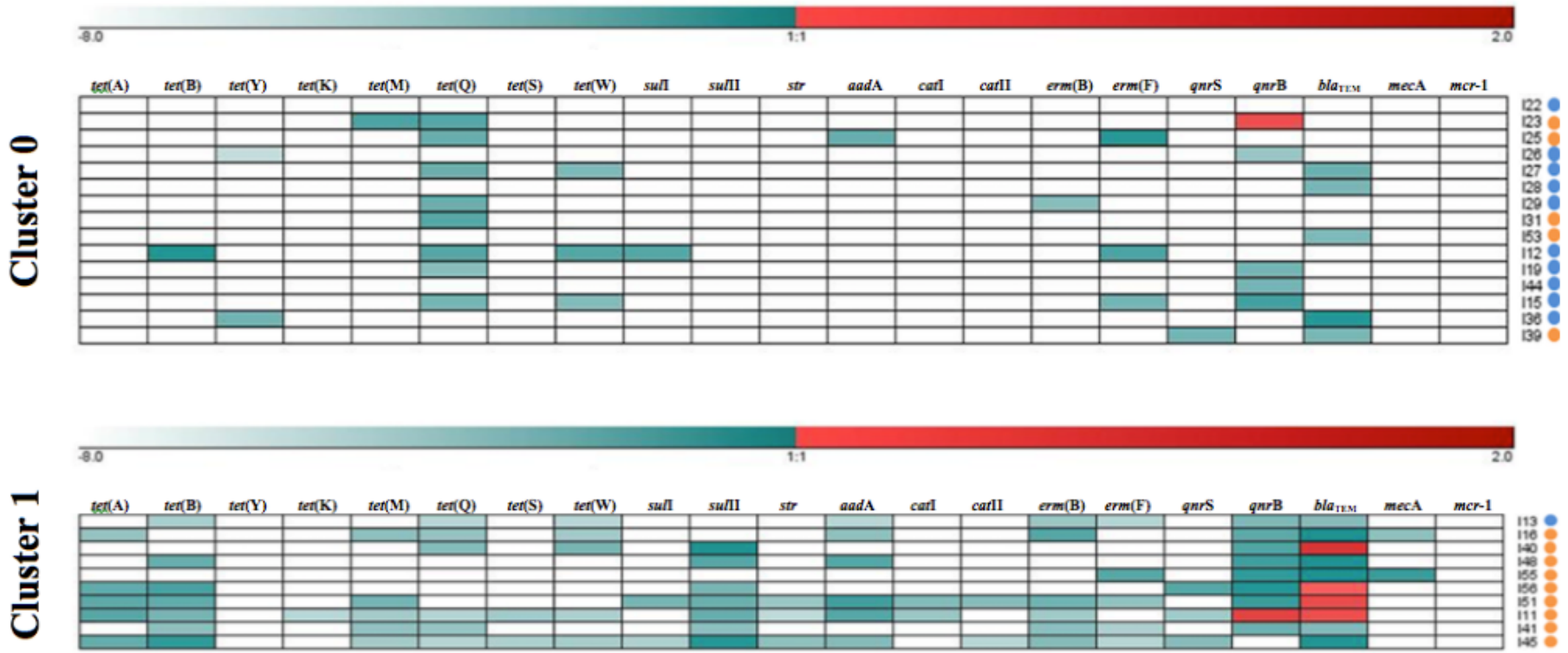
When clustered by antimicrobial class, individuals were positive to one or more genes encoding resistance to tetracyclines (73%, 8/11), fluorquinolones (45%, 5/11), macrolides and betalactams (36%, 4/11), and sulfonamides and aminoglycosides (9%, 1/11). None of the individuals presented ARGs encoding chloramphenicol or polimyxin resistance. The mean number of genes conferring resistance to one or more antimicrobial classes presented in each sample was 2.1. Mutiresistant microbiomes were found in 27% (3/11) of the penguins (Table 1). Although no common patterns were observed, genes conferring resistance to tetracycline and macrolides were present in the microbiomes of the three multiresistant animals.

Table 1. Microbiome patterns, number of detected genes per sample and detected genes according with the animal ID and species (kelp gull *Larus dominicanus* and Magellanic penguin *Spheniscus magellanicus*).

ID	Species	Drug class pattern	Number of detected ARGs	Detected ARGs
I11	kelp gull	TET, SUL, AMINO, PHEN, MACR, FLUOR, BLACT‡	15	*tet*(A), *tet*(B), *tet*(K), *tet*(M), *tet*(Q), *tet*(S) , *tet*(W), *sul*II, *str*, *aad*A, *cat*I, *erm*(B), *qnr*S, *qnr*B, bla_{TEM}
I16	kelp gull	TET, AMINO, MACR, FLUOR, BLACT‡	9	*tet*(A), *tet*(M), *tet*(Q), *tet*(W), *aad*A, *erm*(B), *qnr*B, bla_{TEM}, *mec*A
I23	kelp gull	TET, FLUOR	3	*tet*(M), *tet*(Q), *qnr*B
I25	kelp gull	TET, AMINO, MACR‡	3	*tet*(Q), *aad*A, *erm*(F)
I56	kelp gull	TET, SUL, FLUOR, BLACT‡	6	*tet*(A), *tet*(B), *sul*II, *qnr*S, *qnr*B, bla_{TEM}
I31	kelp gull	TET	1	*tet*(Q)
I39	kelp gull	FLUOR, BLACT	2	*qnr*S, bla_{TEM}
I40	kelp gull	TET, SUL, FLUOR, BLACT‡	5	*tet*(Q), *tet*(W), *sul*II, *qnr*B, bla_{TEM}
I41	kelp gull	TET, SUL, MACR, FLUOR, BLACT‡	8	*tet*(B), *tet*(M), *tet*(Q), *sul*II, *erm*(B), *erm*(F), *qnr*B, bla_{TEM}
I45	kelp gull	TET, SUL, AMINO, PHEN, MACR, FLUOR, BLACT‡	15	*tet*(A), *tet*(B), *tet*(M), *tet*(Q), *tet*(S), *tet*(W), *sul*I, *sul*II, *str*, *aad*A, *cat*II, *erm*(B), *erm*(F), *qnr*S, bla_{TEM}
I48	kelp gull	TET, SUL, AMINO, FLUOR, BLACT‡	5	*tet*(B), *sul*II, *aad*A, *qnrB*, bla_{TEM}
I51	kelp gull	TET, SUL, AMINO, PHEN, MACR, FLUOR, BLACT‡	13	*tet*(A), *tet*(B), *tet*(M), *sul*I, *sul*II, *str*, *aad*A, *cat*I , *cat*II,*erm*(B), *erm*(F), *qnr*B, bla_{TEM}
I53	kelp gull	BLACT	1	bla_{TEM}
I55	kelp gull	MACR, FLUOR, BLACT‡	4	*erm*(F), *qnr*B, bla_{TEM}, *mec*A
I12	Magellanic penguin	TET, SUL, MACR‡	5	*tet*(B), *tet*(Q), *tet*(W), *sul*I, *erm*(F)
I13	Magellanic penguin	TET, AMINO, MACR, FLUOR, BLACT‡	8	*tet*(B), *tet*(Q), *tet*(W), *aad*A, *erm*(B), *erm*(F), *qnr*B, bla_{TEM}
I15	Magellanic penguin	TET, MACR, FLUOR‡	4	*tet*(Q), *tet*(W), *erm*(F), *qnr*B
I19	Magellanic penguin	TET, FLUOR	2	*tet*(Q), *qnr*B
I22	Magellanic penguin	-	0	-

ID	Species	Drug class pattern	Number of detected ARGs	Detected ARGs
I26	Magellanic penguin	TET, FLUOR	2	*tet*(Y), *qnr*B
I27	Magellanic penguin	TET, BLACT	3	*tet*(Q), *tet*(W), bla_{TEM}
I28	Magellanic penguin	BLACT	1	bla_{TEM}
I29	Magellanic penguin	TET, MACR	2	*tet*(Q), *erm*(B)
I36	Magellanic penguin	TET, BLACT	2	*tet*(Y), bla_{TEM}
I44	Magellanic penguin	FLUOR	1	*qnr*B

Figure 1. Resistance patterns of kelp gull (*Larus dominicanus*) and Magellanic penguin (*Spheniscus magellanicus*) samples obtained by k-means clustering of each antimicrobial resistance gene (ARG). Cluster 1 shows samples with high relative load percentage and Cluster 0 shows samples with low relative load percentage. Relative load percentage is expressed in a color scale (white for negative [-8] and dark red for the maximum value [+2]). The species are indicated on the right side (kelp gull [orange dots] and Magellanic penguin [blue dots]).



2.2.3 Qualitative analysis

There were significant differences between species (respectively, kelp gull and Magellanic penguin) in regards to: ARG occurrence (*bla*$_{TEM}$ [79% and 36%. p = .032]; *tet*(M) [43% and 0%. p = .015]; *tet*(A) [36% and 0%. p = .003]; and *sul*II [50% and 0%. p = .007]), mean number of ARGs per sample (6.4 and 2.7. p = .031), ARG mean load percentage (*aad*A [-5.4 and -7.7. p = .045], *tet*(A) [-5.8 and -8. p = .031]; *tet*(M) [-5.8 and -8. p = .016]; *bla*$_{TEM}$ [-2.2 and -5.8. p = .032]; *sul*II [-4.8 and -8. p = .008]), percentage of genes potentially conferring resistance to an antimicrobial class (betalactams [79% and 36%. p = .036] and sulfonamides [50% and 9%. p = .033]), mean number of genes conferring resistance to one or more antimicrobial classes (4 and 2.1. p = .024]), percentage of multiresistant microbiomes (71% and 27%. p = .032]), and clustering (0.6 and 0.1. p = .006]). Statistically significant differences are summarized in Table 2.

Table 2. Statistically significant differences between kelp gull (*Larus dominicanus*) and Magellanic penguin (*Spheniscus magellanicus*): ARG occurrence, mean number of ARGs per sample, mean load percentage of each ARG, the mean number of antimicrobial classes presented in each sample, percentage of multiresistant microbiomes, and resistance patterns. Mann-Whitney U non-parametric test). Numbers in parenthesis indicate the 95% confidence interval (CI).

Parameter	p value	kelp gull (n = 14) 95% CI	Magellanic penguin (n = 11) 95% CI
Occurrence of *tet*(A)	0.03	36% (7, 64%)	0%
Occurrence of *tet*(M)	0.015	43% (13, 73%)	0%
Occurrence of *sul*II	0.007	50% (20, 80%)	0%
Occurrence of *bla*$_{TEM}$	0.036	79% (54, 103%)	36% (2, 70%)
Mean load percentage of *tet*(A)	0.031	-5.8 (-7.6, -4.1)	-8.0
Mean load percentage of *tet*(M)	0.016	-5.8 (-7.4, -4.3)	-8.0
Mean load percentage of *sul*II	0.008	-4.8 (-6.8, -2.9)	-8.0
Mean load percentage of *aad*A	0.045	-5.4 (-7.2, -3.6)	-7.7 (-8.4, -7.0)
Mean load percentage of *bla*$_{TEM}$	0.009	-2.2 (-4.1, -0.2)	-5.8 (-7.9, -3.7)
Percentage of resistance to sulfonamides	0.033	50% (20, 80%)	9% (-11, 29%)
Percentage of resistance to betalactams	0.036	79% (54, 103%)	36% (2, 70%)
Mean number of genes	0.031	6.4 (3.6, 9.2)	2.7 (1.2, 4.2)
Mean number of classes†	0.024	4.0 (2.8, 5.2)	2.1 (1.2, 3.0)
Percentage of multiresistant microbiomes	0.032	71% (44, 98%)	27% (-4, 59%)
Clustering (0=low; 1=high)	0.006	0.6 (0.4, 0.9)	0.1 (-0.1, 0.3)

2.4 DISCUSSION

In accordance with our hypothesis, kelp gulls presented higher occurrence and load of ARGs than Magellanic penguins, findings that may potentially be influenced by the contrasting behaviors of these two seabird species in regard to feeding niches, interaction with human-impacted areas and dispersal. The kelp gull is the most widespread and abundant gull species in the Southern Hemisphere (BURGER; GOCHFELD, 1996; JIGUET et al., 2012; YORIO et al., 2016). Like other gull species, kelp gulls are extremely opportunistic and generalist feeders, very adapted to exploiting a wide variety of human-impacted and highly populated areas, and food subsidies (e.g., fishing discards and refuse disposals) (LUDYNIA et al., 2005; SILVA-COSTA; BUGONI, 2013; YORIO et al., 2016). Such behaviors have been associated with the presence of ARGs in kelp gulls in Argentina (LIAKOPOULOS et al., 2016), as well as in other gull species worldwide (BONNEDAHL et al., 2009; 2015; AHLSTROM et al., 2018). Conversely, the Magellanic penguin is a migratory upper trophic level predator and the most abundant penguin in temperate areas, widely distributed along the southern coast of South America (BIRDLIFE INTERNATIONAL, 2020). Magellanic penguins remain in their colonies during breeding and molting periods, adopting a pelagic behavior while migrating along the continental shelf off the coast of northern Argentina, Uruguay, and southern Brazil (RUOPPOLO et al., 2012; BOERSMA et al., 2013). Although scarce, studies on the presence of ARGs in penguins have associated ARGs occurrence with anthropization in remote locations (RAHMAN et al., 2015; MARCELINO et al., 2019).

The *mec*A gene was detected in 14% (2/14) of kelp gulls, but not in penguins. This gene was reported in other wild bird groups in Brazil (passerines [MATIAS et al., 2018]) and Europe (corvids [LONCARIC et al., 2013; RUIZ-RIPA et al., 2019], storks [GÓMEZ et al., 2016], and vultures [RUIZ-RIPA et al., 2019]). Nevertheless, to the best of our knowledge, this is the first report of *mec*A in seabirds in the Americas, only previously reported in European herring gulls (*Larus argentatus*) in Lithuania through metagenomics (MERKEVICIENE et al., 2017). The *mec*A gene is widely disseminated among *Staphylococcus aureus* and other staphylococcal species (ITO et al., 2012), encoding resistance to methicillin and cross-resistance to other β-lactam antimicrobials (ITO et al., 2012; LAURENT et al., 2012; FIGUEIREDO; FERREIRA, 2014). Methicillin-resistant staphylococci are disseminated worldwide, frequently causing health care- and community-associated infections (TAUBES, 2008; ITO et al., 2012), being considered the leading cause of nosocomial infection in Latin America (GUZMÁN-BLANCO et al., 2009), where it was

also reported in animals, food products and the environment (PATERSON et al., 2012; WAN; CHOU 2014; PAPADOPOULOS et al., 2019).

The *bla*$_{TEM}$ gene was detected in kelp gulls and Magellanic penguins, being the most prevalent gene in the former species (79%; 11/14). *Bla*$_{TEM}$ also presented the highest mean load percentage in this study (-2.2, in kelp gull), indicating an increased dissemination potential in comparison with the other ARGs detected here. Furthermore, the *bla*$_{TEM}$ gene presented significant differences in kelp gull in comparison with Magellanic penguin in regards to occurrence (79% and 36%. p = .032) and mean load percentage (-2.2 and -5.8. p = .032). This gene has been previously described in seabirds in Brazil (EWBANK et al., 2020), the United States (BONNEDAHL et al., 2014; ATTERBY et al., 2016), and Europe (DOLEJSKA et al., 2007; POETA et al., 2008; RADHOUANI et al., 2009; LITERAK et al., 2010; MERKEVICIENE et al., 2017). The TEM betalactamases confer resistance to cephalosporins and penicillins (MROCZKOWSKA; BARLOW, 2008), one of the oldest and most widely used antimicrobial classes in humans and veterinary medicine (GUENTHER et al., 2011; SHARLAND et al., 2018), partialy explaining their dissemination in the tested seabirds. Recently, a similar study in Brazil, that evaluated the microbiome of six species of wild seabirds (overall, 304 individuals), found that the *bla*$_{TEM}$ occurrence and percentage loads ranged from 0% to 25% and -8 to -0.6, respectively, and that the *bla*$_{TEM}$ prevalence was significantly higher in migratory in comparison with non-migratory species (EWBANK et al., 2020). Interestingly, despite the considerable differences regarding species and sampling size, herein we found higher *bla*$_{TEM}$ occurrence and mean load percentages in kelp gull and Magellanic penguin, and higher *bla*$_{TEM}$ occurrence in the non-migratory synanthropic species (kelp gull). Epidemiologically, our findings are very concerning, because while the migratory species evaluated by EWBANK et al., 2020 were using a pristine habitat (Rocas Atoll), kelp gull and Magellanic penguin are using anthropized environments. Kelp gull, especially, are using heavily anthropized areas, which likely influence not only the acquisition and potential transmission of ARGs, but also their development and maintenance, once these individuals are continuously more exposed to ARGs sources (e.g., landfills, wastewater), and consequently, to reinfection.

The genes encoding tetracycline resistance (*tet*) were the most prevalent in this study (79%; 11/14 in kelp gull and 73%; 8/11 in Magellanic penguin): *tet*(A)*, tet*(M) and *tet*(W) in kelp gull, and *tet*(Q) in Magellanic penguin. Additionally, *tet*(Q) was the most prevalent gene in the penguin group (55%, 6/11). Interestingly, EWBANK et al., 2020 found that tetracycline-encoding genes were also the most prevalent antimicrobial class (ranging from

64.5% to 87.9%), significantly greater than the rest of the other ARGs classes (EWBANK et al., 2020). Moreover, we observed significant differences between kelp gull and Magellanic penguin in terms of *tet*(M) and *tet*(A) occurrence (43% and 0%. p = .015, and 36% and 0%. p = .003, respectively), and mean load percentage (-5.8 and -8. p = .016, and -5.8 and -8. p = .031, respectively). The high *tet* occurrence found herein was not surprising, once it had been previously detected in other seabirds in Brazil (EWBANK et al., 2021), and its extensive use in human and veterinary medicine, and in agriculture (CHOPRA; ROBERTS, 2001; DAGHRIR; DROGNI, 2013). *Tet* genes have been reported in gulls in the Americas (MARTINY et al., 2011; TORO et al., 2016; EWBANK et al., 2020) and Europe (DOLEJSKA et al., 2007; POETA et al., 2008; RADHOUANI et al., 2009; LITERAK et al., 2010; RADHOUANI et al., 2011; ANTILLES et al., 2015; MERKEVICIENE et al., 2017), and in wild penguins in Antarctica (RAHMAN et al., 2008; RAHMAN et al., 2015) and Brazil (PRICHULA et al., 2016).

Genes *sul*I and *sul*II were detected in kelp gull (*sul*II: 50% [7/14]) and in a Magellanic penguin (*sul*I: 9% [1/11]). *Sul*I and *sul*II encode resistance to sulfonamides and have been previously reported in wild seabirds in Brazil (EWBANK et al., 2020), with the former also reported in gulls in Europe (DOLEJSKA et al., 2007; POETA et al., 2008; RADHOUANI et al., 2009; ANTILLES et al., 2015). *Sul*II presented significant differences in kelp gulls in comparison with Magellanic penguin regarding its occurrence (50% and 0%. p = .007) and mean load percentage (-4.8 and -8. p = .008). Additionally, resistance to sulfonamides was significantly different in kelp gull in comparison with Magellanic penguin (50% and 9%. p = .033). Interestingly, the prevalences of sulfonamide and *sul*II gene were statistically significant higher in seabirds from a anthropized in comparison with a pristine environment (EWBANK et al., 2020). Sulfonamides are among the oldest synthesized antimicrobials, used in several medical therapies (PARASCA et al., 2013). This antimicrobial class is known to persist in the environment (LAMSHÖFT et al., 2007), and to resist biodegradation in wastewater-treatment processes and in media with elevated microbial activity, such as byproduct sludge (GARCÍA-GALÁN et al., 2008; KÜMMERER, 2008). Thus, the fact that such antimicrobial class presented more significant findings in the synanthropic coastal species (kelp gull), likely indicates higher ARGs pollution of coastal environments due to anthropogenic impact and environmental contamination (e.g., WWTP effluents and wastewater discharge) (SACRISTÁN et al., 2020; EWBANK et al., 2020).

Finally, we also observed significant differences in the *aad*A mean load percentage between kelp gull and Magellanic penguin (respectively, -5.4 and -7.7. p = .045). The *aad*A

gene encodes resistance to two aminoglycosides: streptomycin and spectinomycin (CLARK et al., 1999). Aminoglycosides are used against several aerobic Gram-negative bacilli, many staphylococci, some streptococci, and mycobacteria. Of note, streptomycin is used in multidrug treatments against multidrug-resistant *M. tuberculosis* infections (MAGNET; BLANCHARD, 2005). *Aad*A has been previously reported in gull species (DOLEJSKA et al., 2007; RADHOUANI et al., 2009; ANTILLES et al., 2015), and in little penguins (*Eudyptula minor*) (LUNDBÄCK et al., 2020).

Our findings, especially the detection of the public health relevant *mec*A and bla_{TEM} genes, are very concerning. The present study evaluated samples collected upon the individuals' admission into a rehabilitation center. Thus, the ARGs detected here were acquired in the wild, most likely in the environment (either in anthropized (e.g., landfills, sewage) or natural (e.g., aquatic, continental shelf) epidemiological settings), but potentially from other sources as well, such as infected food items (BRAHMI et al., 2015) and through intra and/or interspecific interactions (e.g., kleptoparasitism). Wildlife is not naturally exposed to antimicrobials in the wild, but once under antimicrobial therapy in rehabilitation centers, the presence of ARGs in their microbiome may interfere, and even prevent, successful therapy. Similarly to nosocomial settings, due to the intense use of antimicrobials, rehabilitation centers may be highly contaminated by these drugs and their metabolites, as well as by ARGs, and exert intense selective pressure over the local resistome (BLYTON et al., 2015; HAENNI et al., 2020). As a consequence, rehabilitation centers may be hot spots for ARGs acquisition, interaction, and development, facilitating resistance exchanges among wildlife, humans (e.g., staff) and the environment, both while in-care and upon release (HAENNI et al., 2020). Thus, rehabilitation centers are very important and informative settings for the study of ARGs within the One Health interface.

Magellanic penguins are a migratory species. Bird migrations may cover great distances, through natural bio-barriers such as oceans, thus considered as holders of a potential central epidemiological role in the dissemination of ARGs, even to remote locations (BONNEDAHL et al., 2009; SMITH et al., 2014; EWBANK et al., 2020). Because migratory birds are capable of acquiring ARGs from humans, domestic animals and the environment (BONNEDAHL et al., 2009, 2010, 2015; HERNANDEZ et al., 2010, 2013; STEDT et al., 2015; ATTERBY et al., 2017; GUENTHER et al., 2017; AHLSTROM et al., 2018; MARCELINO et al., 2019), this group has been largely suggested as reservoirs and dispersers of antimicrobial resistance (GUENTHER et al., 2012; BONNEDAHL et al., 2015; STEDT et al., 2015). Despite a recent experimental study in captive ring-billed gulls (*Larus*

delawarensis) in which the individuals were able to shed and contaminate the artificial environment and infect con-specifics in a controlled setting (FRANKLIN et al., 2020), further studies under natural conditions are necessary to confirm such hypothesis. Herein, migration may have not been a key factor from an epidemiological perspective of ARGs dispersal affecting humans, because despite our significant findings in Magellanic penguin (e.g., detection of ARGs in 10 out of the 11 individuals and of a gene of great public health importance (*bla*$_{TEM}$)), this is a highly pelagic species that spends a great part of its life cycle in the oceans (BOERSMA et al., 2013), sustaining limited direct contact with humans. By contrast, kelp gull are not migratory, only capable of small geographical dislocations (BirdLife International, 2020). Such species presents synanthropic behavior and adaptability to highly anthropized areas, in closer contact with humans and food-producing animals, consequently playing a more relevant role than Magellanic penguin in the epidemiological chain of ARGs within the human-animal-environmental interface. These findings show that all geographical dislocations – from great migrations to small geographical movements, must be considered in the study of ARGs dispersal and epidemiology.

Herein, we showed that the biological and ecological parameters evaluated in this study (i.e., dispersal [migratory and non-migratory], feeding niche [coastal and pelagic], and interaction with human-impacted areas [synanthropic and non-synanthropic]) are key factors in the complex epidemiology of ARGs in wild seabirds. Additionally, we reported the first detection of the *mec*A gene in seabirds in the Americas. Our findings greatly contribute to the current knowledge on ARGs in wild birds both nationally and worldwide, emphasize the importance of ARGs studies in wildlife rehabilitation settings, and reinforce the utility of culture-free highly sensitive molecular diagnostics to assess ARGs in the microbiome of wild birds. Nevertheless, it is important to consider the limitations of our study: (1) our techniques characterize the resistance genotype, not the phenotype, (2) microbiomes were evaluated at the exact point in time of each sample collection, and host-bacteria could eventually lose ARGs-containing plasmids prior to transmission and/or dispersal, and (3) our small sampling size. Admission and pre-release sampling and analysis would allow future assessment of rehabilitation centers as epidemiological settings. Further studies on ARGs in the microbiome of a greater number of seabirds, considering biological and ecological parameters, and the species' natural history (e.g., feeding strategy, habitat, territory), are necessary to broaden our understanding regarding the occurrence and diversity of ARGs in seabirds, and their role as potential sources of infection and dispersal within the One Health chain of ARGs acquisition, interaction and dissemination.

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3 SEABIRDS AS ANTHROPIZATION INDICATORS IN TWO DIFFERENT TROPICAL BIOTOPES: A ONE HEALTH APPROACH TO THE ISSUE OF ANTIMICROBIAL RESISTANCE GENES POLLUTION IN OCEANIC ISLANDS

Ana Carolina Ewbank, Fernando Esperón, Carlos Sacristán, Irene Sacristán, Ricardo Krul, Eduardo Cavalcante de Macedo, Olga Calatayud, Irene Bueno, Ricardo de Francisco Strefezzi, José Luiz Catão-Dias, article published in Science of the Total Environment, 2021, 754, 142141, https://doi.org/10.1016/j.scitotenv.2020.142141.

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Seabirds as anthropization indicators in two different tropical biotopes: A One Health approach to the issue of antimicrobial resistance genes pollution in oceanic islands

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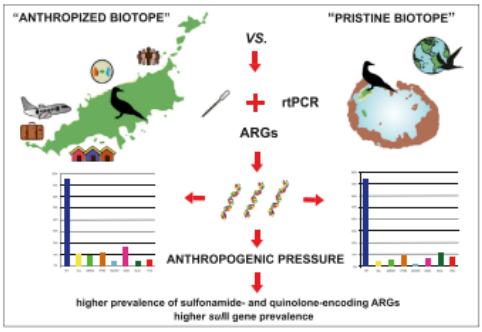
HIGHLIGHTS

- AMR is a quintessential One Health issue and a serious global threat to human health.
- Anthropogenic pressure influenced ARGs prevalence in the anthropized biotope.
- Migratory species had statistically significant higher *mcr*-1 prevalence and percentage load.
- Tetracycline-encoding ARGs were significantly greater in both biotopes.
- Seabirds may be environmental indicators of ARGs pollution, even in a pristine biotope.

GRAPHICAL ABSTRACT



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ABSTRACT

Antimicrobial resistance is a quintessential One Health issue, among the most serious 21st century global threats to human health. Seabirds may act as sentinels of natural and anthropogenic changes in the marine ecosystem health, including pollution by antimicrobial resistance genes (ARGs). We used real time PCR to identify and quantify 22 plasmid-mediated ARGs in the gastrointestinal microbiome of six wild seabird species, comparing an anthropized (Fernando de Noronha Archipelago - FNA) and a pristine biotope (Rocas Atoll - ROA), Brazil. Of 257 birds, 218 (84.8%) were positive to at least one ARG. ARG classes encoding resistance to tetracyclines (75.1%), quinolones (10.5%) and phenicols (10.5%) were the most prevalent, with tetracyclines significantly greater than the remaining classes ($p < 0.05$). Genes *tet*(S) (29.2%), *tet*(A) (28.8%), and *tet*(B) (24.9%) were the most commonly found and had a significantly greater prevalence when compared to the remaining ARGs ($p < 0.05$). The anthropized biotope presented statistically significant higher prevalence of sulfonamide- and

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mcr-1
Migratory birds
Pristine environment
Wildlife

quinolone-encoding ARGs in comparison with the pristine (respectively, $p = 0.01$ and $p = 0.03$), and higher *sul*II gene prevalence ($p = 0.04$), consistent with anthropogenic pressure. Migratory species (only present in ROA) showed statistically significant higher *mcr*-1 (polymyxins) and bla_{TEM} (betalactam) prevalences (respectively, $p = 0.009$ and $p = 0.02$), and *mcr*-1 percentage load ($p = 0.0079$) in comparison with non-migratory. To our knowledge, this is the largest ARGs survey based on direct detection and quantification in seabirds worldwide, and the first to evaluate non-synanthropic species in oceanic islands. This is the first detection of *mcr*-1 in wild free-ranging seabirds in Brazil and in free-ranging migratory non-synanthropic seabirds worldwide. Our findings show the importance of biological and ecological factors, highlighting the role of seabirds as anthropization sentinels and ARGs-pollution environmental indicators (even in a pristine biotope), and their involvement in the One Health epidemiological chain of ARGs.

1. Introduction

Antimicrobial resistance is a quintessential One Health issue and one of the most serious global threats to human health in the 21st century (Liu et al., 2016). Antimicrobial resistance genes (ARGs) result from a naturally occurring phenomenon that predates the existence of humans (D'Costa et al., 2011; Bhullar et al., 2012) and are ubiquitous in the environment (Allen et al., 2009; Forsberg et al., 2012). Despite having ancient origins, the environmental resistomes have been severely impacted by anthropogenic activities (Wright, 2007), considered one of the primary leading forces towards antimicrobial resistance (e.g., use, misuse and overuse of antimicrobials in human and veterinary medicine, aquaculture and agriculture [Yang et al., 2013; Bengtsson and Greko, 2014], pharmaceutical manufacturing waste [Graham et al., 2011], and domestic and agricultural waste releases into the environment [West et al., 2010; Cummings et al., 2011]). As a result, antimicrobial resistance causes major medical challenges, and social and economic losses worldwide (Bengtsson and Greko, 2014; Michael et al., 2014), with potential to eventually become a global crisis and regression to the pre-antibiotic era (Aslam et al., 2018), if not properly addressed and understood from a One Health perspective.

Anthropization - the process of landscape transformation by human impact (Samoilenko et al., 2018), has been suggested as a driving factor regarding ARGs epidemiology in wildlife (Atterby et al., 2017; Ahlstrom et al., 2018; Sacristán et al., 2020). ARGs are considered environmental pollutants (Pruden et al., 2006) and markers of environmental anthropization (Jobbins and Alexander, 2015). The aquatic environment has an important role in the epidemiology of ARGs (Zhang et al., 2009; Marti et al., 2014). These aquatic compartments are important in the transfer, ecology and evolution of ARGs by acting as "melting pots" where antimicrobials and their metabolites and residues, other pollutants, and resistant bacteria from different origins (human [e.g., wastewater from hospitals, sewage], animal [e.g., manure, aquaculture], and environmental [e.g., manure-amended soil]) may interact (Baquero et al., 2008). Seabirds may act as sentinels by reflecting natural and anthropogenic changes in the marine ecosystem health (Rabinowitz et al., 2010), including pollution by ARGs (Atterby et al., 2016, 2017).

In addition to anthropization, it is also important to consider other key factors in the study of ARGs in wildlife, such as host biology and ecology (Dolejska et al., 2016; Dolejska and Literak, 2019; Marcelino et al., 2019). Migratory birds cross vast geographical distances over natural protective bio-barriers and great areas (e.g., oceans), through environments with various degrees of anthropization, which confers them an important epidemiological role in the One Health chain of ARGs, both regarding acquisition and potential dissemination, thus acting as possible indicators of the spectrum of ARGs in the human-wildlife-environment interface (Bonnedahl et al., 2009; Atterby et al., 2017; Ahlstrom et al., 2018). A recent metagenomic study in migratory birds in China, showed that *mcr*-1 is widespread even in species sampled in areas without dense human population and activity (Cao et al., 2020). So far, several studies have shown that migratory birds are capable of acquiring ARGs of human, food-producing animals or environmental origin (Bonnedahl et al., 2009, 2010, 2015; Hernandez et al., 2010, 2013; Stedt et al., 2015; Atterby et al., 2017; Guenther et al., 2017; Ahlstrom et al., 2018; Marcelino et al., 2019), but ARGs dissemination through migration has not yet been confirmed (Guenther et al., 2012; Bonnedahl et al., 2015; Stedt et al., 2015). Nevertheless, in a recent study by Franklin et al. (2020), a migratory seabird species (ring-billed gulls *Larus delawarensis*) experimentally inoculated with an *E. coli* strain harboring plasmid-mediated *mcr-1* gene was able to behave as bridge hosts, shedding these strains in feces for days, contaminating the environment and infecting con-specifics occupying the same artificially controlled environment. Therefore, the potential influence of migratory movements in the dissemination of ARGs should be further investigated.

Feeding behavior has been extensively explored in synanthropic wild seabird species, particularly those adapted to exploiting anthropogenic food subsidies, such as fishing discards and offal, and refuse disposal (e.g., urban trash/garbage dump sites) (Leroux and Loreau, 2008; Bonnedahl et al., 2014; Atterby et al., 2016; Ahlstrom et al., 2018). Nevertheless, feeding behavior has not yet been assessed in non-synanthropic seabird species feeding in coastal or pelagic zones. Regarding to the former, it is also important to consider that not only the birds themselves, but also their prey, may be exposed to an anthropogenically impacted environment (e.g., wastewater discharge, agricultural run-off) (Sellera et al., 2018).

Several studies have used culture-dependent methods to evaluate the prevalence of ARGs in free-ranging seabirds in Europe (gulls [Dolejska et al., 2007; Poeta et al., 2008; Literak et al., 2010; Radhouani et al., 2009, 2011; Antilles et al., 2015; Merkeviciene et al., 2017]), the Americas (gulls [Martiny et al., 2011; Báez et al., 2014; Bonnedahl et al., 2014, 2015; Hasan et al., 2014; Carroll et al., 2015; Atterby et al., 2016; Liakopoulos et al., 2016; Ruzauskas and Vaskeviciute, 2016; Toro et al., 2016; Ahlstrom et al., 2018, 2019] and penguins [Prichula et al., 2016]), Oceania (gulls [Mukerji et al., 2019]), and Antarctica (penguins [Rahman et al., 2015]). Nevertheless, most bacteria are not cultivable (Monier et al., 2011), and culture methods do not favor mobile genetic elements (e.g., plasmids), which encode most ARGs (Nieto-Claudin et al., 2019; Cao et al., 2020).

Herein we employed previously adapted real time PCR (rtPCR) techniques (Sacristán et al., 2020; Cevidanes et al., 2020) to identify and quantify 22 selected plasmid-mediated ARGs in the gastrointestinal microbiome of six wild seabird species from two different oceanic biotopes – an anthropized archipelago (Fernando de Noronha) and a pristine atoll (Rocas), in northeastern Brazil. The goals of this study were to (i) use a One Health approach to evaluate the presence and load of ARGs in wild seabirds from an inhabited archipelago and a pristine uninhabited atoll in the northeastern coast of Brazil and (ii) to use the obtained data to assess and compare the anthropogenic impact on these two marine biotopes, and (iii) to evaluate the presence and load of ARGs according with species and behavior (dispersal and feeding zone). We hypothesized that ARGs prevalence and load would be higher in seabirds (1) inhabiting the anthropized in comparison with the pristine environment; (2) sustaining migratory in comparison with non-migratory behavior, and (3) using coastal in comparison with pelagic feeding zones.

2. Materials and methods

2.1. Study area

The Fernando de Noronha Archipelago - FNA (03°51′S 32°25′W), consists of 21 islands and islets, located at 360 km from the Brazilian coast. FNA is a district of Pernambuco state, and has two Federal Conservation Units: the Fernando de Noronha - Rocas - São Pedro and São Paulo Environmental Protection Area (APA – Área de Proteção Ambiental) and the Fernando de Noronha National Marine Park (PARNAMAR – Parque Nacional Marinho), both under the administration of the Pernambuco State government through the Chico Mendes Institute for Biodiversity Conservation (ICMBio) - Brazilian Ministry of the Environment (ICMBio/MMA, 2017). FNA is an important touristic site in Brazil, receiving at least 100,000 visitors in the study year alone (personal communication, Parque Nacional Marinho, ICMBio, 2020. The only human settlement in the archipelago is located in the Fernando de Noronha Island (inside the APA), and houses the 3101 permanent FNA residents and all visiting tourists (IBGE, 2020).

The Rocas Atoll – ROA (03°51′S 33°48′W), is located at 267 km from the coastline and 148 km from FNA, in Rio Grande do Norte state. It is part of the Rocas Atoll Biological Reserve (ReBio) - a permanent conservation unit established in 1979, which comprises the atoll itself and the surrounding waters as far out as the 1000 m isobath, and is under the jurisdiction of ICMBio (Fischer et al., 2007; Jales et al., 2015). No human-related activities (e.g., fishing, tourism) are allowed in the ReBio area, aside from scientific research, which is limited to a maximum of four researchers and one ICMBio analyst per expedition (a maximum visit rate of approximately 60 visitors a year). All of the food, drinking water, equipment and other necessities are brought by boat from the continent with each new research group. Additionally, all organic waste generated during each expedition is transported back to the continent. Similarly to FNA, ROA is also part of the PARNAMAR.

The PARNAMAR and ReBio are considered World Heritage Sites by UNESCO (WHC, 2001). FNA and ROA are located in the Southern Atlantic Ocean, within the Brazilian territory, and are considered the most important seabird breeding sites in Brazil, both in terms of diversity and number of individuals (Antas, 1991). ROA is also used by migratory and vagrant seabird species for roosting and feeding.

2.2. Samples

We sampled a total of 308 seabirds, comprising six species: 104 from the Fernando de Noronha Archipelago islands (masked boobies [*Sula dactylatra*, n = 48], brown boobies [*Sula leucogaster*, n = 31] and magnificent frigatebirds [*Fregata magnificens*, n = 25]), and 204 from Rocas Atoll (masked boobies [n = 33], brown boobies [n = 33], magnificent frigatebirds [n = 35], red-footed boobies [*Sula sula*, n = 33], sooty terns [*Onychoprion fuscatus*, n = 36], and brown noddies [*Anous stolidus*, n = 34]). FNA samples were collected in September 2018 and ROA samples were collected between December 2018 and January 2019. The number of samples collected in FNA and ROA are described according with species, collection site, behavior (feeding zone [coastal or pelagic], and dispersal [migratory or non-migratory]) in Table 1. All birds were captured with a butterfly net. Fresh fecal samples were immediately obtained by enema during manual immobilization. Briefly, 2 ml of autoclaved phosphate buffered saline were flushed into the cloaca using a Pasteur pipette, immediately retrieved and stored in an autoclaved eppendorf tube placed at −20 °C until analyses. Each recovered enema sample contained a final volume ranging from 1.0 to 1.5 ml. All study samples were collected in full compliance with specific federal permits issued by the Brazilian Ministry of Environment and approved by the Biodiversity Information and Authorization System (SISBIO 59150-4). All procedures were performed in accordance with the Ethical Committee in Animal Research of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (Process numbers 1753110716). The sampled species according with study sites are shown in Fig. 1.

2.3. Molecular analyses

Total DNA extraction was carried out by a pressure filtration technique (QuickGene DNA tissue kit S, Fujifilm, Tokyo, Japan), following the manufacturer's instructions. Subsequently, the 16S rRNA gene was amplified by rtPCR in ten-fold dilutions of each extracted sample (Jiang et al., 2013) to verify the presence of an adequate amount of bacterial DNA. A sample was considered validated if its ten-fold dilution showed a cycle threshold (Ct) less than 25 (Esperón et al., 2018). The number of validated samples is described in Supplementary Table 1. Once validated, samples were analyzed by rtPCR for 22 plasmid-mediated ARGs encoding resistance to eight different antimicrobial classes: tetracyclines (*tet*(A), *tet*(B), *tet*(Y), *tet*(K), *tet*(M), *tet*(Q), *tet*(S), *tet*(W)), aminoglycosides (*aad*A and *str*), sulfonamides (*sul*I and *sul*II), phenicols (*cat*I and *cat*II), macrolides (*erm*(B) and *erm*(F)), quinolones (*qnr*B and *qnr*S), betalactams (bla_{TEM}, bla_{CTX-M} and *mec*A), and polymyxins (*mcr*-1) (Sacristán et al., 2020; Cevidanes et al., 2020). To estimate the percentage of bacteria harboring each ARG (load percentage of each ARG), we used the formula % gene $X = 10^{[2+0.33(ct16S-ctgeneX)]}$, with results expressed in logarithm 10, ranging from −8 (−8 was given to a sample considered negative) to +2 (when 100% of the

Table 1
Multivariable relationships between predictor variables regarding prevalence of classes of antimicrobials in real time PCR-positive birds based on logistic regression analysis (n = 257).

		Regression coefficient	Standard error (SE)	Adjusted odds ratio	95% confidence interval	*p* value
Sulfonamides	Noronha Archipelago	2.02	0.83	7.57	0.55–3.97	0.01
	Magnificent frigatebirds	−0.25	1.03	0.78	−2.4–1.92	0.81
	Sooty terns	0.62	0.91	1.85	−1.09–2.65	0.49
	Masked boobies	−2.10	1.22	0.12	−4.6–0.35	0.087
	Brown boobies	−18.36	1456	0.00	NA–72.93	0.99
	Red-footed boobies	−17.00	1931.5	0.00	NA–104.11	0.99
Quinolones	Noronha archipelago	1.4727	0.6681	4.36	0.27–2.98	0.03
	Magnificent frigatebirds	−1.8360	0.8152	1.59	−3.5–(−0.3)	0.02
	Sooty terns	−0.9694	0.7615	3.79	−2.6–0.47	0.20
	Masked boobies	−1.3750	0.8113	2.52	−3.1–0.15	0.09
	Brown boobies	−2.8404	1.0149	5.84	−5.1–(−1.01)	0.005
	Red-footed boobies	−17.3	1171.5	3.17	−196–45.33	0.99
Betalactams	Magnificent frigatebirds	−0.83	0.59	0.44	−2.01–0.35	0.16
	Sooty terns	−1.2	0.75	0.30	−2.8–0.19	0.11
	Masked boobies	−1.88	0.73	0.15	−3.5–(−0.5)	0.01
	Brown boobies	−2.77	1.10	0.06	−5.7–(−0.96)	0.01
	Red-footed boobies	−1.57	0.85	0.20	−3.5–(−0.04)	0.06
	Migratory	−1.02	0.45	0.36	−1.8–(−0.1)	0.02
Polymyxins	Migratory	−1.28	0.50	0.27	−2.27–(−0.29)	0.009

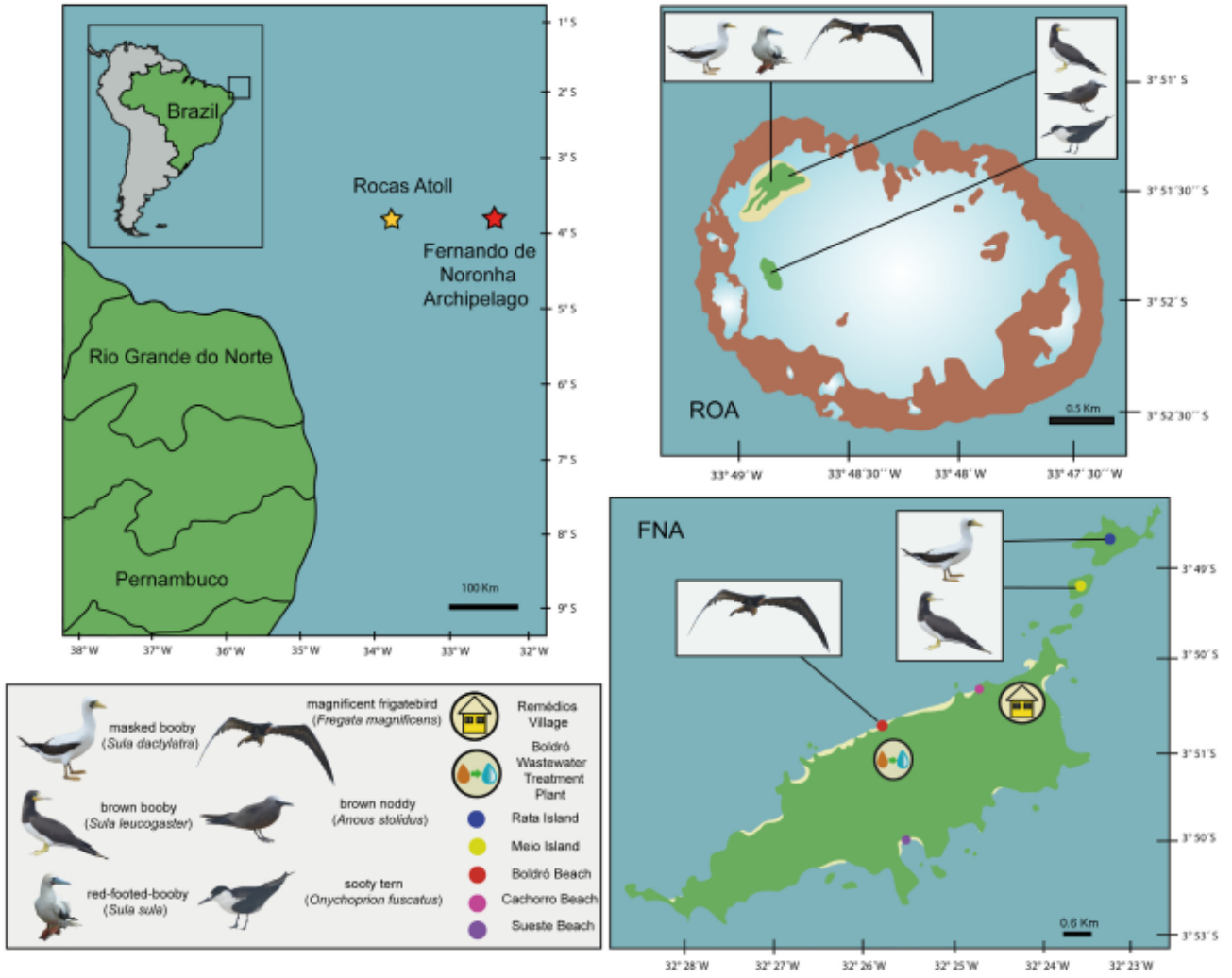

Fig. 1. Geographical location of the study sites and respective states (Rocas Atoll [ROA] – Rio Grande do Norte state [yellow star]; Fernando de Noronha Archipelago [FNA] – Pernambuco state [red star]) on the northeastern Brazilian coast (left), South America (upper left). Scale: 100 km. Sampled species at each study site: Rocas Atoll [ROA] (upper right). Scale: 0.5 km. Fernando de Noronha Archipelago [FNA] (lower right). Scale: 0.6 km. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bacteria in the sample presented the ARG), as per Nieto-Claudin et al. (2019). Our thermal cycle was the same for all the rtPCR reactions (6′ 95 °C, 40× (10″ 95 °C, 30″ 60 °C)), with alignment and extension in the same step, at constant temperature of 60 °C. A melting curve step was performed at the end of the rtPCR reaction (Nieto-Claudin et al., 2019) (further information listed in Supplementary Table 8). Fecal samples presenting at least three ARG-encoding resistance to different classes of antimicrobials were classified as "multiresistant microbiomes" (Sacristán et al., 2014). Thus, samples were classified into "multiresistant microbiomes" and "non-multiresistant microbiomes".

2.4. Statistical analysis

Independent variables (i.e., collection sites, species and behavior (dispersal [migratory or nonmigratory] and feeding zone [coastal or pelagic])) associated with ARG class prevalence, presence of multiresistant profiles, and ARGs prevalence and quantification were assessed with crude and adjusted odd ratios (ORs) calculated by a logistic regression analysis with 95% confidence intervals and corresponding Kruskal-Wallis and Mann-Whitney U tests. Goodness of fit models was evaluated by confidence intervals and an analysis of residuals. All statistical analyses were performed in R software (R Development Core Team 3.0.1., 2013) with a significance level of $p < 0.05$.

3. Results

The majority of the collected samples validated for the 16S rRNA gene (86.5% [90/104] for FNA and 81.9% [167/204] for ROA). From the 257 validated samples, 218 (84.8%) were positive for at least one ARG: 84.4% (76/90) in FNA and 85% (142/167) in ROA (Supplementary Table 1). The statistically significant results of multivariable relationships regarding antimicrobial classes and individual genes prevalence in rtPCR-positive birds based on logistic regression analysis (n = 257), and prevalence of the main classes of ARGs and individual ARGs in species of FNA and ROA, respectively, are shown in Tables 1, 2 and 3. The number of collected and validated samples in FNA and ROA according with species, collection site, behavior (dispersal [migratory or nonmigratory] and feeding zone [coastal or pelagic]), prevalence of each class of ARGs and multiresistant profiles, and of ARGs in the sampled birds in this study, and per study site (FNA and ROA), are listed in Supplementary materials.

Table 2
Multivariable relationships regarding individual genes prevalence between predictor variables in real time PCR-positive birds based on logistic regression analysis (n = 257).

		Regression coefficient	Standard error (SE)	Adjusted odds ratio	95% confidence interval	*p* value
*sul*II	Noronha Archipelago	1.04	0.511	2.8	0.05–2.09	0.04
	Magnificent frigatebirds	−1.24	0.69	0.2	−2.6–0.09	0.07
	Sooty terns	−1.0	0.76	0.3	−2.6–0.43	0.18
	Masked boobies	−0.2	0.56	0.7	−1.3–0.9	0.67
	Brown boobies	−1.85	0.85	0.15	−3.8–(−0.3)	0.03
	Red-footed boobies	−17.0	1171.5	0.0	NA–46.34	0.98
bla_{TEM}	Magnificent frigatebirds	−0.82	0.59	0.43	−2.0–0.3	0.16
	Sooty terns	−1.2	0.74	0.3	−2.0–0.19	0.10
	Masked boobies	−1.8	0.73	0.15	−3.4–(−0.5)	0.01
	Brown boobies	−2.7	1.1	0.06	−5.7–(−0.9)	0.01
	Red-footed boobies	−1.5	0.85	0.2	−3.5–(−0.04)	0.06
	Migratory	−1.0	0.44	0.3	0.14–0.88	0.02
*qnr*S	Magnificent frigatebirds	−1,2	0.69	0.28	−2.6–0.09	0.07
	Sooty terns	−1.0	0.76	0.36	−2.6–0.43	0.18
	Masked boobies	−0.2	0.56	0.79	−1.3–0.93	0.67
	Brown boobies	−1.8	0.85	0.15	−3.8–(−0.3)	0.03
	Red-footed boobies	−17.2	1171.5	0.000	NA–46.3	0.98

3.1. Qualitative descriptive results

Gene classes able to confer resistance to tetracyclines (75.1%), quinolone (10.5%) and phenicols (10.5%) were the most prevalent in this study, with tetracyclines significantly greater than the rest of the ARGs classes ($K = 761.0$, $p < 0.05$). Multiresistant microbiomes were observed in 8.6% of the samples (22/257) (Supplementary Table 2). Genes *tet*(S) (29.2%), *tet*(A) (28.8%), and *tet*(B) (24.9%) were the most commonly found ARGs, and had a significantly greater prevalence when compared to the remaining ARGs ($K = 2071.0$, $p < 0.05$) (Supplementary Table 3).

Genes encoding resistance to tetracyclines were the most prevalent in all studied species, especially in sooty terns (90.6%; 29/32). Masked and brown boobies presented significant lower prevalence of ARGs encoding resistance to betalactams, and brown boobies presented significant lower prevalence of ARGs encoding resistance to quinolones (Tables 1 and 3). *Tet*(S) (53.1%; 17/32) in sooty terns was the most commonly detected gene. The bla_{TEM} prevalence was statistically significant lower in masked and brown boobies ($p = 0.01$), while the prevalence of *qnr*S was statistically significant lower only in brown boobies ($p = 0.03$) (Table 2).

The ARGs class comprising genes encoding tetracycline resistance presented similar prevalences in the species that occurred in both study sites (magnificent frigatebirds, masked boobies and brown boobies): FNA (75.5%; 68/90) and ROA (73.7%; 56/76). Nevertheless, there were statistically significant differences regarding the prevalence of sulfonamide and quinolone ARGs classes between FNA and ROA birds (respectively, $p = 0.01$ and $p = 0.03$), which were higher in FNA (Table 1). Multiresistant microbiomes were present in FNA (11.1%; 10/90) and in ROA (3.9%; 3/76), although not presenting significant statistical differences in terms of prevalence. Genes *tet*(A) (FNA [31.1%; 28/90] and ROA [26.3%; 20/76]), *tet*(B) (FNA [17.8%; 16/90] and ROA [31.6%; 24/76]) and *tet*(S) (FNA [28.9%; 22/76] and ROA [26.3%; 20/76]) were the most prevalent ARGs in both sites. The *sul*II gene prevalence was statistically significant higher in birds in FNA ($p = 0.04$) (Table 2).

In terms of dispersal, tetracycline-encoding genes were also high in both migratory (81.7%; 49/60) and non-migratory species (73.1%; 144/197). Although not statistically significant in terms of prevalence, multiresistant microbiomes were present in migratory and non-migratory species (respectively, 11.7% [7/60] and 7.6% [15/197]). The prevalence of the colistin-encoding resistance gene*mcr*-1 was higher in migratory in comparison with non-migratory species, with statistically significant differences between them ($p = 0.009$) (Table 1). Additionally, bla_{TEM} prevalence was statistically significant higher in migratory species ($p = 0.02$) (Table 2).

When comparing feeding zones, the gene class able to cause resistance to tetracyclines presented similar prevalences in coastal (75%; 78/104) and in pelagic species (75.2%; 115/153). Multiresistant microbiomes were present in both groups (pelagic [11%; 17/154] and coastal [4.8%; 5/104]), however, no significant statistical differences were observed in regard to their prevalence. *Tet*(S) in pelagic species was the most prevalent gene (34.4%; 53/154). The colistin-encoding gene (*mcr*-1) was only detected in pelagic species, at a prevalence of 11.8% (18/153).

Table 3
Prevalence of the main classes of ARGs and individual ARGs found in the bird species of the Fernando de Noronha Archipelago and the Rocas Atoll.

	Noronha n = 90 Prevalence (%) (95% CI)	Atol n = 167 Prevalence (%) (95% CI)	Brown noddies n = 28 Prevalence (%) (95% CI)	Magnificent frigatebirds n = 55 Prevalence (%) (95% CI)	Sooty terns n = 32 Prevalence (%) (95% CI)	Masked boobies n = 62 Prevalence (%) (95% CI)	Brown boobies n = 49 Prevalence (%) (95% CI)	Red-footed boobies n = 31 Prevalence (%) (95% CI)	Migratory n = 60 Prevalence (%) (95% CI)	Non-migratory n = 197 Prevalence (%) (95% CI)
Sulfonamides	11.1	4.7	7.1	16.4	12.5	4.8	0.0	0.0	1.0	6.1
class	4.5–17.7	1.5–8.0	−3.0–17.3	6.3–26.5	0.3–24.6	−0.6–10	0.0–0.0	0.0–0.0	2.1–17.8	2.7–9.5
Quinolones	16.7	7.1	21.4	9.1	9.3	17.7	4.1	0.0	15.0	9.0
class	8.8–24.5	3.2–11.1	5.2–37.6	1.2–16.9	−1.3–20.05	7.9–27.5	−1.6–9.8	0.0–0.0	5.6–24.3	5.0–13.2
Betalactams	4.4	11.3	25.0	12.7	9.3	4.8	2.0	6.4	16.7	6.6
class	0.1–8.7	6.5–16.2	7.9–42.1	3.6–21.8	−1.3–20.0	−0.6–10.3	−2.0–6.1	0.1–8.7	6.9–26.4	3.1–10.1
Polymixins	5.5	7.8	21.4	21.4	10.0	12.9	0.0	3.2	15.0	4.6
class	0.7–10.4	3.6–11.9	5.2–37.6	5.2–37.6	−1.4–21.4	4.3–21.5	0.0–0.0	−3.3–9.8	5.6–24.3	1.6–7.5
*sul*II	11.1	4.2	7.1	16.36	9.3	4.8	0.0	0.0	8.3	1.9
	4.5–17.7	1.1–7.2	−3.0–17.3	6.3–16.5	−1.3–20.0	−0.6–10.3	0.0–0.0	0.0–0.0	1.1–15.5	−0.7–4.5
*qnr*S	15.6	7.2	21.4	7.2	9.1	17.7	4.1	0.0	17.8	8.6
	7.9–23.2	3.3–11.1	5.2–37.6	0.2–14.4	−1.3–19.4	8.0–27.5	−1.7–9.8	0.0–0.0	5.6–23.9	6.7–12.6
bla_{TEM}	4.4	11.3	25.0	12.7	9.1	4.8	2.0	6.4	16.4	6.6
	0.1–8.8	6.5–16.2	7.9–4.2	3.6–21.8	−1.2–19.4	−0.6–10.3	−2.0–6.1	−2.7–15.6	6.8–25.9	3.1–10-1

3.2. Quantitative descriptive results

The percentage load in FNA and ROA was similar despite slightly different ranges (respectively, −6.2 [−8.0 to −0.3] and −6.2 [−8.0 to −1.0]). Among all evaluated species, sooty terns presented the highest percentage load (*tet*(S): −5.1 [ranging from −8.0 to −1.1]). The highest percentage load found in species occurring on both sites was observed in FNA magnificent frigatebirds (*tet*(W): −5.5 [ranging from −8.0 to −1.1]). In regard to dispersal, the percentage load varied between migratory (*tet*(S): −5.4 [ranging from −8.0 to −1.1]) and non-migratory species, although not statistically significant. The *mcr*-1 percentage load was statistically significant higher in migratory species ($p = 0.0079$) (percentage load in migratory species = −7.113, SD = 2.18; percentage load in non-migratory species = −7.692, SD = 1.42). Although the percentage load was similar (−6.3) regarding feeding zone, ranges varied slightly between coastal (−8.0 to −0.3) and pelagic species (−8.0 to −0.9).

4. Discussion

The prevalence of ARGs-encoding sulfonamide and quinolone resistance - antimicrobial classes widely used in human and veterinary medicine for decades (Baran et al., 2011; Poirel et al., 2012; Liu et al., 2017), was significantly higher in FNA, the study site under the highest anthropic pressure. The prevalence of the *sul*II gene was also statistically significant higher in birds in FNA ($p = 0.04$). These findings corroborate with our first hypothesis and are in accordance with previous wildlife studies that correlated ARGs with anthropogenic impact (Miller et al., 2009; Atterby et al., 2016; Sacristán et al., 2020).

For years, FNA has been dealing with serious infrastructural and environmental issues caused by disorderly and disproportionate tourism. In the year of the field study alone (2018), FNA received over 100,000 tourists from all over the world, an increment of 12% over the estimated archipelago's capacity (ICMBio/MMA, 2017). The archipelago presents chronic basic sanitation problems; the wastewater treatment system is deficient and obsolete, wastewater treatment plants (WWTPs) effluents are irregularly disposed at the beaches (Boldró and Cachorro; northeastern face of the island), and untreated sewage from the Air Force Base is disposed in the mangrove (Sueste beach; southern area) (Andrade et al., 2007; Da Costa Cristiano et al., 2020) (Fig. 1). WWTPs are considered potential hot spots of ARGs exchange and dissemination (Szczepanowski et al., 2004; Karkman et al., 2018), potentially acting as important ARGs reservoirs (Czekalski et al., 2012; Fahrenfeld et al., 2013), by releasing into the environment many ARGs not effectively removed by traditional treatment processes (Michael et al., 2013; Czekalski et al., 2014; Tang et al., 2016). Wastewater may combine promoters of selection pressure for antimicrobial resistance (e.g., antimicrobials and its residues, disinfectants, and metals) and antimicrobial-resistant bacteria from many different sources (i.e., hospitals, animal husbandry and urban wastewaters) (Zhang et al., 2009; Karkman et al., 2018; Gao et al., 2018).

Sulfonamides and quinolones are persistent in the environment (Lamshöft et al., 2007), biodegradation-resistant in wastewater-treatment processes and even in media with high microbial activity (e.g., byproduct sludge) (García-Galán et al., 2008; Kümmerer, 2008). Genes encoding resistance to these two antimicrobial classes have been detected in several studies related to WWTP effluents (Watkinson et al., 2009; Gao et al., 2012), sometimes even at higher rates than in the influent (Mao et al., 2015). Additionally, international travel knowingly contributes to the worldwide dissemination of antimicrobial resistance (MacPherson et al., 2009; Arcilla et al., 2017). Thus, we suggest that the prevalence of ARGs-encoding resistance to sulfonamide and quinolone was significantly higher in FNA due to the high year round influx of tourists associated with the insufficient wastewater management system of the archipelago, that likely contaminated the aquatic environment (Dolejska et al., 2007, 2009; Atterby et al., 2016, 2017; Marcelino et al., 2019) and/or the birds' food sources (fish and seafood) (Nesse et al., 2005; Sellera et al., 2018). Future studies are necessary to evaluate the occurrence, prevalence and diversity of ARGs in the wastewater, WWTP's influents and effluents, and local fish/seafood of the archipelago of Noronha in order to further assess this issue.

As described above, *sul*II was statistically significant higher in birds in FNA. This gene encodes resistance to sulfonamides through ribosomal protection (Jiang et al., 2013), and has been widely reported in humans (Hammerum et al., 2006; Wu et al., 2010), wastewater (Hoa et al., 2008; Mao et al., 2015), WWTPs (Du et al., 2014; Laht et al., 2014), and in the aquatic environment (Na et al., 2014; Zhang et al., 2018). In seabirds, *sul*II has mostly been reported in gulls from Europe (Dolejska et al., 2007; Poeta et al., 2008; Radhouani et al., 2009; Merkeviciene et al., 2017) and North America (Ahlstrom et al., 2019). Gulls are synanthropic species and opportunistic feeders (e.g., human refuse) - thus more likely exposed to ARGs, which may or may not sustain migratory behavior depending on the species and population (Weiser and Powell, 2010; Atterby et al., 2016; Birdlife International, 2004). We suggest that the high *sul*II prevalence observed in FNA was possibly due to environmental pollution (e.g., WWTP effluents and wastewater discharge into the aquatic environment).

Interestingly, although not statistically relevant, phenicols - represented by chloramphenicol-encoding genes *cat*I and *cat*II – were the third most prevalent antimicrobial class in this study, with an overall prevalence of 10.5%. The phenicols class was, respectively, the first and third most prevalent ARGs class in free-living pigeons (29.1%) and in Andean foxes (36.1%) from Costa Rica and Chile analyzed by similar techniques (Blanco-Peña et al., 2017; Cevidanes et al., 2020). Due to its well known adverse effects (e.g., aplastic anemia, bone-marrow suppression, gray baby syndrome, and hypersensitivity), chloramphenicol is seldomly used in humans, mostly limited to the therapy of a small number of life threatening infections (e.g., meningitis), on account of its ability to cross the blood-brain barrier (Schwarz et al., 2004). Chloramphenicol was banned from use as therapy and growth promoter in food-producing animals in Brazil, Chile and Costa Rica, among others; however, its use is still permitted in companion animals (Brasil, 2003; Rojas Martínez, 2009; ISP, 2019; SAG, 2019). Our findings are possibly due to its use, although limited, in humans and non-food-producing animals, but also to the fact that plasmids carrying these genes also frequently carry several additional genes encoding for resistance to other antimicrobial agents, ultimately favoring ARGs co-selection (Schwarz et al., 2004).

Tetracycline resistance-encoding genes were the most prevalent in both study areas, as described in similar studies in Galapagos tortoises (*Chelonoidis porteri*) in the Galapagos Archipelago, Ecuador (Nieto-Claudin et al., 2019), and in guignas (Sacristán et al., 2020) and Andean foxes (*Lycalopex culpaeus*) in Chile (Cevidanes et al., 2020). Interestingly, *tet*(S) was the most prevalent *tet* gene found in this study, in contrast to *tet*(Q) and *tet*(W), reported by Nieto-Claudin et al., 2019, Cevidanes et al., 2020 and Sacristán et al., 2020.

The high tetracycline prevalence is not surprising, once these are low cost broad spectrum antimicrobials extensively used for decades in human therapy, in veterinary medicine, in aquaculture, and agriculture (Chopra and Roberts, 2001; Daghrir and Drogui, 2013). Nevertheless, considering all 22 evaluated genes conferring resistance to eight different antimicrobial classes, the overall ARGs prevalence and percentage load were low in this study when compared with previous ones (Blanco-Peña et al., 2017; Nieto-Claudin et al., 2019; Sacristán et al., 2020; Cevidanes et al., 2020). Interestingly, genes of marked Public Health importance were observed on both sites: *mcr*-1 (FNA [masked boobies] and ROA [masked and red-footed boobies, sooty terns, and brown noddies]) and bla_{TEM} (FNA [masked boobies and magnificent frigatebirds] and ROA [all evaluated species]).

The *mcr*-1 gene confers resistance to colistin (class polymyxin) (Liu et al., 2016). Despite being discontinued in humans in the

1970s' due to its toxicity and low renal clearance (Wang et al., 2018), the use of colistin has been recovered in the last years as a last resource antibiotic for the treatment of multidrug-resistant Gram-negative bacteria (Michalopoulos and Karatza, 2010). In agriculture and aquaculture, however, this antibiotic has been extensively used as therapy and growth promoter (Kempf et al., 2016; Wang et al., 2018; Shen et al., 2016), despite being banned in several countries, including those from the European Union, and more recently China and India (Laxminarayan et al., 2020). In December 2018, the use of colistin for livestock was also banned in Brazil (Ação Civil Pública, proc. n° 5026342-78.2017.4.03.6100, Ministério Público Federal, 2018). The bla_{TEM} gene confers resistance to cephalosporins and penicillins (class betalactam) (Mroczkowska and Barlow, 2008). Betalactams are one of the oldest and most widely used antibiotic classes in humans and veterinary medicine (Guenther et al., 2011; Sharland et al., 2018). Certain betalactams (e.g., cefepime), and other antimicrobials such as polymyxins (e.g. colistin), are now commonly used as 'last resort' antibiotics against multidrug-resistant infections (MDR) in humans (Wang et al., 2018; Shen et al., 2020). The presence of these two Public Health relevant genes is worrisome, especially for *mcr*-1, which is capable of spreading in hospital environments and in the community even in the absence of colistin use (Tian et al., 2017; Wang et al., 2017). The worldwide presence of these genes, previously reported in humans (Torpdahl et al., 2013; Liu et al., 2016), animals (Yuan et al., 2009; Anjum et al., 2016; Zhang et al., 2016), animal food-products (Matsumoto et al., 2014; Monte et al., 2017), and the environment (Lachmayr et al., 2009; Sacramento et al., 2018), is an alarming indication that we may be approaching the inevitable progression to pan-drug resistance (Liu et al., 2016). The vast majority of bla_{TEM} and *mcr*-1 reports in wild free-ranging seabirds has been in gulls in Europe (Dolejska et al., 2007; Poeta et al., 2008; Radhouani et al., 2009; Literak et al., 2010; Ruzauskas and Vaskeviciute, 2016), North America (Bonnedahl et al., 2014; Atterby et al., 2016; Ahlstrom et al., 2019), Oceania (Mukerji et al., 2019), and South America (Liakopoulos et al., 2016), aside from recent reports of bla_{TEM} in gentoo penguins (*Pygoscelis papua*) in Antarctica (Marcelino et al., 2019).

The *mcr*-1 and bla_{TEM} prevalences (respectively, $p = 0.009$ and $p = 0.02$), as well as the *mcr*-1 percentage load ($p = 0.0079$) were significantly higher in migratory in comparison with non-migratory seabirds, corroborating with our second hypothesis. Migratory species were only sampled in ROA, the most important breeding site for brown noddies in the Brazilian territory and also home to the largest sooty tern population in the Southern Atlantic (Del Hoyo et al., 1996; Higgins and Davies, 1996; Fischer et al., 2007). Additionally, ROA is a highly populated key seabird breeding site in the Southern Atlantic, with up to thousands of breeding pairs in the peak of the breeding season (November–February) (Antas, 1991; Schulz-Neto, 2004). Migratory birds cross vast geographical distances over natural protective bio-barriers and great areas (e.g., oceans), which confers them an important epidemiological role in the One Health chain of ARGs, both in terms of acquisition and potential dissemination, thus acting as possible indicators of the spectrum of ARGs in the human-wildlife-environment interface (Bonnedahl et al., 2009; Atterby et al., 2017; Ahlstrom et al., 2018). A recent metagenomic study in migratory birds in China, showed that *mcr*-1 is widespread even in species sampled in areas without dense human population and activity (Cao et al., 2020). So far, several studies have shown that migratory birds are capable of acquiring ARGs of human, food-producing animals or environmental origin (Bonnedahl et al., 2009, 2010, 2015; Hernandez et al., 2010, 2013; Stedt et al., 2015; Atterby et al., 2017; Guenther et al., 2017; Ahlstrom et al., 2018; Marcelino et al., 2019), but ARGs dissemination through migration has not yet been confirmed (Guenther et al., 2012; Bonnedahl et al., 2015; Stedt et al., 2015). Therefore, the significantly higher *mcr*-1 percentage load observed in the migratory species ($p = 0.0079$) may be a very important finding from an epidemiological perspective, as it suggests that they have a higher potential of *mcr*-1 dissemination to the environment, wildlife, and even humans. We hypothesize that although *mcr*-1 and bla_{TEM} were likely acquired somewhere else (possibly in the territories used during non-breeding season) by the migratory species using ROA as breeding grounds, *mcr*-1 and bla_{TEM} could potentially contaminate the atoll environment and resident species. Nevertheless, it is important to carefully consider that (1) our technique characterizes the resistance genotype and not the phenotype, and (2) that such evaluations are limited to the time of sampling, and that host-bacteria could eventually lose plasmids containing ARGs before their transmission and/or dispersal. Therefore, our findings safely suggest that seabirds carry and may potentially spread ARGs to humans, animals and the environment. Such interpretation applies to all discussions regarding the potential spread of ARGs by migrating seabirds, which are often suggested as disseminators and reservoirs of ARGs (Bonnedahl et al., 2009; Stedt et al., 2015; Atterby et al., 2016; Hernández and González-Acuña, 2016; Ruzauskas and Vaskeviciute, 2016). Further rt-PCR-based studies comparing these two species in their breeding and non-breeding grounds would be able to shed some light on the potential sources and hot spots of contamination in such areas, and ARGs dissemination potential through geographical movements (e.g., individual satellite telemetry tracking devices).

Our findings did not corroborate with our third hypothesis - that higher ARGs prevalence and loads would be observed in seabirds using coastal in comparison with pelagic feeding zones. Nonetheless, it was interesting to observe that in comparison with the other four evaluated species in both study sites, masked and brown boobies presented significant lower prevalence of ARGs encoding resistance to betalactams, and brown boobies presented significant lower prevalence of ARGs encoding resistance to quinolones. Additionally, in terms of specific ARGs, we observed statistically significant lower bla_{TEM} prevalence in both species ($p = 0.01$) and statistically significant lower *qnrS* prevalence in brown boobies ($p = 0.03$). The ARGs differences found in this study were potentially linked to these species' gut microbiome composition and their ecological relationship with environmental resistomes (Forsberg et al., 2014; Gibson et al., 2015). Avian gut microbiota composition are influenced mainly by host taxonomy (Waite and Taylor, 2014; Hird et al., 2015; Marcelino et al., 2019) and ecological factors (e.g., dietary specialization [Blanco et al., 2006; Roggenbuck et al., 2014], host age [Godoy-Vitorino et al., 2010; Van Dongen et al., 2013], anthropization and variety of foraging sites [Fuirst et al., 2018]), but also by geographic distribution (Green and Bohannan, 2006; Marcelino et al., 2019). Among the six evaluated species, only boobies are taxonomically close, within the same genera. Although using different feeding zones, all six species shared similar diets, mostly based on fish and squid (Del Hoyo et al., 1996). As stated above, two of the booby species - masked and brown boobies - had similar resistance profiles. Of note, they were resident species sampled in nesting mixed colonies in FNA and ROA, which could partially explain such similarity and the differences observed when compared with red-footed boobies. Although in the currently available studies, taxonomy seems to be a strong determinant of gut microbiota, it does not explain why masked and brown boobies have similar results between them, but different from those observed in red-footed boobies. These findings highlight the importance of considering host biology and ecology in the study of ARGs in wildlife, aside from the level of anthropogenic impact in the studied area (Dolejska et al., 2016; Dolejska and Literak, 2019; Marcelino et al., 2019). Further studies on the gut microbiota composition and biology of the herein evaluated seabird species (e.g., fishing grounds, specific feeding habits) are needed to clarify these findings.

Although not detected in this study, the fact that the selected ARGs conferring resistance to aminoglycoside (*aadA* and *str*) and macrolide (*ermB* and *ermF*) were not detected in our samples does not necessarily rule out the possibility of other ARGs encoding resistance for such classes being present in the evaluated microbiomes. The same applies to the $bla_{CTX\text{-}M}$ and *mecA* genes, selected among ARGs encoding resistance to the betalactam class. Most specifically, the CTX-M enzyme family is

very wide, limiting the ability of the employed primers to identify all possible variants of the $bla_{CTX\text{-}M}$ gene (Supplementary Table 8).

5. Conclusions

To the authors' knowledge, this is the largest ARGs survey based on direct detection and quantification in seabirds worldwide, and the first to evaluate nonsynanthropic species living in oceanic islands – including in a pristine one (ROA). This is the largest report of *mcr*-1 in free-ranging wild birds worldwide and the first detection of *mcr*-1 in free-ranging seabirds in Brazil. In order to test our hypotheses, our statistical analyses were based on comparisons between sampling sites, species, feeding behavior, and dispersal for 22 different ARGs. A *p*-value adjustment method was not used in this study because such approach would have increased the Type II error (Perneger, 1998; Nakagawa, 2004; Rothman, 1990). Moreover, to provide epidemiological context to environmental antimicrobial resistance, we informed the biological significance of ARGs using logistic regression, which provides effect measures (ORs) with confidence intervals, instead of relying solely on significant *p*-values. According with our first hypothesis, the anthropized biotope (FNA) presented results consistent with anthropogenic pressure, with significantly higher prevalence of ARGs encoding sulfonamide and quinolone resistance, and *sul*II as the most prevalent gene. The *mcr*-1 and bla_{TEM} prevalences, and the *mcr*-1 percentage load were significantly higher in migratory seabirds, corroborating with our second hypothesis. Nevertheless, we were not able to confirm our third hypothesis. It is not clear why masked and brown boobies presented statistically significant lower prevalence of ARGs. Our findings show that, although anthropogenic pressure is an important factor in the study of ARGs prevalence and load, other biological and ecological factors (e.g., dispersal, feeding zones and diet) should also be assessed in order to broaden our understanding of ARGs epidemiology. Additionally, our findings reinforce the involvement of seabirds in the complex One Health epidemiological chain of ARGs, highlighting the role of seabirds as environmental indicators of ARGs pollution and sentinels of anthropization, even in a pristine biotope (Miller et al., 2009; Hernandez et al., 2010). Further understanding the magnitude of gene transfer in the human-animal-environmental interface is crucial to realistically assess the ARGs-spreading potential of wildlife and consequent risk to Public Health. Mitigation measures to decrease the release of ARGs into the environment (e.g., WWTPs, wastewater) and promote a more responsible use of antimicrobials in human medicine and animal husbandry are also crucial, and should be enforced in a governmental level worldwide, especially in oceanic islands that are key seabird reproductive sites such as the ones evaluated in this study.

CRediT authorship contribution statement

Ana Carolina Ewbank: Conceptualization, Methodology, Investigation, Writing - original draft, Funding acquisition, Project administration. **Fernando Esperón Fajardo:** Conceptualization, Methodology, Resources, Writing - review & editing, Funding acquisition. **Carlos Sacristán:** Conceptualization, Methodology, Investigation, Writing - original draft, Funding acquisition. **Irene Sacristán:** Formal analysis, Writing - review & editing. **Ricardo Krul:** Investigation, Resources. **Eduardo Macedo Cavalcanti:** Investigation, Resources. **Olga Calatayud:** Methodology, Resources. **Irene Bueno:** Formal analysis, Writing - review & editing. **Ricardo de Francisco Strefezzi:** Formal analysis, Writing - review & editing, Funding acquisition. **José Luiz Catão-Dias:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary data

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4 WILD SEABIRDS CARRYING O25b-ST131-*fim*H22 HARBORING *bla*$_{\text{CTX-M-8}}$ AND A NOVEL ST11350 HARBORING *bla*$_{\text{CTX-M-55}}$ AND *fos*A3 IN A PRISTINE ATOLL, BRAZIL

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ABSTRACT

Antimicrobial resistance is among the most serious public health threats of the 21st century, with great impact in terms of One Health. Among antimicrobial resistant bacteria (ARB), extended-spectrum ß-lactamase (ESBL)-producing *Escherichia coli* (ESBL-EC) represent major challenges to human healthcare. Wild birds have been commonly used as environmental bioindicators of ESBL-EC. Remote locations represent a unique opportunity to evaluate the occurrence, dissemination and epidemiology of ARB in the environment. Herein we surveyed ESBL-EC in 204 cloacal swabs from six nonsynanthropic seabird species at the pristine Rocas Atoll, Brazil. We identified ESBL-EC isolates in 2.4% (5/204) of the tested seabirds, all in magnificent frigatebirds (*Fregata magnificens*). We isolated strains of O25b-ST131-*fim*H22 harborring *bla*$_{\text{CTX-M-8}}$ (3 clones), ST117 harboring *bla*$_{\text{SHV-12}}$, and a novel ST11350 (clonal complex 349) harboring genes *bla*$_{\text{CTX-M-55}}$ and *fos*A3. All the isolates presented Extraintestinal pathogenic *E. coli* (ExPEC) virulence profiles. We suggest that magnificent frigatebirds may act as "flying bridges", transporting ESBL-EC and ARGs from an anthropogenically-impacted environment to a pristine and remote location. The characteristics of our isolates warrant zoonotic concern and, despite the apparent good health of all the evaluated birds, be a potential risk to the avian population using the atoll. To our knowledge, this is the first description of: (1) the pandemic and public health relevant ST131-O25b harboring *bla*$_{\text{CTX-M-8}}$ worldwide; (2) ST131-*fim*H22 in wild birds; and (3); *fos*A3 in wildlife. Our findings expand the current epidemiological knowledge regarding host and geographical distribution of ESBL-EC and ARGs in wild birds, and emphasize the disseminating characteristics and adaptability of ST131 and ST117 strains within the human-animal-interface. Herein we confirm the importance of considering biological and ecological species-specific factors in the study of ARB and ARGs in wildlife and that nonsynanthropic wild birds may have a role in the epidemiology of antimicrobial resistance.

Keywords: antibiotic resistance, antimicrobial resistance, extended-spectrum cephalosporin–resistant *Escherichia coli,* remote, South America, ST117, wild birds, wildlife.

4.1 INTRODUCTION

Antimicrobial resistance (AMR) is among the most serious global clinical and public health threats of the 21st century, with great impact on the One Health interface (WORLD HEALTH ORGANIZATION, 2014; LIU et al., 2016; QUEENAN et al., 2016). The development of bacterial resistance results from bacterial genetic plasticity and interactions among microbial agents, host organisms and the environment (DA COSTA et al., 2013; FINLEY et al., 2013), and predates the existence of humans (D'COSTA et al., 2011; BHULLAR et al., 2012). Nevertheless, in recent decades, the wide use of antimicrobials in human healthcare and veterinary medicine, agriculture and food production systems (DA COSTA et al., 2013; HIDASI et al., 2013; ROCA et al., 2015), in addition to waste disposal and spillover of antimicrobials and their metabolites into the environment (GRAHAM et al., 2011; WEST et al., 2011; GUO et al., 2018), have altered bacterial ecosystem dynamics, leading to a significantly increased selective pressure (WRIGHT 2007; WORLD HEALTH ORGANIZATION, 2014). Human, animal and environmental bacteria share great portions of their resistome (FORSBERG et al. 2012; FINLEY et al. 2013; BENGTSSON-PALME et al., 2015). Anthropogenic pressure over the environment, through habitat fragmentation and pollution (e.g., landfills, wastewater treatment plants) intensifies the contact among bacterial communities of wildlife, domestic animals and humans, increasing the opportunities for transmission of antimicrobial resistant genes (ARGs) and antimicrobial resistant bacteria (ARB) (AHLSTROM et al., 2018; MARCELINO et al., 2019; SACRISTÁN et al., 2020). The resulting AMR challenge and even preclude treatment of common infections caused by resistant pathogens, and undermine and/or prevent advances in human and veterinary health and medicine, resulting in increased mortality, and social and economic burdens (BENGTSSON; GREKO 2014; WORLD HEALTH ORGANIZATION, 2014).

Escherichia coli (family Enterobacterales) are Gram-negative, non-sporulating facultative anaerobes that inhabit the intestines and faeces of warm-blooded animals and reptiles (BERG 1996; GORDON; COWLING 2003). *E. coli* can be easily disseminated into different ecosystems through water, soil, food, and other media (SKURNIK et al., 2006; RADHOUANI et al., 2009); therefore, its presence is largely used as an indicator of environmental contamination (BONNEDAHL et al., 2009; PESAPANE et al., 2013) and

anthropogenic activity (TENAILLON et al., 2010). Extended-spectrum ß-lactamase (ESBL)-producing Enterobacterales, especially *E. coli* (ESBL-EC), are a relevant public health issue and represent major human health care challenges (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL, 2011a,b; STEDT et al., 2015), being extensively used as key indicators in tracing the evolution of multidrug-resistant bacteria (MDR) in wildlife and the environment (GUENTHER et al., 2011). Although widely reported in nosocomial and community-acquired infections (RUSSO; JOHNSON 2003; NICOLAS-CHANOINE et al., 2014), livestock, animal-derived food products, and companion animals worldwide (GESER et al., 2012; EWERS et al., 2012), the occurrence, prevalence and epidemiology of ESBL-EC in wildlife and the environment is still poorly understood, especially in remote areas (ALLEN et al., 2009; HERNANDEZ et al., 2010; GUENTHER et al., 2017).

Due to their relative abundance and varied geographic range, wild birds have been commonly used as bioindicators of the environmental presence and dissemination of ESBL-EC (GUENTHER et al., 2012; HERNANDEZ et al., 2013; BONNEDAHL et al., 2015; STEDT et al., 2015; AHLSTROM et al., 2018), including in remote locations (SJÖLUND et al., 2008; HERNANDEZ et al., 2010; ARDILES-VILLEGAS et al., 2011; ATTERBY et al., 2016; HERNANDÉZ; GONZÁLEZ-ACUÑA 2016; GUENTHER et al., 2017). Nevertheless, as seen in other wildlife studies (COLE et al., 2005; FURNESS et al., 2017; KOZAK et al., 2009), investigations regarding wild birds have mostly evaluated synanthropic species and those inhabiting anthropized or agricultural areas (BONNEDAHL et al., 2009; 2014; 2015; STEDT et al., 2015; ATTERBY et al., 2016; AHLSTROM et al., 2018; 2019).

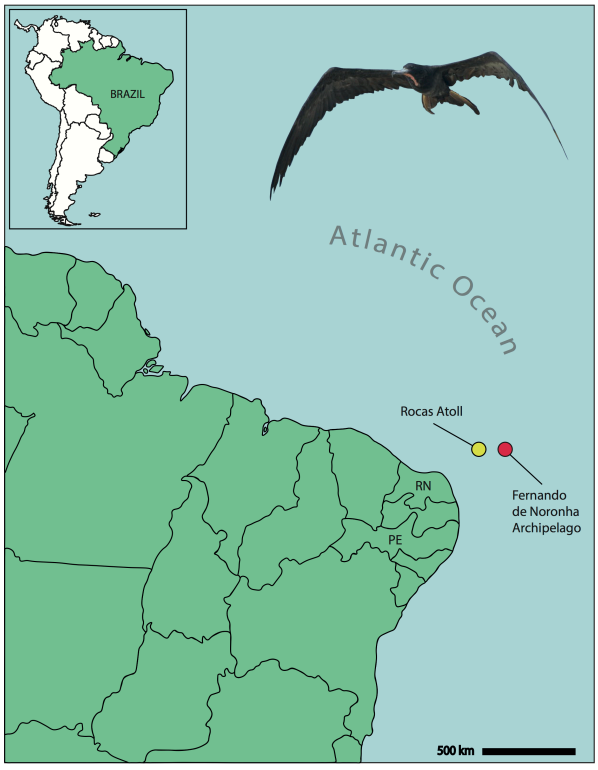
Pristine and remote habitats are under limited anthropogenic influence and direct antimicrobial exposure (e.g., sewage and wastewater discharge), thus representing a unique opportunity to evaluate the occurrence and dissemination of ARB and ARGs, their epidemiology and persistence in the environment. Considering the zoonotic potential and abundance of *E. coli* in different epidemiological settings, this study aimed on evaluating the prevalence and characteristics of ESBL-EC in cloacal swabs of 204 wild seabirds from six nonsynanthropic wild seabird species of a pristine atoll off the northeastern coast of Brazil, and discussing the potential environmental and public health consequences arising thereof. We used microbiological techniques and whole genome sequencing (WGS) to further identify and characterize the bacterial lineages, serotypes, resistome, plasmidome and virulome of the ESBL-EC present in the evaluated microbiomes.

4.2 MATERIALS AND METHODS

4.2.1 Study area

The Rocas Atoll – ROA (03º51'S 33º48'W) - the only atoll of the Southern Atlantic Ocean, is located at 267 km from Rio Grande do Norte state, northeastern Brazil (Fig. 1). It is part of the Rocas Atoll Biological Reserve (ReBio), under the jurisdiction of the Chico Mendes Institute for Biodiversity Conservation (ICMBio) - Brazilian Ministry of the Environment (FISCHER et al., 2007; JALES et al., 2015). Access to ROA is only permitted for research purposes, limited to a maximum of four researchers and one ICMBio analyst per expedition, which usually varies between 30 to 45 days (at a maximum yearly visiting rate of approximately 60 visitors). Food, potable water, equipment and other necessities are brought in from the continent with each new expedition group. Additionally, after every expedition, all generated organic waste is transported to the continent, where it is discarted. ROA is classified as a World Heritage Site by UNESCO (WORLD HERITAGE CENTER, 2001) and is one of the most important seabird breeding sites in Brazil (ANTAS, 1991), also used by migratory and vagrant seabird species.

Figure 1. Geographical location of Rocas Atoll (Rio Grande do Norte state – RN [yellow dot]) and Fernando de Noronha Archipelago (Pernambuco state – PE [red dot]), northeastern Brazilian coast. Brazil (green), South America (upper left). Scale: 500 km.



4.2.2 Sampling and bacterial identification

We sampled a total of 204 seabirds, comprising six species, as part of a surveillance study: sooty terns (*Onychoprion fuscatus*, n = 36), magnificent frigatebirds (*Fregata magnificens*, n = 35), brown noddies (*Anous stolidus*, n = 34), masked boobies (*Sula dactylatra*, n = 33), brown boobies (*Sula leucogaster*, n = 33), and red-footed boobies (*Sula sula*, n = 33). All samples were collected between December 2018 and January 2019. All birds were captured with a butterfly net and manually restrained, being immediately released after sampling. Banding numbers were recorded when present. Cloacal swabs were maintained in Amies transport medium with charcoal, at room temperature, until processed. In order to detect ESBL-EC, cloacal samples were streaked onto ceftriaxone (CRO)-supplemented MacConkey agar plates (CRO: 2 mg/L), and incubated overnight at 35 ± 2 °C. Bacterial isolates were identified by MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry, Bruker Daltonik, Leipzig, Germany). Clonal relatedness among samples of interest was identified by ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus - PCR) (DA SILVEIRA et al., 2002).

All samples were collected in full compliance and approved by the Biodiversity Information and Authorization System (SISBIO 59150-4), Brazilian Ministry of Environment. All procedures were performed in accordance with the Ethical Committee in Animal Research of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (Process Process number 1753110716).

4.2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by the disc diffusion method using human and veterinary antibiotics (CLSI, 2018, 2019), including amoxicillin/clavulanate, ampicillin, aztreonam, ceftriaxone, cefotaxime, ceftiofur, ceftazidime, cefepime, cefoxitin, cephalexin, cephalotin, imipenem, meropenem, ertapenem, nalidixic acid, ciprofloxacin, gentamicin, amikacin, chloramphenicol, fosfomycin, trimethoprim-sulfamethoxazole, and tetracycline. ESBL production was screened using the double-disc synergy test (DDST) (JARLIER et al., 1988).

4.2.4 Whole genome sequence analysis

Genomic DNA was extracted from the selected ESBL-EC strains using a PureLinkTM Quick Gel Extraction Kit (Life Technologies, Carlsbad, CA, USA), followed by the preparation of a genomic paired-end library (75 x 2 bp) using the Nextera DNA Flex library Preparation Kit (Illumina Inc., San Diego, CA), according to the manufacturer's instructions. The whole genome was sequenced on the NextSeq platform (Illumina). *De novo* genome assembly and contig annotation were carried out using CLC Genomics Workbench 12.0.3. The MLST 2.0, PlasmidFinder 2.1, ResFinder 4.1, VirulenceFinder 2.0 and SerotypeFinder 2.0 databases available from the Centre for Genomic Epidemiology (http://genomicepidemiology.org/) were used to identify, respectively, the multilocus sequence type (MLST), plasmid replicons, resistome, virulome and serotype. ESBL genes were screened by polymerase chain reaction (PCR) in the ESBL-EC isolate not selected for WGS (DROPA, et al., 2016).

Minimum spanning trees based on the wgMLST scheme and MSTree V2 tool from Enterobase (http://enterobase.warwick.ac.uk/species/index/ecoli) including the *E. coli* isolates found in this study and international collections of 198 and 138 *E. coli* strains belonging to, respectively, ST131 and ST117, were ellaborated. The phylogenetic trees were generated with iTOL v.6 (https://itol.embl.de), and their interactive versions are available at: https://itol.embl.de/tree/191197714816903161785032 4 (ST131) and https://itol.embl.de/tree/177102823915901619127015 (ST117).

4.3 RESULTS

4.3.1 Bacterial isolation, antimicrobial resistance profile and clonal relatedness

Overall, ESBL-EC were identified in 2.4% (5/204) of the evaluated individuals (one isolate per individual: FM17, FM18, FM21, FM25 and FM32), and only in magnificent frigatebirds (14.3%, 5/35 of the individuals). Among the five isolates, antimicrobial resistance against broad-spectrum β-lactams, tetracycline, sulphonamide, trimethoprim, and fosfomycin were detected (Table 1). ERIC-PCR identified that FM17, FM21 and FM32 strains were clonally related. Isolates FM18, FM21, FM25, and FM32 were selected for WGS analysis. Isolate FM17 was not sequenced because it showed identical antimicrobial resistance profile and was clonally related to FM32 strain.

Table 1. Phenotypical characteristics of the ESBL-producing *Escherichia coli* isolates identified in this study

Species	Bacterial strain	Antimicrobial resistance profile [a]
Magnificent frigatebird (*Fregata magnificens*)	*Escherichia coli* FM17	Amp[1], Cfe[1], Cfl[1], Cpm[1], Ctf [1], Ctx[1], Cro[1], Tet[1]
	Escherichia coli FM18	Amp[1], Atm[1], Caz[1], Cfe[1], Cfo[2], Cfl[1], Cpm[1], Ctx[1], Cro[1], Ctf[l], Sut[1], Tet[1]
	Escherichia coli FM21	Amc[2], Amp[1], Cfe[1], Cfl[1], Cpm[1], Ctx[1], Cro[1], Ctf [1], Tet[1]
	Escherichia coli FM 25	Amp[1], Atm[1], Caz[2], Cfe[1], Cfl[1], Cfo[2], Cpm[1], Ctx[1], Cro[1], Ctf[l], Fos[1]
	Escherichia coli FM 32	Amp[1], Cfe[1], Cfl[1], Cpm[1], Ctf [1], Ctx[1], Cro[1], Tet[1]

[a]Amc: amoxicilin/clavulanate, Amp: ampicillin, Atm: aztreonam, Caz: ceftazidime, Cfe: cephalexin, Cfl: Cephalotin; Cfo: Cefoxitin, Cpm: cefepime, Ctf: ceftiofur, Ctx: cefotaxime; Cro: Ceftriaxona; Fos: fosfomycin, Tet: tetracycline; Sut, trimethoprim-sulfamethoxazole
[1]Resistant, [2] Intermediate.

4.3.2 WGS analysis, antibiotic resistome, serotype prediction and MLST

WGS showed that FM21 and FM32 isolates harbored genes $bla_{\text{CTX-M-8}}$ and *tet*(B) in their resistome. Isolate FM17 was positive for $bla_{\text{CTX-M-8}}$ gene by conventional PCR. FM18 harbored genes $bla_{\text{SHV-12}}$, *tet*(A), *sul*I, *sul*II, while FM25 carried genes $bla_{\text{CTX-M-55,}}$ bla_{TEM}, and *fos*A3. Multilocus sequence typing (MLST) analysis revealed that FM21 and FM32 corresponded to ST131 clones (serotype O25:H4), FM18 was identified as ST117 (serotype H51:O18), and FM25 belonged to a novel ST11350 clone (clonal complex ST349), serotype O166:H15 (Tables 2 and 3).

4.3.3 Virulome and plasmidome

Several virulence genes (VGs) of concern, characteristic of ExPEC, were identified in this study: *cva*C (microcin C), *fim*H (type I fimbriae), *fyu*A (siderophore receptor), *hly*F (hemolysin F), *ibe*ABC (invasin of brain endothelial cells), *ire*A (siderophore receptor), *iro*N (enterobactin siderophore receptor protein), *iss* (increased serum survival), *kps*E (capsule polysaccharide export inner-membrane protein), *kps*MII (polysialic acid transport protein),

*mch*F (ABC transporter protein), *neu*C (polysialic acid capsule biosynthesis protein), *omp*T (outer membrane protein), *pic* (serine protease autotransporters of Enterobacteriaceae (SPATE)), *trat*T (serum resistance associated), *tsh* (temperature-sensitive hemagglutinin), *usp* (uropathogen-specific protein), and *vat* (vacuolating autotransporter toxin). The IncF plasmid incompatibility (Inc) group was the most frequently identified in this study, followed by IncI, IncN and IncQ1. All findings, according with isolates, are shown in Table 3.

4.3.4 Phylogenetic analysis

Phylogenetically, samples FM21 and FM32 (ST131) clustered with isolates from humans (isolated in Argentina, Canada and Spain), poultry (isolated in the United States and Europe [Denmark, Germany, Norway, Spain and Sweden]), animal-derived food products (in the US) and wildlife (wild turkey [*Meleagris gallopavo*]) (Fig. 2). FM18 (ST117) clustered with samples isolated from human (in Denmark), food (ground turkey in the US), livestock (pig in Ireland, Spain, and Hungary), companion animal (dog in the US), and wildlife (European herring gull [*Larus argentatus*] in the US and mew gull [*Larus canus*] in the United Kingdom, and grey-headed flying fox [*Pteropus poliocephalus*] in Australia) (Fig. 3).

Table 2. Resistome, plasmidome, virulome and MLST analysis of ESBL-producing *Escherichia coli* colonizing magnificent frigatebirds (*Fregata magnificens*) in Rocas Atoll, northeastern Brazil

Isolate	MLST [a]	Serotype	Antimicrobial resistance genes [b]	Plasmid Inc. groups	Virulome [d]
FM18	ST117	O51:H18	*bla*$_{SHV-12}$ [1] *tet*(A) [2] *sul*I [3] *sul*II [3] *aad*A2b* *aad*A1* *aph*(6)-Id* *aph*(3")-Ib* *cat*A1* *dfr*A1* *Inu*(F)* *mdf*(A)* *mph*(B)*	IncFIB IncFIC(FII) IncQ1	*chu*A *fim*H97 *gad* *hra* *lpf*A *omp*T *pic* *sitA* *ter*C, *tra*T *vat*
FM21	ST131	O25:H4	*bla*$_{CTX-M-8}$ [1] *tet*(B) [2] *mdf*(A)*	Incl1-I IncFIB IncFII	*bcp*ABC *chu*ASTUWXY, *cia*, *cva*C *eae*H, *ecp*ABCDER, *eha*B, *esp*C, *ets*C *fig*C, *fim*ABCDEFGHI, *fim*H22, *fyu*A *hly*E/*cly*A, *hly*F, *hra* *ibe*ABC,*ire*A, *iro*BCDEN, *irp*1, 2, *iss* *Kps*E, *kps*MII_K1 *mch*F *neu*C *omp*T *sit*ABCD

Isolate	MLST [a]	Serotype	Antimicrobial resistance genes [b]	Plasmid Inc. groups	Virulome [d]
					*ter*C, *tia, tra*T, *tsh* *upa*G/*eha*G, *usp* *ybt*AEPQSTUX, *yfc*V
FM25A	Novel ST11350 (clonal complex ST349)	O166:H15	$bla_{\text{CTX-M-55}}$ [1]	IncFIB	*aat*A
			bla_{TEM} [1,‡]	IncFII	*cah, chu*ASTUW
			*fof*A3 [4]	IncN	*eae*h, *eha*B, *eil*A, *esp*L1, 4, *esp*R1, *esp*X1,2,4,5,6, *esp*Y1,3,4,5
			mdf(A)*		*fim*H93 *gal*E *hcp*BC, *hly*E/*cly*A, *hly*F *ibe*BC, *iss* *kps*E, *kps*MII_K5 *omp*T *sit*ABCD *ter*C, *tra*T
FM32	ST131	O25:H4	$bla_{\text{CTX-M-8}}$ [1]	IncFIB	*chu*ASTUWXY, *cia, cva*C
			tet(B) [2]	IncFII	*eae*H, *ecp*ABCDE, *eha*B, *esp*C, *ets*C
			mdf(A)*	IncI1-I	*fig*C, *fim*ABCDEFGHI, *fim*H22, *fyu*A *hcp*ABC, *hly*E/*cly*A, *hly*F, *hra* *ibe*ABC, *ire*A, *iro*BCDEN, *irp*1,2, *iss* kps*E*, kps*MII_K1* mch*F* neu*C* ompT *pil*QRSV

Isolate	MLST [a]	Serotype	Antimicrobial resistance genes [b]	Plasmid Inc. groups	Virulome [d]
					*sit*A *ter*C, *tia, tra*T, *tsh* *upa*G/*eha*G, *usp* *ybt*AEPQSTUX

[a] MLST, Multilocus sequence typing. Novel ST (adk 34, fumC 36, gyrB 39, icd 87, mdh 67, purA 16, recA 215).

[b] Genes conferring resistance to antimicrobials: 1, cephalosporins; 2, tetracyclines; 3, fosfomycin; 4, sulfonamides.

[c] Virulence factor genes: *aat*A (autotransporter adhesin AIDA-I type); *bcp*ABC (bacterioferritin comigratory protein); *cah* (calcium-binding antigen 43 homologue); *chu*ASTUWXY(outer membrane hemin receptor), *cia* (colicin ia); *cva*C (microcin C); *eae*H (putative attaching and effacing protein homolog); *ecp*ABCDE (*E. coli* common pillus); *eha*B EHEC autotransporter B; eilA (*Salmonella* HilA homolog); etsC (putative type I secretion outer membrane protein); *esp*C *(E. coli secreted protein C); esp*L1, R1, X1 , X4, X6, Y1, Y2, Y4 (type III system-secreted proteins); *fig*C (lateral flagellae); *fim*ABCDEFGHI (type I fimbriae); fyuA (siderophore receptor); *gal*E (UDP-glucose 4-epimerase); *hcp*ABC (hemolysin co-regulated protein); *hly*E/*cly*A (hemolysin/cytolysin E); *hly*F (hemolysin F); *hra* (heat-resistant agglutinin); *ibe*ABC (invasin of brain endothelial cells); *ire*A (siderophore receptor); *iro*BCDEN(enterobactin siderophore receptor protein); irp1,2 (high molecular weight protein 2 non-ribosomal peptide synthetase); iss (increased serum survival); *lpf*A (long polar fimbriae); *kps*E (capsule polysaccharide export inner-membrane protein); *kps*MII (polysialic acid transport protein); *mch*F (ABC transporter protein MchF); *neu*C (polysialic acid capsule biosynthesis protein); *omp*T (outer membrane protease (protein protease 7)); *pic* (serine protease autotransporters of Enterobacteriaceae (SPATE)); *pil*QRSV (type IV pili); *sit*ABCD (Iron transport protein); *ter*C (tellurium ion resistance protein); *tia* (adhesins); *tra*T (outer membrane protein complement resistance); *tsh* (temperature-sensitive hemagglutinin); *usp* (uropathogenic specific protein); *upa*G/*eha*G (UpaG adhesin, trimeric AT); *vat* (vacuolating autotransporter toxin); *ybt*AEPQSTUX (siderophore yersiniabactin); *yfc*V (fimbrial protein).

[‡] Unfortunately, the obtained sequences were not long enough to accurately deferentiate among genes $bla_{\text{TEM-1B}}$, $bla_{\text{TEM-141}}$, $bla_{\text{TEM-206}}$ and $bla_{\text{TEM-214}}$.

***** Unactive genes found in the whole genome sequencing (WGS).

4.3.5 Banding recovery

Banding information was recovered from seven individuals, including one of the five in which ESBL-EC was isolated (FM18; Table 3) (personal communication, CEMAVE/ICMBio, 2020)[1].

Table 3. Banding information available for the individuals evaluated in this study, according with band number, individual ID#, species, banding site (ROA = Rocas Atoll; FNA = Fernando de Noronha Archipelago), coordinates (latitude and longitude), year of banding, age (AD = Adult; JUV = Juvenile), and sex (M = Male; U = Undetermined)

Band Number	Individual ID#	Species	Banding Site	Coordinates		Year of Banding	Age	Sex
				Latitude	Longitude			
J-85514	OF3	sooty tern	ROA	03º 51’ 50.00” S	033º 48’ 48.00” W	Jun 2017	AD	U
J-85552	OF15	sooty tern	ROA	03º 51’ 50.00” S	033º 48’ 48.00” W	Jun 2017	AD	U
J-85508	VM6	brown noddy	ROA	03º 51’ 50.00” S	033º 48’ 48.00” W	Jun 2017	AD	U
J-85507	VM11	brown noddy	ROA	03º 51’ 50.00” S	033º 48’ 48.00” W	Jun 2017	AD	U
L-144014	VM15	brown noddy	ROA	03º 51’ 50.00” S	033º 48’ 48.00” W	Jun 2017	AD	U
U-56034	FM18	magnificent frigatebird	FNA	03º 50’ 30.00” S	032º 25’ 06.00” W	Nov 2016	JUV	U
U-63169	FM33	magnificent frigatebird	ROA	03º 51’ 50.00” S	033º 48’ 48.00” W	Jul 2017	AD	M

[1] Information provided by Centro Nacional de Pesquisas Para Conservação das Aves Silvestres (CEMAVE) – Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), on July 21st 2020.

4.4 DISCUSSION

Overall, five ESBL-EC (prevalence of 2.45%; 5/204) were isolated in magnificent frigatebirds: three clones of the globally disseminated O25b-ST131 harboring *bla*$_{\text{CTX-M-8}}$, a ST117 clone harboring *bla*$_{\text{SHV-12,}}$ and a novel ST11350 (ST349 clonal complex) harboring *bla*$_{\text{CTX-M-55}}$ and *fos*A3.

The emerging pandemic ST131 clone is a public health threat implicated in multidrug-resistant extraintestinal infections worldwide (EWERS et al., 2010; LAVIGNE et al., 2012; TAUSOVA et al., 2012; BLANCO et al., 2013; DAHBI et al., 2014; NICOLAS-CHANOINE et al., 2014), frequently described in community- and hospital-acquired urinary tract infections and bacteremia (RUSSO; JOHNSON 2003; NICOLAS-CHANOINE et al., 2014), companion animals, food products, and the environment (EWERS et al., 2010; DOLEJSKA et al., 2011; HU et al., 2013; KAWAMURA et al., 2014). The globally disseminated O25b:H4-B2-ST131 clonal group is of particular concern, combining resistance and multiple virulence factors (BLANCO et al., 2013). ST131 is the most commonly identified clone in wild animals (WANG et al., 2017). In wild birds, especifically, this clone has been described in South America (HERNÁNDEZ et al., 2010; SAVIOLLI et al., 2016), Europe (TAUSOVA et al., 2012; JAMBOROVA et al., 2015; 2017), Eurasia (HERNANDÉZ et al., 2010), Asia (HASAN et al., 2014; 2015), and Oceania (MUKERJI et al., 2019). To the authors'knowledge, despite its numerous reports, this is the first description of a 25b-ST131 clone harboring *bla*$_{\text{CTX-M-8}}$. Interestingly, CTX-M-8 is considered one of the most prevalent CTX-M enzymes in Brazil, where it was first described in 2000 (BONNET et al., 2000; Rocha et al., 2016). Since then, CTX-M-8 has been extensively identified in the country, in several epidemiological settings within the human-animal-environmental interface (MINARINI et al., 2009; PEIRANO et al., 2011; FERREIRA et al., 2014; 2016; CASELLA et al., 2015; MELO et al., 2018; SILVA et al., 2018; DROPA et al., 2016; SACRAMENTO et al., 2018). Nevertheless, this is the first detection of *bla*$_{\text{CTX-M-8}}$ in wild birds in Brazil, previously described in owls (Magellanic horned owl (*Bubo magellanicus*) and rufous-legged owl (*Strix rufipes*)) in Chile (FUENTES-CASTILLO et al., 2019), and rooks (*Corvus frugilegus*) (JAMBOROVA et al. 2015) and yellow-legged gulls (*Larus michaellis*) in Europe (STEDT et al., 2015).

The novel ST11350 (ST349 clonal complex) harbored genes *bla*$_{\text{CTX-M-55,}}$ *fos*A3*,* and *bla*$_{\text{TEM}}$. CTX-M-55 is mostly restricted to Asia, where it is the second most common ESBL enzyme described in Enterobacterales (LUPO et al., 2018; JONES et al., 2008; XIA et al.,

2014), and has recently emerged as a dominant genotype in Chinese hospitals (ZHANG et al., 2014). As expected, *bla*$_{\text{CTX-M-55}}$ has been described in *E. coli* isolates from several wild bird species in Asia (GUENTHER et al., 2012; HASAN et al., 2015), but also in Europe (JAMBOROVA et al., 2015; STEDT et al., 2015), the Americas (BONNEDAHL et al., 2015; AHLSTROM et al., 2019; DE CARVALHO et al., 2020), and Oceania (MUKERJI et al., 2019). Similarly, *fos*A3 - the most disseminated among fosfomycin-modifying enzymes (*fos*), is also endemic and widespread in Asia (REHMAN et al., 2017; YANG et al., 2017, ZURFHLU et al., 2019). Fosfomycin resistance is particularly concerning because due to its broad spectrum and limited use in recent decades, fosfomycin has become a great therapeutic choice against multidrug-resistant bacteria, especially ESBL- and carbapenemase-producing Enterobacteriaceae (POPOVIC et al., 2010; RAZ, 2012; SASTRY; DOI, 2017; ZURFHLU et al., 2019). For instance, fosfomycin is broadly used in the treatment of uncomplicated urinary infections worldwide (MICHALOPOULOS; LIVADITIS; GOUGOUTAS, 2011; RAZ, 2012; DIJKMANS et al., 2017; Zurfhlu et al., 2019), including in Brazil, where it is also largely used as therapy for multidrug-resistant nosocomial infections (PERDIGÃO-NETO et al., 2014). In contrast, although not approved for use in veterinary medicine in the majority of countries (e.g., China and Europe) (WANG et al., 2017), fosfomycin is widely used in Argentina, Brazil, and Central America, especially for the treatment of infectious diseases in broilers and pigs (PÉREZ et al., 2014). The *fos*A3 gene is commonly associated with *bla*$_{\text{CTX-M}}$ (as seen here) and *rmt*B genes, especially in enterobacteria isolated from animals of southern Asia, but also in humans (YANG et al., 2017). Interestingly, associations of *bla*$_{\text{CTX-M-55}}$ and *fos*A3 genes have been reported in Brazil: in poultry (Cunha et al., 2017) and in an assimptomatic person (FERNANDES et al., 2018). Nevertheless, to the authors'knowledge, this is the first description of *fos*A3 and of its association with the *bla*$_{\text{CTX-M-55}}$ in wild birds worldwide. In regard to *bla*$_{\text{TEM}}$, the obtained sequence was unfortunatelly not long enough to accurately defferentiate among genes *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{TEM-141}}$, *bla*$_{\text{TEM-206}}$ and *bla*$_{\text{TEM-214}}$.

The ST117 clone is an emerging pathogen (Masella et al., 2021), described in community and hospital-acquired infections, companion animals, and livestock - where it is of special concern in poultry (Maluta et al., 2014; Masella et al., 2021; Cunha et al., 2017). In wild birds, ST117 has been reported in Europe (corvids [JAMBOROVA et al., 2015] and raptors [GUENTHER et al., 2012]) and in the Americas (corvids [JAMBOROVA et al., 2017] and gulls [LIAKOPOULOS et al., 2016]). Of note, among several other resistance genes, our isolate harbored *bla*$_{\text{SHV-12}}$, a gene extensively described in numerous epidemiological settings worlwide (DOLEJSKA et al., 2011; ALVES et al., 2014; OJER-USOZ et al., 2017;

MAMANI et al., 2019), and in several wild bird groups (COSTA et al., 2006; LITERAK et al., 2010; JAMBOROVA et al., 2015; ALCALÁ et al., 2016), including seabirds (gulls [DOLEJSKA et al., 2009; HERNANDEZ et al., 2013]).

All the isolates described in this study presented a variety of virulence genes of concern characteristic of Extraintestinal pathogenic *E. coli* (ExPEC) (Table 2) (MELLATA et al., 2013; SAROWSKA et al., 2019). ExPEC are capable of causing a variety of diseases in humans and animals, with consequent economic and social health burdens worldwide (RILEY 2004; KEMMETT et al., 2014). ExPEC are subdivided into avian pathogenic (APEC), neonatal meningitis (NMEC), sepsis-associated (SEPEC) and uropathogenic *E. coli* (UPEC) pathotypes that share many features, including virulence-associated genes and serotypes (KAPER et al., 2004; MELLATA et al., 2013; SAROWSKA et al., 2019). Strains ST131 (FM21 and FM32) and the novel ST11350 (FM25), presented highly virulent genes found in APEC, and to a lesser extent, in UPEC and NMEC *E. coli* (MELLATA et al., 2013; SAROWSKA et al., 2019). The ST131 isolated here presented virulent profiles very similar to previously described ST131-*fim*H22 (REID et al., 2019; DÍAZ-JIMÉNEZ et al., 2020; SAIDENBERG et al., 2020; LOPES et al., 2021). This potential foodborne uropathogen (LIU et al., 2018; REID et al., 2019; SAIDENBERG et al., 2020) sustains zoonotic transmission and although mainly described in livestock (GARCÍA-MENIÑO et al., 2018; SAIDENBERG et al., 2020) and associated meat products (DÍAZ-JIMÉNEZ et al., 2020), has recently been suggested as a pathogen capable of transmission through the human-animal-environmental interface, due to recent reports in wildlife (pinnipeds in Antartica) (MORA et al., 2018) and soil (Brazil) (LOPES et al., 2021). Our findings emphasize such potential and, to the authors' knowledge, are the first description of the ST131-*fim*H22 sublineage in wild birds worldwide. Finally, although our ST117 isolate presented a serotype (O51) different from those commonly considered potentially pathogenic to humans and poultry (O1, O2, O18, O25b, O78, O111) (MORA et al., 2012; MOULIN-SCHOULEUR et al., 2007), its virulence genotype is commonly shared between APEC and UPEC strains (i.e., *pic*, *vat*, *lpf*A, *tra*T) (MALUTA et al., 2014; FERNANDES et al., 2018; SAROWSKA et al., 2019). APEC and ExPEC strains causing infections in humans can be closely phylogenetically related (e.g., ST131, ST117), with the former being suggested as a reservoir of virulence genes to ExPEC, and therefore, a potential health risk to humans (RODRIGUEZ-SIEK et al., 2005; BÉLANGER et al., 2011; MANGES 2016). In birds, APEC may cause multiple systemic infections refered to as colibacillosis, that in poultry, leads to significant economic losses worldwide (MELLATA et al., 2013; KEMMETT et al., 2014; SAIDENBERG; KNÖBL

2005). Thus, in spite of the apparently good health condition of the magnificent frigatebirds individuals carrying these isolates, our findings warrant concerns in terms of zoonotic risk, and to a lesser extent, potential health challenges to the local avian population using the Atoll.

Phylogenetically, ST131 and ST117 clustered with isolates from a variety of epidemiological settings and geographic locations, in accordance with their pandemic profiles (REF). Of note, ST117 (FM18) grouped closer with strains from migratory and opportunistic (e.g., scavenging in human subsidies) gull species in Europe and the United States (mew and European herring gulls, respectively) (BIRDLIFE INTERNATIONAL, 2020), previously reported to carry ARGs and ARB (WALLENSTEN et al., 2011; STEDT et al., 2015; ATTERBY et al, 2016; 2017). In terms of geographical distribution, both species overlap in northern Europe; mew gulls are distributed throughout most of the Northern Hemisphere (North America, Asia and Northern Europe) and European herring gulls also occur in southwestern Europe (BIRDLIFE INTERNATIONAL, 2020). Nevertheless, none of the seabird species evaluated herein share their territories with these gull species (REF).

Figure 2. Phylogeny of CTX-M-8-producing *E. coli* isolate (FM21 and FM32) from magnificent frigatebirds (*Fregata magnificens*), in comparison with an international *E. coli* collection, regarding country and source of isolation (colored circles). Isolates clustering with our samples are seen in the blue clade. Tree scale: 1000

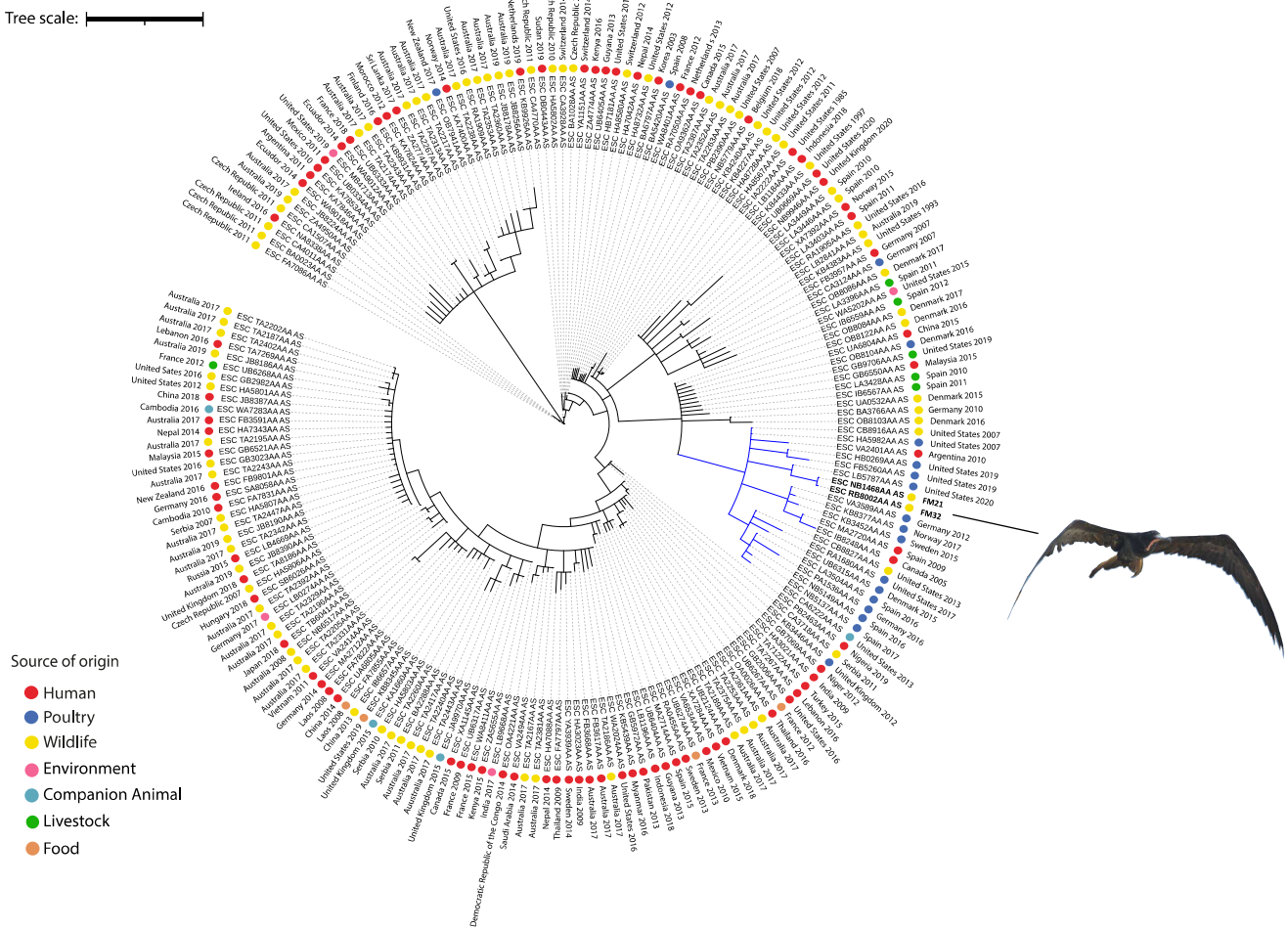
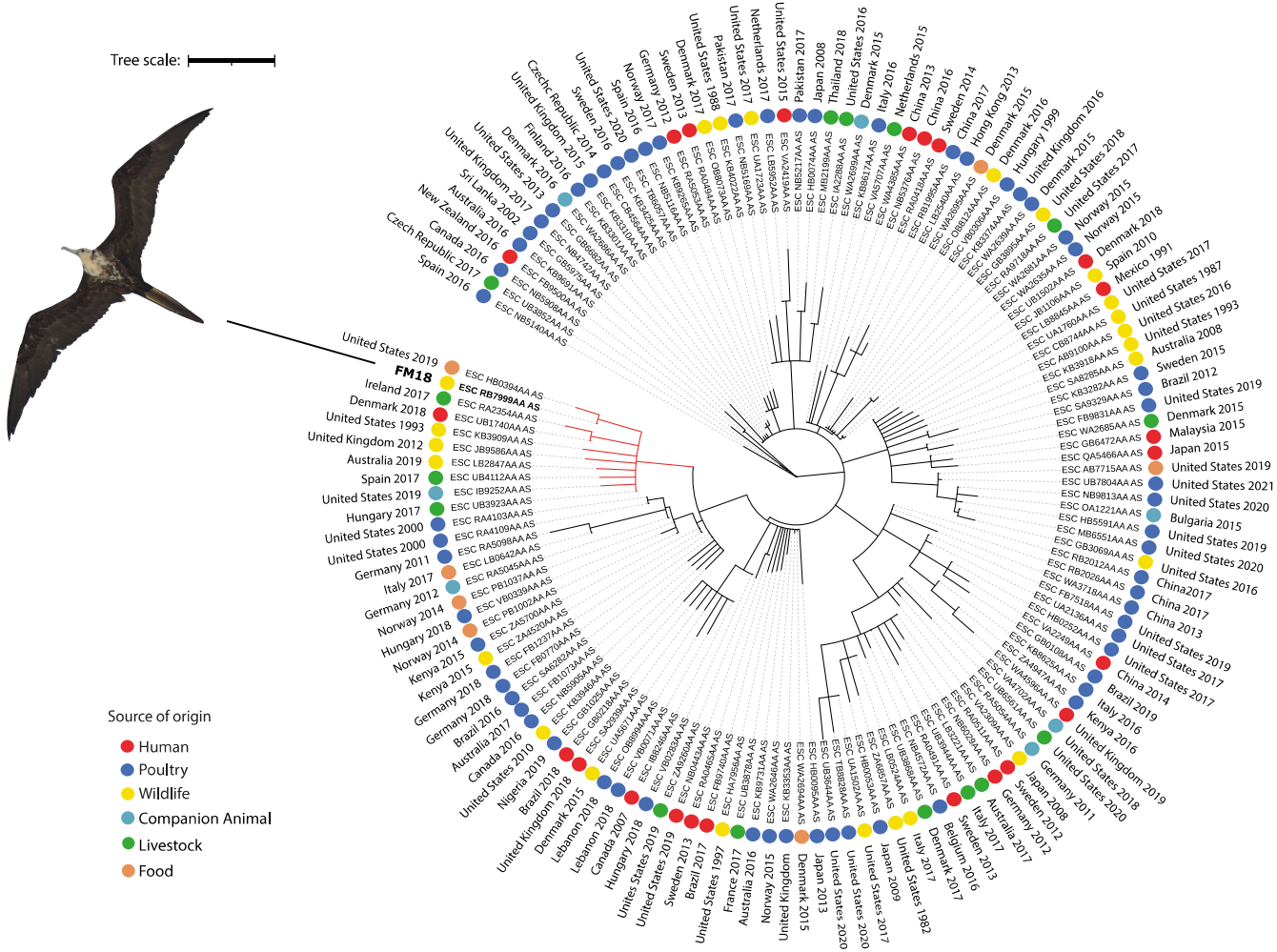


Figure 3. Phylogeny of CTX-M-8-producing *E. coli* isolate (FM18) from magnificent frigatebirds (*Fregata magnificens*), in comparison with an international *E. coli* collection, regarding country and source of isolation (colored circles). Isolates clustering with our samples are seen in the red clade. Tree scale: 1000



All our ESBL-EC isolates were detected in magnificent frigatebirds (order Pelicaniformes, family Fregatidae). This seabird species is highly colonial, nonmigratory and nonsynathropic, and sustains vast foraging territories (WEIMERSKIRCH et al., 2001; 2003; BIRDLIFE INTERNATIONAL, 2020), feeding in surface prey but also relying on kleptoparasitism and fishing interaction (NELSON, 1976; CORRE; JOUVENTIN, 1997; ZALUSKI et al., 2019). This species does not breed in ROA, using the atoll only for overnight roosting (ANTAS, 1991). The magnificent frigatebirds sampled in this study most likely came from the large year-round breeding colony of Sela Gineta Island, in Fernando de Noronha Archipelago (FNA) (03º45'S to 03º57'S and 32º19'W to 32º41'W), located at 360 km off the Brazilian coast and 148 km from ROA. Such suggestion is reinforced by the banding information recovered from two specimens, including FM18, which carried a ST117 isolate harboring *bla*$_{\text{SHV-12}}$ (Table 3). Due to its geological formation, Sela Gineta has never been inhabited or opened to tourist visitation; however, it is less than one kilometer from Fernando de Noronha Island, the only human settlement in the archipelago. This island houses the 3101 permanent FNA residents (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2021) and all tourists, estimated at 100,000 visitors in the study year alone (personal communication, Parque Nacional Marinho, ICMBio, 2020).

International travel has been shown to contribute to the worldwide dissemination of ESBL-EC (BENGTSSON-PALME et al., 2015; ARCILLA et al., 2017). Additionally, some of the ESBL-EC strains described herein (i.e., ST131 and ST117), have been previously reported in the mainland (CAMPOS et al., 2018; FERNANDES et al., 2018). Thus, we believe that national and international tourism may have played a role in the acquisition of these strains. The disproportionate and intense tourism causes serious infrastructural and basic sanitation problems to FNA, such as direct release of untreated sewage, and deficient wastewater treatment and release of wastewater into the sea (ANDRADE et al., 2007; DA COSTA CRISTIANO et al., 2020). Once in the aquatic environment, ARGs and ARB may disseminate further, eventually to humans (ZHANG et al, 2009) and domestic and wild animals (TAUSOVA et al., 2012; BRAHMI et al., 2015), including wild birds (DOLEJSKA et al., 2009; ATTERBY et al., 2016; 2017; MARCELINO et al., 2019) and their prey (fish and seafood) (BRAHMI et al., 2015; SELLERA et al., 2018). We hypothesize that the magnificent frigatebirds carrying ESBL-EC likely acquired these isolates in FNA, as a consequence of one or more of the following factors: the high year-round influx of tourists, inadequate wastewater management system of the archipelago and influx of raw sewage and wastewater treatment plant effluents into the aquatic environment, and ingestion of

contaminated food items (fish and seafood). Thus, magnificent frigatebirds could have acted as a "flying bridge", transporting these isolates from an intensely anthropized environment (FNA) to a pristine one (ROA), as previously suggested in other wild bird species using isolated biomes (SJÖLUND et al., 2008; HERNÁNDEZ et al., 2010; 2016). Such hypothesis is especially concerning considering the combined use of FNA and ROA by several seabird species, as shown in our banding records (Table 3) and previous literature (ANTAS, 1991).

Alternatively, the research base and the presence of researchers in ROA, albeit strictly controlled to minimize potential environmental impact (e.g., minimal number of researchers per expedition, short-term expeditions, transport of organic waste back to the continent), could have served as potential sources for the ESBL-EC strains identified in this study, as previously suggested in other remote locations (HERNÁNDEZ et al., 2016; MARCELINO et al., 2019). Nevertheless, in that case, one would expect to also find ESBL-EC isolates in resident species (masked and brown boobies), which breed and spend most of their life cycles in the atoll (SCHULZ-NETO, 1998). A recent direct molecular detection study in the microbiome of the same ROA individuals evaluated herein and seabird species from FNA (i.e., masked and brown boobies, and magnificent frigatebirds), found that masked and brown boobies presented significant lower prevalence of ARGs encoding resistance to beta-lactams (*bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX}}$), and statistically significant lower *bla*$_{\text{TEM}}$ prevalence (EWBANK et al., 2020). Interestingly though, *bla*$_{\text{TEM}}$ prevalence was statistically significant higher in sooty terns and brown noddies (EWBANK et al., 2020). ROA is an intensely populated seabird breeding site (ANTAS, 1991; SCHULZ-NETO, 2004) - the main one for brown noddies in Brazil, and home of the largest sooty tern population in the Southern Atlantic (DEL HOYO et al., 1996; HIGGINS; DAVIES, 1996; FISCHER et al., 2007). Based on our banding records (Table 3), individuals of both species do return to ROA and, most importantly, are highly migratory and hypothetical transporters of AMR with far greater potential than non-migratory species such as magnificent frigatebirds (BONNEDAHL et al., 2009; ATTERBY et al., 2017; AHLSTROM et al., 2018; EWBANK et al., 2020), possibly serving as local sources of ARB. Finally, the direct intra and interspecies contact (i.e., colonial behavior and kleptoparasitism) and broad foraging territories sustained by magnificent frigatebirds could have potentially favored the spread of the isolates found in this study. This hypothesis is especially supported by the isolation of the O25b-ST131 clones, which possibly originated from a common source and were circulating within the magnificent frigatebird colony. Of note, this species sustains mixed colonies in FNA and ROA with other seabirds analyzed here: red-footed and brown boobies (MANCINI et al., 2016). Particularly in the atoll, such interspecific contact is

especially close, due to kleptoparasitism, and competion over and sharing of the very limited number of perching sites for overnight roosting (A. C. Ewbank, personal observation; Fig. 4). Nevertheless, ESBL-EC were found solely in magnificent frigatebirds and not in these contacting species. The cause(s) behind all the above-mentioned factors remain(s) to be determined and warrant further studies, but may be related to differences in host taxonomy, age, diet and feeding ecology, variety of foraging sites, gut morphology, gastrointestinal environment (which affect the structure of *E. coli* populations), microbiota and microbiome (ESCOBAR-PARAMO et al., 2006; NELSON et al., 2008; GODOY-VITORINO et al., 2010; FUIRST et al., 2018; MARCELINO et al., 2019). Additionally, one must also consider species-specific differences in exposure to potential sources and differences in strains with varying capabilities to persist in the source environments and in each species gut (NELSON et al., 2008).

Figure 4. Area intensilly cohabitated by frigatebird (*Fregata magnificens*), red-footed-booby (*Sula sula*) and brown booby (*Sula leucogaster*)

Although able to acquire ARGs and ARB of human, food-producing animals and environmental origin (BONNEDAHL et al., 2009; 2010; 2015; HERNANDEZ et al., 2010; 2013; STEDT et al., 2015; ATTERBY et al., 2017; GUENTHER et al., 2017; AHLSTROM et al., 2018; MARCELINO et al., 2019), the role of wild birds as natural dispersers has not yet been confirmed (GUENTHER et al., 2012; BONNEDAHL et al., 2015; STEDT et al., 2015). Despite previous suggestions (BONNEDAHL et al., 2009; SIMÕES et al., 2010; ATTERBY et al., 2016; ZENDRI et al., 2019), there is no current scientific evidence that wild bird populations act as reservoirs of infection (HAYDON et al., 2002; VIANA et al., 2014), able to permanently maintain ARGs and ARB in their microbiota and transmit them to a target population (e.g., humans, animals) in real world conditions. Nevertheless, a migratory seabird species (ring-billed gulls [*Larus delawarensis*]) experimentally inoculated with an *E. coli* strain harboring plasmid-mediated *mcr*-1 gene was able to shed these strains in feces for days, contaminate the environment and infect con-specifics in an artificially controlled environment (FRANKLIN et al., 2020). Thus, the potential influence of migratory movements in the dissemination of ARB and ARGs should be further investigated. In spite of that, wild birds have a relevant epidemiological role in the One Health chain of ARGs and ARB, acting as bioindicators at the human-wildlife-environment interface (BONNEDAHL et al., 2009; ATTERBY et al., 2017; AHLSTROM et al., 2018; EWBANK et al., 2020). Finally, the complex ESBL-EC transmission dynamics in natural environments is believed to be driven by plasmid transfer in commensal and pathogenic strains, and by the clonal spread of certain lineages in local areas (i.e., O25b-ST131) (MOHSIN et al., 2017).

Finally, despite the considerable number of birds evaluated in this study, the low ESBL-EC prevalence was not surprising, considering the low anthropogenic influence over the atoll (e.g., geographical isolation and characteristics of local research expeditions). In spite of that, such ESBL-EC prevalence is still higher than those described in previous studies in wild birds at remote places, which ranged from no ESBL-EC isolates to 0.14% (HERNANDÉZ et al., 2010; ATTERBY et al., 2016; HERNANDÉZ et al., 2016; GUENTHER et al., 2017; RAMEY et al., 2018). Regardless, the presence of critically important isolates and genes (conferring resistance to cephalosporins) (TACCONELLI et al., 2018), in such a pristine environment is not only concerning from a One Health perspective, but also in face of the disruptive potential of AMR introduction into bacterial communities of fragile ecosystems (ALLEN et al., 2010; LO GIUDICE et al., 2019).

To the authors' knowledge, our study describes the first detection of: (1) the pandemic and public health relevant ST131-O25b harboring $bla_{\text{CTX-M-8}}$ worldwide; (2) ST131-*fim*H22 in

wild birds; and (3); *fos*A3 in wildlife. Our findings expand the current epidemiological knowledge regarding host and geographical distribution of ESBL-EC and ARGs in wild birds, and although it was not possible to identify the exact epidemiological route(s) of our strains, emphasize the disseminating characteristics and adaptability of ST131 and ST117 strains within the human-animal-interface, highlighting the complex and interconnected One Health nature of antimicrobial resistance. Herein we confirm the importance of considering biological and ecological species-specific factors in the study of ARB and ARGs in wildlife and that nonsynanthropic wild birds may have a role in the epidemiology of antimicrobial resistance (DOLEJSKA; LITERAK, 2009; AHLSTROM et al., 2018; EWBANK et al., 2020). We suggest that magnificent frigatebirds may act as "flying bridges", transporting ESBL-EC and ARGs from an anthropogenically-impacted environment to a pristine and remote location. We suggest that inter and intraspecific interactions may serve as infection routes and have increased epidemiological relevance in key seabird breeding insular territories with limited space availability sustaining high population densities, such as ROA. Additionally, the atoll is also used by highly migratory species (e.g., brown noddies and sooty terns), which could hipothetically participate in the potencial dissemination of resistance. Most of all, the characteristics of our isolates warrant zoonotic concern and, despite the apparent good health of all the evaluated birds, be a potential risk to the avian population using the atoll. Continuous spatial and temporal studies on the prevalence and characteristics of ARB and ARGs in the pristine and remote Rocas Atoll are necessary to further assess potential sources of infection. In order to confirm our hypothesis about the origin of the ESBL-EC isolates, further studies are required to survey their presence in the seabird species concomitantly using FNA and ROA, and potential local antimicrobial resistance sources (e.g., wastewater effluents and sewage) in the archipelago. All future studies would greatly benefit from longitudinal sample collection to assess the abundance, colonization and shedding of ARB throughout a span of time, and of devices such as satellite trackers, assessing the potential transport of resistance from anthropized to pristine biomes by wild birds, effectively investigating if wild birds could act as reservoirs and disseminators of ARGs and ARB.

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5 HIGHLY VIRULENT *ESCHERICHIA COLI* ST648 HARBORING CMY-2 AND CTX-M-2 ß-LACTAMASES IN MAGNIFICENT FRIGATEBIRD (*FREGATA MAGNIFICENS*) OF AN UNINHABITED INSULAR ENVIRONMENT, SOUTHEASTERN BRAZIL

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ABSTRACT

Antimicrobial resistance result from a naturally occurring ancient phenomenon increasingly pressured by anthropogenic activities. *Escherichia coli* (family Enterobacterales) has been used as markers of environmental contamination and anthropogenic activity. Seabirds may behave as bioindicators of the environmental presence and dissemination of extended-spectrum-beta-lactamase (ESBL)-AmpC-producing *E. coli* (ESBL/AmpC-EC) in remote locations. We surveyed cloacal swabs of 20 wild magnificent frigatebird (*Fregata magnificens*) in the uninhabited Alcatrazes Archipelago, located in the highly anthropized southeastern Brazilian coast. We found an ESBL/AmpC-EC prevalence of 5% (1/20). The isolate belonged to a highly virulent MDR ST648 (serotype O153:H9) pandemic clone harboring antimicrobial resistance genes $bla_{\text{CTX-M-2}}$, $bla_{\text{CMY-2}}$, *qnr*B, *tet*(A), *tet*(B), *sul*I, *sul*II and *aac*(3)-Via. Additionally, the isolate carried virulence genes (VGs) characteristic of avian pathogenic *E. coli* (APEC) (*hly*F, *iro*N, *iss*, *iut*A, and *omp*T) and other extraintestinal *E. coli* (ExPEC) pathotypes (e.g., *kps*MII [K1 capsule virulence factor], *ibe*ABC [invasion brain endothelium gene], *sit*ABCD [iron transport protein], and *iro*BCDEN [enterobactin siderophore receptor protein]). To the authors' knowledge, this is the first report of ST648 clone and ESBL/AmpC-EC in wild birds inhabiting insular environments. We suggest that this potentially zoonotic and avian pathogenic isolate was likely acquired through indirect contamination by human sources released into the marine environment (e.g., sewage), ingestion of contaminated seafood, or direct intra and/or interspecies contact. Our findings highlight the public health importance of wildlife studies on pathogenic bacteria, and the role of wild birds as anthropization sentinels in insular environments and their involvement in the One Health chain of antimicrobial resistance, even in uninhabited sites.

Keywords: Amp-C, avian pathogenic *Escherichia coli* (APEC), antibiotic, antimicrobial resistance, ESBL, island, wild bird.

5.1 INTRODUCTION

Antimicrobial resistance result from a naturally occurring ancient phenomenon that have been severely affected by anthropogenic activities such as use, misuse and overuse of antimicrobials in human and veterinary medicine, aquaculture and agriculture, and release of pharmaceutical manufacturing, domestic and agricultural waste into the environment (WRIGHT, 2007; WEST et al., 2010; CUMMINGS et al., 2011; GRAHAM et al., 2011; YANG et al., 2013; BENGTSSON; GRECO, 2014). *Escherichia coli* (family Enterobacterales) is broadly suggested and used as a marker of environmental contamination and anthropogenic activity (BONNEDAHL et al., 2009; TENAILLON et al., 2010; PESAPANE et al., 2013). Extended-spectrum-ß-lactamase (ESBL)-producing *E. coli* (ESBL-EC) and AmpC-producing *E. coli* (AmpC-EC) are a rapidly emerging public health issue (WORLD HEALTH ORGANIZATION, 2014), described in several epidemiological settings within the human-animal-environmental interface; from nosocomial to community-acquired infections (EWERS et al., 2012; EGERVÄRN et al., 2017; MUGHINI-GRAS et al., 2019). Consequently, antimicrobial resistance lead to great healthcare, social and economical burdens worldwide (BENGTSSON; GRECO, 2014; MICHAEL et al., 2014), thus considered a quintessential One Health issue (LIU et al., 2016).

Seabirds have been used as bioindicators of the ESBL/AmpC-EC environmental presence and dissemination in remote locations (HERNANDEZ et al., 2010; ARDILES-VILLEGAS et al., 2011; ATTERBY et al., 2016; HERNANDÉZ; GONZÁLEZ-ACUÑA 2016; RAMEY et al., 2018) due to their potential as sentinels of natural and anthropogenic-related changes to the marine ecosystem health (RABINOWITZ et al., 2010). Given that antimicrobial resistance genes are considered environmental pollutants and markers of environmental anthropization (PRUDEN et al., 2006; JOBBINS; ALEXANDER, 2015), most ESBL/AmpC-EC seabird studies have focused in synanthropic species inhabiting anthropized environments (e.g., urban areas and dumpsites) (BONNEDAHL et al., 2009; 2014; ATTERBY et al., 2016; AHLSTROM et al., 2018; 2019). Yet, insular biomes not inhabited by humans represent an informative setting in the study of the One Health chain of antimicrobial resistance by providing valuable insight into the occurrence, diversity, and dissemination of antimicrobial resistance genes (ARGs) and antimicrobial resistant bacteria

(ARB), such as ESBL/AmpC-EC, the indirect anthropogenic effects over the environment (e.g., marine pollution), and potential influence of biological and ecological characteristics of their local avian fauna (e.g., migration, use of coastal areas) (HERNANDEZ ET AL., 2010; EWBANK ET AL., 2020)

This study surveyed the occurrence, phenotypic and genotypic characteristics of ESBL/AmpC-EC in cloacal swabs of 20 wild magnificent frigatebird (*Fregata magnificens*; family Fregatidae) from an uninhabited archipelago in southeastern Brazil. We used microbiological techniques and whole genome sequencing (WGS) to further identify and characterize the bacterial lineages, serotypes, resistome, plasmidome and virulome.

5.2 MATERIALS AND METHODS

5.2.1 Study area

The Alcatrazes Island is the principal, among the five islands and four islates forming the Alcatrazes Archipelago (24° 05' 44.69" S 45° 41' 52.92" W), located at 36 km off the coast of São Sebastião district, in São Paulo state, southeastern Brazil (ROCHA; BONNET 2009; ICMBio 2017). Human occupation and tourist visitation to the archipelago have been historically restricted. In 1979, the Brazilian Navy started using the northeastern face of Alcatrazes Island as target practice. Later on, in 1987, the Tupinambás Ecological Station (Esec Tupinambás) was created, partially including the archipelago, and restricting visitation even more. In 2013, the Brazilian Navy moved its training grounds to a smaller island of Alcatrazes. Finally, in 2016, the archipelago and adjacent marine area (approximatelly 700 km^2) were declared a conservation area - the Alcatrazes Archipelago Wildlife Refuge (Refúgio de Vida Silvestre do Arquipélago de Alcatrazes - Refúgio de Alcatrazes), focused specifically on the conservation of its local wildlife and flora, administered by the Chico Mendes Institute for Biodiversity Conservation (ICMBio), Brazilian Ministry of Environment (ICMBio, 2017).

5.2.2 Sampling and bacterial identification

Overall, 20 magnificent frigatebirds were sampled in the main island (Alcatrazes Island), in January 2020. All birds were captured with the aid of a butterfly net, manually restrained and immediately released after sample collection. The cloacal swabs were

maintained in Amies transport medium containing charcoal and maintained at room temperature until processed. Aiming on ESBL- and AmpC-EC detection, cloacal samples were streaked onto ceftriaxone (CRO)-supplemented MacConkey agar plates (CRO: 2 mg/L) and incubated overnight at 35 ± 2 °C. Bacterial isolates were identified by MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry, Bruker Daltonik, Leipzig, Germany). All samples were collected in full compliance with the Biodiversity Information and Authorization System (SISBIO 59150-4), Brazilian Ministry of Environment. All procedures were performed in accordance with the Ethical Committee in Animal Research of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (Process number 1753110716).

5.2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility was evaluated by the disc diffusion method using the following human and veterinary antimicrobials (CLSI, 2018, 2019): amoxicillin/clavulanate, ceftriaxone, cefotaxime, ceftiofur, ceftazidime, cefepime, cefoxitin, imipenem, meropenem, ertapenem, enrofloxacin, ciprofloxacin, gentamycin, amikacin, chloramphenicol, trimethoprim-sulfamethoxazole, and tetracycline. The double-disc synergy test (DDST) was used to screen for ESBL (JARLIER et al., 1988).

5.2.4 Whole genome sequence analysis

The genomic DNA of a single ESBL/AmpC-EC strain was extracted using a PureLinkTM Quick Gel Extraction Kit (Life Technologies, Carlsbad, CA, USA) and a genomic paired-end library (75 x 2 bp), prepared using a Nextera XT DNA Library Preparation Kit (Illumina Inc., Cambridge, UK), according to the manufacturer's instructions. The whole genome was sequenced on the NextSeq platform (Illumina). *De novo* genome assembly and contig annotation was performed with CLC Genomics Workbench 12.0.3. The MLST 2.0, PlasmidFinder 2.0, ResFinder 4.1, VirulenceFinder 2.0 and SerotypeFinder 2.0 databases available at the Centre for Genomic Epidemiology (http://genomicepidemiology.org/) were used to identify, respectively, the multilocus sequence type (MLST), plasmid replicons, resistome, virulome and serotype.

A minimum spanning tree based on the wgMLST scheme and MSTree V2 tool from Enterobase (http://enterobase.warwick.ac.uk/species/index/ecoli) was constructed including

the *E. coli* isolate found in this study and an international collection of 107 *E. coli* strains belonging to ST648. The phylogenetic tree was generated using iTOL v.6 (https://itol.embl.de). An interactive version of the tree can be found at https://itol.embl.de/tree/179113768110067161722950 8.

5.3 RESULTS

Overall, we found an ESBL/AmpC-EC prevalence of 5% (1/20) in the evaluated individuals. Phenotypically, the isolate presented a multidrug resistance (MDR) profile, being resistant to amoxicillin/clavulanic acid, ceftiofur, cefoxitin, cefepime, aztreonam, trimethoprim-sulfametoxazol, gentamicin, and tetracycline. Genotypically, the isolate harboured genes *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CMY-2}}$, *qnr*B, *tet*(A), *tet*(B), *sul*I, *sul*II and *aac*(3)-VIa in its resistome. Multilocus sequence typing (MLST) analysis revealed that the isolate corresponded to ST648 (serotype O153:H9). The resistome, plasmidome and virulome are listed in Table 1.

Upon phylogenetic analysis, our isolate clustered with strains recovered from human (Australia), livestock (Spain and United States), poultry (United States), and gull (*Larus argentatus*; United States) (Figure 1).

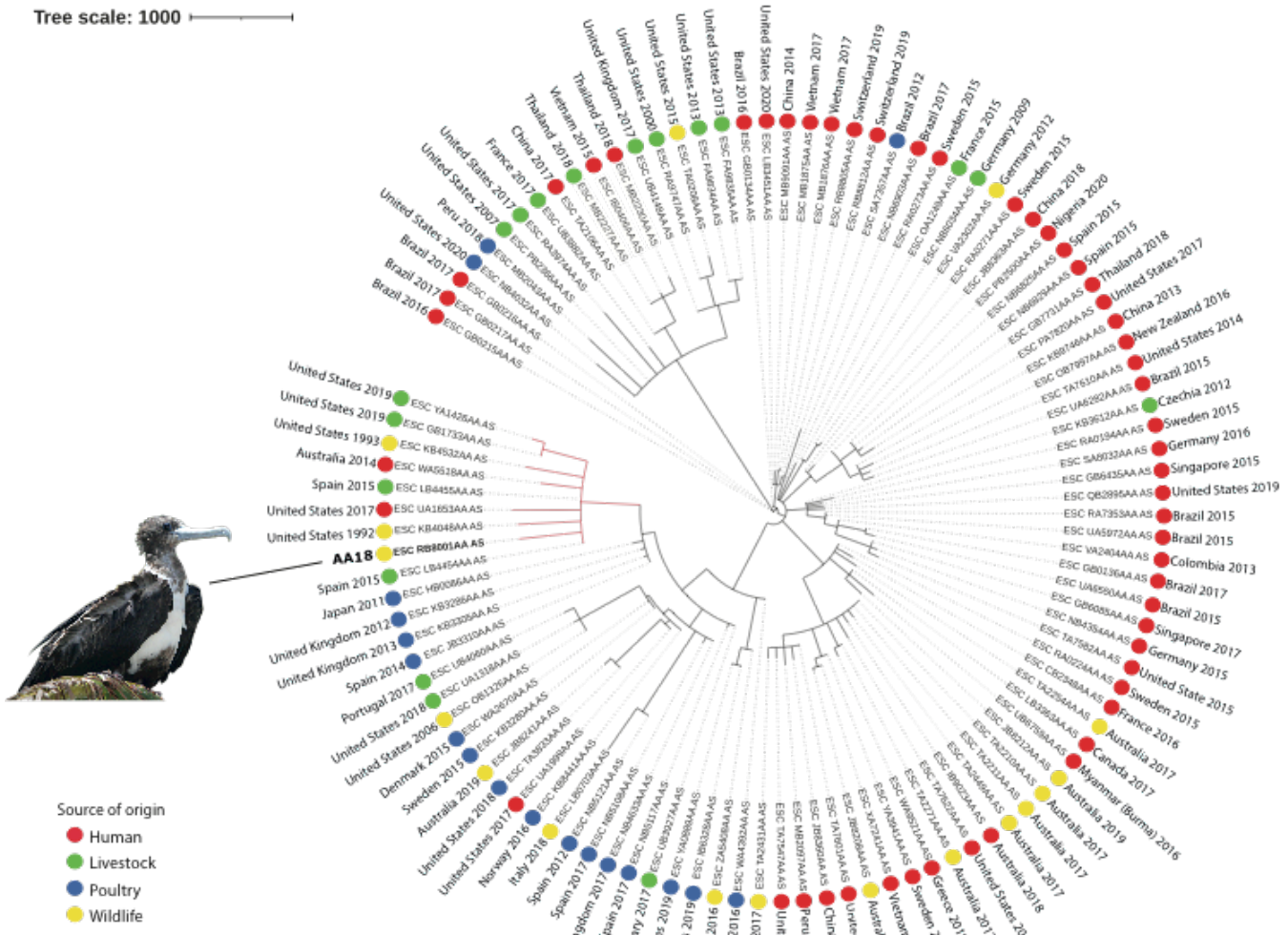
Table 1. Resistome, plasmidome, virulome and MLST analysis an ESBL-producing *Escherichia coli* ST648 (O153:H9) clone colonizing a magnificent frigatebird (*Fregata magnificens*) in Alcatrazes Archipelago, southeastern Brazil.

Antimicrobial resistance[a]	Plasmid Inc. groups	Virulome[b]
bla$_{CXT-M-2}$ [1]	Col	*aec*15, 19, 22, 23, 24, 25, 26, 27/clpV, 28, 29, 30, 31, 32
bla$_{CMY-2}$ [1]	IncFIB	*cba*, *cfa*BC, *chu*ASTUWXY, *cia*, *cma*, *cva*C
aa(3)-VIa [2]	IncFIB	*eae*H, *ecp*ABCDE, *eha*B, *eil*A, *elf*ACDG, *esp*L1, R1, X1 , X4, X6, Y1, Y2, Y4, *ets*C
*cat*I [3]	IncFII	*fim*ACDEFGHI
*qnr*B [4]		*gad*, *glf*
*sul*I [5]		*hly*F
*sul*II [5]		*ibe*BC, *ire*A, *iro*BCDEN, *iss*, *iuc*ABCD, *iut*A
tet(A) [6]		*kps*E, *kps*MII
tet(B) [6]		*lpf*A
		*mch*F
		*omp*T
		*pap*CDH, *pil*UW
		*sit*ABC
		*ter*C, *tia*, *tra*T, *tsh*
		vat
		*yfc*V

[a] Genes conferring resistance to: 1, cephalosporins; 2, aminoglycosides; 3, phenicols; 4, quinolones; 5, sulfonamides; 6, tetracyclines.

[b] Virulence factor genes: *aec* (auxin efflux carrier); *cba* (colicin ia); *cfa*BC (cyclopropane-fatty-acyl-phospholipid synthase); *chu*ASTUWXY (outer membrane hemin receptor), *cia* (colicin ia); *cma* (ColM activity); *cva*C (microcin C); *eae*H (putative attaching and effacing protein homolog); *ecp*ABCDE (*E. coli* common pillus); *eil*A (*Salmonella* HilA homolog); *elf*ACDG (*E. coli* laminin-binding fimbriae); *esp*L1, R1, X1 , X4, X6, Y1, Y2, Y4 (ype III system-secreted proteins); *ets*C (putative type I secretion outer membrane protein); *fim*ACDEFGHI (Type I fimbriae); *gad* (glutamate decarboxylase); *glf* (UDP-galactopyranose mutase); *hly*F (hemolysin F); *ibe*BC (invasion brain endothelium), *ire*A (siderophore receptor); *iro*BCDEN (enterobactin siderophore receptor protein); *iss* (increased serum survival); *iuc*ABCD (aerobactin production); *iut*A(aerobactin siderophore receptor gene); *kps*E (capsule polysaccharide export inner-membrane protein), *kps*MII (polysialic acid transport protein; Group 2 capsule); *lpf*A (long polar fimbriae); *mch*F (ABC transporter protein MchF); *omp*T (outer membrane protease (protein protease 7); *pap*CDH (P fimbriae); *pil*UW (Type I fimbriae); *sit*ABC (iron transport protein); *ter*C (tellurium ion resistance protein); *tia* (adhesin); *tra*T (outer membrane protein complement resistance); *tsh* (temperature-sensitive hemagglutinin); *sit*ABC (iron transport protein); *vat* (vacuolating autotransporter toxin); *yfc*V (major subunit of a putative chaperone-usher fimbria).

Figure 1. Phylogeny of CTX-M-2 and CMY-2-producing ST648 *E. coli* isolate of a magnificent frigatebird (*Fregata magnificens*) in comparison with an international *E. coli* collection, regarding source of origin (colored circles), origin of the isolate (country), and year of isolation. Tree scales: 1000.



5.4 DISCUSSION

In this study, we found an overall prevalence of 5% (1/20) ESBL/AmpC-EC isolates in magnificent frigatebirds of Alcatrazes Archipelago, southeastern coast of Brazil: a MDR highly virulent avian pathogenic *E. coli* (APEC) isolate of the pandemic lineage ST648 (serotype O153:H9) harboring *bla*$_{\text{CTX-M-2}}$ and *bla*$_{\text{CMY-2}}$. To the authors' knowledge, this is the first report of the ST648 clone and Amp-EC in wild birds inhabiting insular environments.

Escherichia coli ST648 is a predominantly MDR highly virulent emerging clone and one of the most commonly reported international sequence types (STs) in the human–animal–environment interface worldwide, suggesting great host adaptation (HU et al., 2013; MÜLLER et al., 2016; FERNANDES et al., 2018; PAULSHUS et al., 2019; DE CARVALHO et al., 2020). Similarly, ST648 has been detected in wild birds from almost all continents: Europe (passerines and waterfowl [GUENTHER et al., 2010], birds of prey and cranes [GUENTHER et al., 2012], and corvids [SCHAUFLER et al., 2019]), the Americas (gulls [POIREL et al., 2012; BÁEZ et al., 2014), Asia (gulls [HASAN et al., 2012] and waterfowl [YANG et al., 2016]), and Oceania (gulls and penguins [MUKERJI et al., 2019]). To this date, in Brazil, this clone had been described solely in wild birds of prey (BATALHA DE JESUS et al., 2018; DE CARVALHO et al., 2020).

The CTX-M-2 and CMY-2 enzymes are, respectively, the most prevalent CTX-M ESBL-encoding family in South America and AmpC beta-lactamase worldwide (JACOBY 2009; ROCHA et al., 2016; WANG et al., 2017), broadly reported in all epidemiological settings, including in Brazil (PIETSCH et al., 2018; ROCHA et al., 2016, MELO et al., 2018; FERNANDES et al., 2020b; CUNHA et al., 2017; CONTE et al., 2017; DE CARVALHO et al., 2020). In wild birds, the *bla*$_{\text{CTX-M-2}}$ and *bla*$_{\text{CMY-2}}$ genes have been described in Europe (gulls [STEDT et al., 2015; ALCALÁ et al., 2016], corvids [LONCARIC et al., 2013; JAMBOROVA et al., 2017], and in an Eurasian magpie [ATHANASAKOPOULOU et al., 2021]), and in the Americas (gulls [POIREL et al., 2012; ATTERBY et al., 2016; BÁEZ et al., 2015; LIAKOPOULOS et al., 2016; AHLSTROM et al., 2018] and bald eagles [AHLSTROM et al., 2018]). In Brazil, *bla*$_{\text{CTX-M-2}}$ has been detected in wild birds of prey (BATALHA DE JESUS et al., 2018; DE CARVALHO et al., 2020) and parrots (BATALHA DE JESUS et al., 2018), and in bumblefoot lesions of a wild Magellanic penguin (*Spheniscus magellanicus*) undergoing rehabilitation (SELLERA et al., 2017); while *bla*$_{\text{CMY-2}}$ has been described only in raptors (BATALHA DE JESUS et al., 2018).

Our isolate presented several virulence genes of concern, characteristic of Extraintestinal pathogenic *E. coli* (ExPEC); an emerging pathogen responsible for increasing socio-economic burdens, with pandemic strains reportedly causing community and healthcare-associated outbreaks, also affecting livestock (especially poultry), companion animals and wildlife worldwide (RILEY 2004; EWERS et al., 2010; GUENTHER et al., 2010; KEMMET et al., 2014; MASELLA et al., 2020). The ExPEC pathotype is subdivided into avian pathogenic *E. coli* (APEC), neonatal meningitis *E. coli* (NMEC), sepsis-associated *E. coli* (SEPEC), and uropathogenic *E. coli* (UPEC) (MELLATA et al., 2013; SAROWSKA et al., 2019). We identified plasmid-borne virulence factors typical of highly pathogenic APEC isolates: *cva*C (colicin V), *fim*C (fimbriae type I), *hly*F (hemolysin F), *ibe*ABC (invasion brain endothelium gene), *iro*N (salmochelin), *iss* (increased serum survival), *iuc*C (aerobactin production) *iut*A (ferric aerobactin receptor), *omp*T (outer membrane protein), *sit*A (iron transport protein), *tsh* (temperature-sensitive hemagglutinin) and *tra*T (transfer protein) (EWERS et al., 2007; 2009; SAROWSKA et al., 2019). APEC strains cause multiple systemic and localized infections in birds, generally referred to as avian colibacillosis, leading to high mortality and decreased production, consequently imposing severe economic losses to the poultry industry worldwide (SAIDENBERG; KNÖBL 2005; KEMMET et al., 2014; MARKLAND et al., 2015). Worryingly, we also found virulence genes characteristic of the other ExPEC: *chu*A (outer membrane hemin receptor), *kps*MII (polysialic acid transport protein), *sit*ABCD (iron transport protein), *iro*BCDEN (enterobactin siderophore receptor protein), *tra*T, *vat* (vacuolating autotransporter toxin), *pap*C (outer membrane usher protein), and *yfc*V (major subunit of a putative chaperone-usher fimbria) (KIM 2002; GRIMWOOD et al., 2000; SAROWSKA et al., 2019). Of note, some of the virulence factors found in our isolate were previously reported in magificent frigatebirds from Alcatrazes Archipelago: *cva*C, *fim*H, *hly*F, *ibe*A, *iro*N, *iss*, *iut*A, *omp*T, *pap*C, and *tra*T (SAVIOLLI et al., 2016). Although APEC and ExPEC strains are phylogenetically close, sharing some of the same virulence genes, APEC may carry others not common in ExPEC isolates, such as those present in the colicin V (ColV) plasmid (RODRIGUEZ-SIEK et al., 2005; BÉLANGER et al., 2011; MELATTA et al., 2013; MANGES 2016). These characteristics suggest that APEC strains are potentially zoonotic, and could be a reservoir and source of virulence genes for ExPEC strains (EWERS et al., 2007; JOHNSON et al., 2008; BÉLANGER et al., 2011). APEC infections in humans could take place through consumption of undercooked food from animal origin (especially retail poultry products), and direct contact with birds and their feces (DZVA; STEVENS 2008; OJENIYI 1989).

Anthropization has been suggested as a driving factor in the epidemiology of ARGs in wildlife (ATTERBY et al., 2017; AHLSTROM et al., 2018; SACRISTÁN et al., 2020; EWBANK et al., 2020). Although occasionally visited or exploited for commercial guano harvesting until the mid-20th century (GIBRAN et al. 2012), to this date, there are no reports of human occupation or settlements in the archipelago (Alcatrazes Archipelago Wildlife Refuge, Chico Mendes Institute for Biodiversity Conservation, personnal communication). Nevertheless, Alcatrazes is located in the highly anthropized southeastern Brazilian coast, subjected to intense tourism activities, fishing, oil exploitation, harbouring a commercial port and the largest oil and derivatives terminal in Latin America (Terminal Marítimo Almirante Barroso - TEBAR). The aquatic environment is very relevant in the epidemiology of ARGs (ZHANG et al., 2009; MARTI; BALCAZAR et al., 2014), by promoting the interaction among antimicrobials, their metabolites and residues, other pollutants (e.g., disinfectants, metals), and resistant bacteria from distinct settings (human [e.g., wastewater, sewage], animal [e.g., aquaculture, manure], and environmental [e.g., manure-amended soil]) (BAQUERO et al., 2008). Recent studies assessing antimicrobial resistance pollution in the marine ecosystem of the southeastern Brazilian coast showed that the local resistome is under severe anthropogenic pressure (FERNANDES et al., 2017; SELLERA et al., 2018; FERNANDES et al., 2020a; FERNANDES et al., 2020b; Ewbank et al., 2021).

Despite the hypothetical zoonotic and pathogenic potential of our isolate (Ewers et al., 2014; Maluta et al., 2014; Sarowska et al., 2019), one must also carefully consider the low prevalence of ESBL-/AmpC-EC found in this study (5%; 1/20), and species-specific biological and ecological factors - key in the discussion of antimicrobial resistance in wild birds (DOLEJSKA et al., 2019; EWBANK et al., 2020). The Alcatrazes Archipelago is the largest insular bird breeding site of the southeastern Brazilian coast and the biggest breeding colony of magnificent frigatebirds in the southern Atlantic (Alcatrazes Island) (MUSCAT et al., 2014, ICMBio 2017). Magnificent frigatebirds are nonsynanthropic, nonmigratory, highly colonial seabird species that prefer insular over coastal environment and known for their particular feeding techniques (e.g., kleptoparasitism and fisheries interaction) (BIRDLIFE INTERNATIONAL, 2020; SAVIOLLI et al., 2016). Such characteristics infer that the studied individuals most likely sustain very limited to no direct contact with humans, and that due to their philopatric (site fidelity) behavior are continuously interacting with other birds (especially with brown boobies (*Sula leucogaster*) and black vultures (*Coragyps atratus*), (A.C. Ewbank, personal observation), thus actively exchanging body fluids, a possible route of infection by ESBL-EC, as seen in other avian pathogens (DE THOISY et al., 2009;

NIEMEYER et al., 2017). Furthermore, all the magnificent frigatebirds of Alcatrazes evaluated in this study and by Saviolli et al. (2016) were apparently healthy, showing no sign of disease. We suggest that our isolate was likely acquired through indirect infection by human sources released into the local marine environment (e.g., sewage) (FERNANDES et al., 2017; FERNANDES et al., 2018; FERNANDES et al., 2020a; FERNANDES et al., 2020b), ingestion of contaminated seafood (BRAHMI et al., 2015; SELLERA et al., 2018a; SELLERA et al., 2018b), or direct intra and/or interspecies contact.

Previous studies have suggested wild birds as reservoirs and disseminators of ARGs and ARB to insular biomes (SJÖLUND et a., 2008; HERNANDEZ et al., 2010; ARDILES-VILLEGAS et al., 2011; RAMEY et al., 2018; EWBANK et al., 2020). In spite of experimental studies assessing the shedding, contamination and potential transmission of ARGs and ARB by wild birds (SANDEGREN et al., 2018; FRANKLIN et al., 2020), their potential role as dispersers under real-world conditions has not yet been confirmed (GUENTHER et al., 2012; BONNEDAHL et al., 2015; STEDT et al., 2015). Our findings demonstrate that even in the absence of regular human presence, insular resistomes are indirectly pressured by anthropogenic activities, suggesting that contamination of the marine ecosystem should also be considered in the study of antimicrobial resistance in these biomes.

Herein we report a highly virulent potentially zoonotic and avian pathogenic strain of the emerging pandemic ST648 *E.coli* clone harboring genes $bla_{\text{CTX-M-2}}$ and $bla_{\text{CMY-2}}$ in a wild magnificent frigatebird from an insular biome (Alcatrazes Archipelago, southeastern coast of Brazil). Our findings highlight the public health importance of studies on antimicrobial resistance and pathogenic bacteria in wildlife, and the role of wild birds as anthropization sentinels of insular environments and their involvement in the One Health chain of antimicrobial resistance, even in uninhabited sites. Future studies evaluating the occurrence and diversity of ESBL/AmpC-EC in magnificent frigatebirds on the Alcatrazes Archipelago should rely on continuous temporal sampling to assess a larger number of specimens, evaluate interacting species (i.e., brown boobies and black vultures), and environmental samples (i.e., sea water and soil), including local marine life (i.e., fish), in order to monitor these populations through a One Health approach and further elucidate the epidemiology of ESBL/AmpC-EC in this insular environment.

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6 FINAL COMMENTS

In this study, we combined molecular (real time PCR and whole genome sequencing - WSG) and classic microbiological techniques (bacterial culture and antibiotic susceptibility testing) to analyze the microbiome and microbiota of seabirds in Brazil in, respectively, enema and cloacal swab samples. By sampling enemas and clocal swabs from each individual instead of collecting droppings (i.e., fecal pools), we were able to avoid "environmental contamination" and evaluate each bird, ultimately providing more reliable data. Despite their different approaches and resulting information, these techniques are complementary, and should be employed according with the goals of each study. The direct real time PCR (rtPCR) detection and quantification provides genotypic characterization, and relies on the fact that most bacteria are not cultivable and that culture methods do not favor mobile genetic elements (e.g., plasmids), which encode most antimicrobial resistance genes (ARGs). Because this method does not rely on bacterial culture, it allowed us to directly assess the whole microbiome, ultimately enabling a broader epidemiological assessment and discussion. Moreover, it yielded quantitative data, and consequently, more substantial comparisons. On the other hand, the combination of classical microbiology and whole genome sequencing (WGS) generated a different data set, identifying the phenotype and genotype of antimicrobial resistant bacteria, and providing information (e.g., multilocus sequence typing [MLST], plasmid replicons, resistome, virulome, serotype) that could be compared with previous isolates deposited in electronic international data sets. Once combined, the above-mentioned studies ultimately provided solid ground information for future clinical and epidemiological studies on the issue of AMR in wild birds, not only in Brazil, but also worldwide.

In the first chapter, we used rtPCR to identify and quantify selected ARGs in the microbiome of kelp seagulls (*Larus dominicanus*) and Magellanic penguins (*Spheniscus magellanicus*) upon their admission to a rehabilitation center in Florianópolis, Southern Brazil. In general, seagulls are highly synanthropic and non-migratory, while Magellanic penguins are strictly migratory pelagic seabird species. In this scenario, we assessed the ARGs that would be introduced into the rehabilitation setting, which potentially and indirectly, could informe us on their presence in their environment of origin. We observed significant differences between species, higher in kelp gull in comparison to Magellanic penguin, likely due to these species' contrasting biology and ecology, key in studies of AMR in wildlife (i.e., dispersal [migratory and non-migratory], feeding niche [coastal and pelagic], and interaction with human-impacted areas [synanthropic and non-synanthropic]).

Furthermore, to our knowledge, this study is the first to report the *mec*A gene in seabirds in the Americas. We discussed the importance of ARGs in wild individuals, which are usually not directly exposed to antimicrobials in the wild. However, when ARGs are present in this particular epidemiological setting (i.e., rehabilitation center), if active and promoting antimicrobial resistance may compromise and even prevent treatment. Moreover, we also discussed the biosafety, antimicrobial discard and decontamination policies of rehabilitation centers, where both patients and staff may be exposed to these ARGs. Finally, we emphasized the importance of performing complete physical examinations and diagnostic testing (e.g., complete blood count an hemogram) to evaluate the need of antimicrobial prescription, that when deemed necessary, should be conducted responsibly (i.e., appropriate drug of choice, dosage and term), to prevent local contamination and circulation within the other epidemiological settings (e.g., staff), and ultimately, the (re)introduction of ARGs into the environment upon these individuals' release.

In the following chapters – 2, 3 and 4, we assessed different epidemiological settings: sebirds using the shore (both beach and marine), and seabird colonies in insular ecosystems. Island populations, communities, and ecosystems maintain the fundamental processes, properties, and interactions of ecological continental systems, but often in a more restricted scenario. For seabirds, especially pelagic and/or migratory species, oceanic islands are excellent areas for breeding, and gaining weight and energy during their geographical movements. The reproductive season, in particular, is characterized by a high-energy demand. Islands usually have restricted surface areas and/or appropriate nesting sites, forcing birds to congregate and compete for space and resources, increasing their stress and influencing their immune system. Thus, islands are often challenging environments, and from an epidemiological perspective, may behave like "melting pots", where ARGs and ARB from various locations and hosts conjoin, promoting countless combinations and exchange.

In the second chapter, we assessed the microbiome of wild seabirds using the previously described direct molecular methods to evaluate the presence of selected ARGs in birds of two different biotopes: a highly touristic and anthropized archipelago (Fernando de Noronha Archipelago, FNA, Pernambuco: (masked boobies [*Sula dactylatra*], brown boobies [*Sula leucogaster*] and magnificent frigatebirds [*Fregata magnificens*]) and a pristine atoll (Rocas Atoll, ROA, Rio Grande do Norte: (masked boobies, brown boobies, magnificent frigatebirds, red-footed boobies, sooty terns [*Onychoprion fuscatus*], and brown noddies [*Anous stolidus*]) with minimal historical human presence and activity, both in the northeastern Brazilian coast. To the best of our knowledge, this study was the largest to report

the *mcr*-1 gene in free-ranging wild birds worldwide. Our results showed that ARGs prevalence and load were higher in seabirds inhabiting the anthropized in comparison with the pristine environment. The results obtained in the anthropized biotope were consistent with anthropogenic pressure: significantly higher prevalence of sulfonamide- and quinolone-encoding ARGs, and *sul*II as the most prevalent gene. Additionally, we observed significantly higher *mcr*-1 and bla_{TEM} prevalences, and *mcr*-1 percentage load in migratory when compared to resident seabird species. Epidemiologically, the first finding highlights the important epidemiological role of migratory species, while the second suggests this group has a higher potential of *mcr*-1 dissemination into the human-wildlife-environment interface.

The third and fourth chapters described the use of classical microbiological techniques and WGS to identify and characterize bacteria with One Health relevance present in the microbiota of wild seabirds inhabiting and/or using a pristine atoll and a little anthropogenically-impacted archipelago inserted in the most anthropized area in the country, respectively, in the northern and southeastern Brazilian coasts. To the authors' knowledge, these are, respectively, the first detection of the pandemic and public health relevant ST131 and ST648 strains in seabirds of Brazilian insular biomes, also novel in magnificent frigatebirds [*Fregata magnificens*]) . In the third chapter we hypothesize that frigatebirds may have acted as "flying bridges", transporting ARB and ARGs from an anthropogenically-impacted environment (FNA) to the study site (ROA). Additionally, the identification of three ST131 clones indicated its circulation within the evaluated population. In the fourth chapter we described the highly virulent multidrug resistant (MDR) pandemic ST648 carrying virulence genes related to avian pathogenic *E. coli* (APEC). Although all evaluated birds were apparently healthy, these findings are very relevant from a One Health perspective. In both cases we hypothesize that the isolates were likely acquired through one or more of the following routes: indirect contamination by human sources released into the marine environment (e.g., sewage), ingestion of seafood contaminated with these bacteria, and direct intra and/or interspecies interaction.

Our findings corroborate that seabirds are anthropization and environmental sentinels of AMR. Additionally, we showed that seabirds are involved in the One Health epidemiological chain of AMR – even in environments minimally exposed to human presence and related activities. Although not possible to identify specific epidemiological routes, we described the occurrence and diversity of ARGs and ARB throughout different epidemiological and geographical settings, highlighting the complex and interconnected One Health nature of antimicrobial resistance. Finally, our results emphasized that although

anthropization is important, other biological and ecological factors (e.g., dispersal, feeding zones, inter-/intra-species behavior) must always be considered in order to further understand the epidemiology of AMR.

In light of the above findings, one could argue that the increasingly close contact with wildlife represents a potential source of human infection by ARGs and ARBs. Yet, the available literature clearly shows that the other way around is much more significant; after all, humans are the ones producing, misusing, overusing, and inappropriately disposing antimicrobials (along with their metabolites) into the environment, creating the perfect media for new combinations and exchange among ARGs, ARB, their hosts and the environment. We live in a globalized and highly connected world, with millions of yearly national and international travelers, and thousands of tons of food products traded worldwide. Additionally, other pressing factors such as deforestation, pollution, climate change, and overpopulation – to name a few - are also changing the ways we interact with wildlife and the "environmental footprints" left by us (e.g., disposal of pollutants into the sea, soil and air).

To this day, it has been greatly discussed and suggested that wild birds may transport and spread AMR during geographical movements and/or close contact with humans and domestic animals. Studies have shown that, indeed, wild birds are capable of acquiring ARGs and ARB from humans and domestic animals. Nevertheless, few experimental reports have shown that they are capable of maintaining and shedding AMR, ultimately infecting other specimens and the environment. Furthermore, these same studies relied on a very limited number of specimens and have been reproduced in extremely controlled environments, and not in natural conditions, subjected to the dynamic balance of microbial communities and their hosts and environment. Regardless, this raises an important epidemiological question that we all must contextualize in a bigger picture: even if wild birds could acquire, maintain and shed AMR, from an epidemiological approach, how significant could their dispersal be in terms of One Health? As opinion makers, we scientists and researchers must continuously work together on further investigating the potential of AMR development and spread by wild birds, identifying hot spots, and monitoring congregation and resting areas (e.g., breeding grounds) throughout their migratory routes. All whilst avoiding the anthropocentric view that humans are victims of ARGs and ARB transmitted and carried by wildlife, which unnecessarily villainizes wild animals and, most importantly, prevent us from truly focusing and debating the real issues (e.g., infection routes).

Antimicrobial resistance is, unquestionably, one of the most urgent global health issues of our lifetime, with great social, economic and political determinants and

consequences. Understanding that antimicrobial resistance is a natural phenomenon intrinsic of bacteria life is the cornerstone of the matter; it entails it is an ever evolving process, and that as such, requires continuous surveillance and adaptations that might be able to mitigate, but not “definitely resolve” or “avoid” the problem. Most importantly, AMR must be recognized as a quintessential One Health issue and be analyzed and discussed as such. In order to move forward, further AMR studies in wildlife must also focus beyond the human-related presence and activities, and more in the environment (especially in the marine ecosystem and fauna), on microbiome function and composition, and species-specific characteristics - all likely to play a very important role in the development and spread of AMR within the human-animal-environment interface. Such complex framework will provide valuable information regarding AMR development, maintenance and spread in wildlife, while indirectly assessing its dynamics of environmental presence and dispersal.

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APPENDIX A - Supplementary materials published in Science of the Total Environment, 754, Ana Carolina Ewbank, Fernando Esperón, Carlos Sacristán, Irene Sacristán, Ricardo Krul, Eduardo Cavalcante de Macedo, Olga Calatayud, Irene Bueno, Ricardo de Francisco Strefezzi, José Luiz Catão-Dias, Seabirds as anthropization indicators in two different tropical biotopes: "A One Health approach to the issue of antimicrobial resistance genes pollution in oceanic islands, 142141, Copyright Elsevier (or appropriate Society name) (2021)."

Supplementary table 1. Number of collected and validated samples in Fernando de Noronha Archipelago and Rocas Atoll according with species, collection site, behavior (feeding zone [coastal or pelagic], and dispersal [migratory or non-migratory])

Species	Fernando de Noronha Archipelago					Rocas Atoll		Behavior	
	Collection site			Number of samples		Number of samples			
	Fernando de Noronha Island	Meio Island	Rata Island	Number of collected samples	Number of validated samples	Number of collected samples	Number of validated samples	Feeding Zone	Dispersal
Magnificent frigatebird (*Fregata magnificens*)	25	-	-	25	23	35	32	Coastal	Non-migratory
Masked booby (*Sula dactylatra*)	-	25	23	48	42	33	20	Pelagic	Non-migratory
Brown booby (*Sula leucogaster*)	12	19	-	31	25	33	24	Coastal	Non-migratory
Red-footed booby (*Sula sula*)	-	-	-	-	-	33	31	Pelagic	Non-migratory
Sooty tern (*Onychoprion fuscatus*)	-	-	-	-	-	36	32	Pelagic	Migratory
Brown noddy (*Anous stolidus*)	-	-	-	-	-	34	28	Pelagic	Migratory
Total	37	44	23	104	90	204	167		

Supplementary table 2. Prevalence of each class of antimicrobial resistance gene and prevalence of multiresistant profiles in the sampled birds.

	Tetracyclines	Sulfonamides	Aminoglycosides	Phenicols	Macrolides	Quinolones	Betalactams	Polymixins	Multiresistance profile
Number of samples (n)	257	257	257	257	257	257	257	257	257
Mean	75.10	7.00	7.00	10.51	3.11	10.51	8.94	7.00	8.56
Lower 95% confidence interval of mean	69.77	3.86	3.86	6.73	0.97	6.73	5.43	3.86	5.11
Upper 95% confidence interval of mean	80.42	10.15	10.15	14.28	5.25	14.28	12.46	10.15	12.00

Supplementary table 3. Prevalence of antimicrobial resistance genes in the sampled birds.

	tet(A)	*tet*(B)	*tet*(Y)	*tet*(K)	*tet*(M)	*tet*(Q)	*tet*(S)	*tet*(W)	*sul*I	*sul*II	*str*	*aad*A	*cat*I	*cat*II	*erm*(B)	*erm*(F)	*qnr*S	*qnr*B	bla_{TEM}	bla_{CTX-M}	*mec*A	*m*
Number of values (n)	257	257	257	257	257	257	257	257	257	257	257	257	257	257	257	257	257	257	257	257	257	2
Mean	29.0	25.0	19.0	1.6	3.5	5.0	29.0	5.4	0.39	6.6	1.5	5.4	1.2	9.0	0.8	2.3	10.0	0.8	8.9	0.0	0.0	7
Lower 95% confidence interval of mean	23.22	19.58	13.88	0.03	1.23	2.36	23.59	2.65	-0.37	3.55	0.03	2.65	-0.15	5.75	-0.30	0.47	6.40	-0.30	5.43	0.0	0.0	3.
Upper 95% confidence interval of mean	34.37	30.23	23.47	3.08	5.76	7.75	34.78	8.24	1.15	9.67	3.08	8.24	2.48	12.92	1.86	4.19	13.83	1.86	12.46	0.0	0.0	10

Supplementary table 4. Bird species sampled in FNA according with antimicrobial class, evaluated genes and number of validated samples (n).

Antimicrobial class	ARGs	Species (n)		
		magnificent frigatebird (*Fregata magnificens*) (23)	**masked booby (*Sula dactylatra*) (42)**	**brown booby (*Sula leucogaster*) (25)**
Tetracyclines	***tet*(A)**	39%[a] / -5.6[b] (-8, -0.3)[c]	33%[a] / -6.1[b] (-8, -0.9)[c]	20%[a] / -6.8[b] (-8, -1)[c]
	***tet*(B)**	22%[a] / -6.8[b] (-8, -1.5)[c]	26%[a] / -6.5[b] (-8, -1)[c]	16%[a] / -7.1[b] (-8, -0.9)[c]
	***tet*(Y)**	17%[a] / -7[2] (-8, -0.6)[c]	12%[a] / -7.3[b] (-8, -0.8)[c]	28%[a] / -6.3[b] (-8, -0.6)[c]
	***tet*(K)**	9%[a] / -7.5[b] (-8, -2.5)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8.0)[c]
	***tet*(M)**	0%[a] / -8[b] (-8, -8.0)[c]	7%[a] / -7.6[b] (-8, -0.8)[c]	0%[a] / -8[b] (-8, -8.0)[c]
	***tet*(Q)**	4%[a] / -7.7[b] (-8, -1.4)[c]	5%[a] / -7.7[b] (-8, -1.5)[c]	4%[a] / -7.8[b] (-8, -2.8)[c]
	***tet*(S)**	17%[a]/ -7.1[b] (-8, -1.9)[c]	31%[a] / -6.4[b] (-8, -1.1)[c]	20%[a] / -6.9[b] (-8, -2.1)[c]
	***tet*(W)**	43%[a]/ -5.5[b] (-8, -1.1)[c]	2%[a] / -7.9[b] (-8, -1.8)[c]	4%[a] / -7.8[b] (-8, -1.8)[c]
Sulfonamides	***sul*I**	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]
	***sul*II**	35%[a] / -5.8[b] (-8, -1.1)[c]	5%[a] / -7.7[b] (-8, -2.4)[c]	0%[a] / -8[b] (-8, -8)[c]
Aminoglycosides	***str***	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]
	***aad*A**	4%[a] / -7.7[b] (-8, -1.4)[c]	12%[a] / -7.3[b] (-8, -1.2)[c]	8%[a] / -7.6[b] (-8, -1.8)[c]

Phenicols	***cat*I**	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]
	***cat*II**	26%[a] / -6.6[b]	7%[a] / -7.6[b]	8%[a] / -7.6[b]
		(-8, -1.6)[c]	(-8, -1.5)[c]	(-8, -2)[c]
Macrolides	***erm*B**	4%[a] / -7.8[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -3.1)[c]	(-8, -8)[c]	(-8, -8)[c]
	***erm*F**	0%[a] / -8[b]	2%[a] / -7.8[b]	8%[a] / -7.5[b]
		(-8, -8)[c]	(-8, -1.1)[c]	(-8, -1.9)[c]
Quinolones	***qnr*S**	13%[a] / -7.1[b]	24%[a] / -6.5[b]	4%[a] / -7.8[b]
		(-8, -1.1)[c]	(-8, -1.2)[c]	(-8, -2.4)[c]
	***qnr*B**	9%[a] / -7.4[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -0.8)[c]	(-8, -8)[c]	(-8, -8)[c]
Betalactams	***bla*$_{TEM}$**	9%[a] / -7.3[b]	5%[a] / -7.6[b]	0%[a] / -8[b]
		(-8, -1.5)[c]	(-8, -0.8)[c]	(-8, -8)[c]
	***bla*$_{CTX-M}$**	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]
	***mec*A**	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]
Polymyxin	***mcr*-1**	0%[a] / -8[b]	12%[a] / -7.2[b]	0%[a] / -8[b]
		(-8, -8)[c]	(-8, -0.2)[c]	(-8, -8)[c]

[a] % of individuals of species X positive to gene Y.
[b] Percentage load, expressed in logarithmic scale, of each antimicrobial gene regarding gene 16SrRNA.
[c] Range of minimum (-8) and maximum (+2) percentage load of genes detected in species X.

Supplemental table 5. Antimicrobial classes and multiresistant microbiomes found in FNA species.

Species	n	Antimicrobial class								Multiresistant microbiomes
		Tetracyclines	Sulfonamides	Aminoglycosides	Phenicols	Macrolides	Quinolones	Betalactams	Polymyxin	
magnificent frigatebird (*Fregata magnificens*)	23	82.6%	34.8%	4.3%	26.1%	4.3%	17.4%	8.7%	0%	13%
masked booby (*Sula dactylatra*)	42	76.2%	4.8%	11.9%	7.1%	2.4%	23.8%	4.8%	11.9%	16.7%
brown booby (*Sula leucogaster*)	25	68%	0%	8%	8%	8%	4%	0%	0%	0%

Supplemental table 6. Bird species sampled in ROA according with with antimicrobial class, evaluated genes and number of validated samples (n).

Antimicrobial class	ARGs	Species (n)					
		magnificent frigatebird (*Fregata magnificens*) (32)	**masked booby (*Sula dactylatra*) (20)**	**brown booby (*Sula leucogaster*) (24)**	**red-footed booby (*Sula sula*) (31)**	**sooty tern (*Onychoprion fuscatus*) (32)**	**brown noddy (*Anous stolidus*) (28)**
Tetracyclines	***tet*(A)**	34%[a] / -6.2[b] (-8, -1.7)[c]	20%[a] / -6.7[b] (-8, -1)[c]	21%[a] / -6.7[b] (-8, -0.3)[c]	29%[a] / -6.3[b] (-8, -0.9)[c]	33%[a] / -5.9[b] (-8, -1)[c]	21%[a] / -6.7[b] (-8, -1.3)[c]
	***tet*(B)**	38%[a] / -5.8[b] (-8, -0.8)[c]	30%[a] / -6.3[b] (-8, -0.2)[c]	25%[a] / -6.4[b] (-8, -0.6)[c]	26%[a] / -6.5[b] (-8, -1.2)[c]	18%[a] / -6.8[b] (-8, -1.1)[c]	21%[a] / -6.7[b] (-8, -1.4)[c]
	***tet*(Y)**	13%[a] / -7.3[b] (-8, -0.9)[c]	5%[a] / -7.7[b] (-8, -1.8)[c]	29%[a] / -6.4[b] (-8, -1.3)[c]	23%[a] / -6.8[b] (-8, -1.4)[c]	27%[a] / -6.5[b] (-8, -1.7)[c]	14%[a] / -7.2[b] (-8, -1.6)[c]
	***tet*(K)**	3%[1] / -7.8[b] (-8, -1.9)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	3%[a] / -7.8[b] (-8, -2.1)[c]	0%[a] / -8[b] (-8, -8)[c]
	***tet*(M)**	3%[a] / -7.8[b] (-8, -2.3)[c]	5%[a] / -7.8[b] (-8, -3.8)[c]	8%[a] / -7.5[b] (-8, -1.8)[c]	0%[a] / -8[b] (-8, -8)[c]	3%[a] / -7.9[b] (-8, -5.9)[c]	4%[a] / -7.8[b] (-8, -2.1)[c]
	***tet*(Q)**	13%[a] / -7.3[b] (-8, -1.4)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	3%[a] / -7.9[b] (-8, -3.6)[c]	6%[a] / -7.6[b] (-8, -1.2)[c]	7%[a] / -7.6[b] (-8, -1.9)[c]
	***tet*(S)**	28%[a] / -6.4[b] (-8, -1.3)[c]	35%[a] / -6.1[b] (-8, -1)[c]	17%[a] / -7[b] (-8, -1.4)[c]	19%[a] / -7[b] (-8, -1.7)[c]	52%[a] / -5.2[b] (-8, -1.1)[c]	36%[a] / -5.8[b] (-8, -1.2)[c]
	***tet*(W)**	6%[a] / -7.6[b] (-8, -1.4)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]
Sulfonamides	***sul*I**	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	3%[a] / -7.8[b] (-8, -1.8)[c]	0%[a] / -8[b] (-8, -8)[c]
	***sul*II**	3%[a] / -7.8[b] (-8, -2.3)[c]	5%[a] / -7.7[b] (-8, -2.7)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]	9%[a] / -7.4[b] (-8, -1.1)[c]	7%[a] / -7.6[b] (-8, -2.1)[c]
Aminoglycosides	***str***	0%[a] / -8[b] (-8, -8)[c]	5%[a] / -7.7[b] (-8, -2.2)[c]	0%[a] / -8[b] (-8, -8)[c]	10%[a] / -7.4[b] (-8, -0.9)[c]	0%[a] / -8[b] (-8, -8)[c]	0%[a] / -8[b] (-8, -8)[c]
	***aad*A**	3%[a] / -7.9[b]	5%[a] / -7.9[b]	4%[a] / -7.7[b]	10%[a] / -7.4[b]	0%[a] / -8[b]	0%[a] / -8[b]

		(-8, -3.7)[c]	(-8, -5.4)[c]	(-8, -0.8)[c]	(-8, -1.6)[c]	(-8, -8)[c]	(-8, -8)[c]
Phenicols	***cat*I**	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	3%[a] / -7.8[b]	0%[a] / -8[b]	7%[a] / -7.6[b]
		(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -1.7)[c]	(-8, -8)[c]	(-8, -1.4)[c]
	***cat*II**	9%[a] / -7.5[b]	5%[a] / -7.7[b]	4%[a] / -7.8[b]	6%[a] / -7.6[b]	6%[a] / -7.6[b]	14%[a] / -7.1[b]
		(-8, -2.4)[c]	(-8, -2)[c]	(-8, -2.2)[c]	(-8, -1.6)[c]	(-8, -1.9)[c]	(-8, -1.8)[c]
Macrolides	***erm*B**	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	3%[a] / -7.8[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -1.5)[c]	(-8, -8)[c]	(-8, -8)[c]
	***erm*F**	3%[a] / -7.8[b]	0%[a] / -8[b]	0%[a] / -8[b]	3%[a] / -7.8[b]	3%[a] / -7.8[b]	0%[a] / -8[b]
		(-8, -2)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -2)[c]	(-8, -2.2)[c]	(-8, -8)[c]
Quinolones	***qnr*S**	3%[a] / -7.8[b]	5%[a] / -7.7[b]	4%[a] / -7.7[b]	0%[a] / -8[b]	9%[a] / -7.5[b]	21%[a] / -6.6[b]
		(-8, -1.4)[c]	(-8, -2)[c]	(-8, -0.9)[c]	(-8, -8)[c]	(-8, -1.9)[c]	(-8, -1)[c]
	***qnr*B**	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]
Betalactams	***bla*$_{TEM}$**	16%[a] / -6.7[b]	5%[a] / -7.7[b]	4%[a] / -7.6[b]	6%[a] / -7.4[b]	9%[a] / -7.4[b]	25%[a] / -5.9[b]
		(-8, -0.8)[c]	(-8, -2.1)[c]	(-8, -1.7)[c]	(-8, -1.6)[c]	(-8, -0.6)[c]	(-8, -1.3)[c]
	***bla*$_{CTX-M}$**	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]
	***mec*A**	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]	0%[a] / -8[b]
		(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]	(-8, -8)[c]
Polymyxin	***mcr*-1**	0%[a] / -8[b]	15%[a] / -7[b]	0%[a] / -8[b]	3%[a] / -7.8[b]	9%[a] / -7.5[b]	21%[a] / -6.6[b]
		(-8, -8)[c]	(-8, 0.5)[c]	(-8, -8)[c]	(-8, -2)[c]	(-8, -1.7)[c]	(-8, -1.2)[c]

[a] % of individuals of species X positive to gene Y.
[b] Percentage load, expressed in logarithmic scale, of each antimicrobial gene regarding gene 16SrRNA.
[c] Range of minimum (-8) and maximum (+2) percentage load of genes detected in species X.

Supplemental table 7. Antimicrobial classes and multiresistant microbiomes found in ROA species.

Species	n	Antimicrobial class								Multiresistant microbiomes
		Tetracyclines	Sulfonamides	Aminoglycosides	Phenicols	Macrolides	Quinolones	Betalactams	Polymyxin	
magnificent frigatebird (*Fregata magnificens*)	32	75%	3.1%	3.1%	9.4%	3.1%	3.1%	15.6%	0%	6.3%
masked booby (*Sula dactylatra*)	20	70%	5%	10%	5%	0%	5%	5%	15%	5%
brown booby (*Sula leucogaster*)	24	75%	0%	4.2%	4.2%	0%	4.2%	4.2%	0%	0%
red-footed booby (*Sula sula*)	31	64.5%	0%	19.4%	9.7%	6.5%	0%	6.5%	3.2%	6.5%
sooty tern (*Onychoprion fuscatus*)	32	87.9%	12.1%	0%	6.1%	3%	9.1%	9.1%	9.1%	6.1%
brown noddy (*Anous stolidus*)	28	71.4%	7.1%	0%	21.4%	0%	21.4%	25%	21.4%	17.9%

Supplementary table 8. Primers used for real time PCR detection of selected ARGs.

Antimicrobial class	Gene	Sequence (5'-3')	Sense	Amplicon size (bp)	Reference
Tetracycline	*tet*(A)	GCGCTNTATGCGTTGATGCA	+	387	Jiang et al. 2013
		ACAGCCCGTCAGGAAATT	-		
	tet(B)	TACGTGAATTTATTGCTTCGG	+	206	Jiang et al. 2013
		ATACAGCATCCAAAGCGCAC	-		
	tet(Y)	ATTTGTACCGGCAGAGCAAAC	+	181	Jiang et al. 2013
		GGCGCTGCCGCCATTATGC	-		
	tet(K)	TCGATAGGAACAGCAGTA	+	169	Jiang et al. 2013
		CAGCAGATCCTACTCCTT	-		
	tet(M)	ACAGAAAGCTTATTATATAAC	+	171	Jiang et al. 2013
		TGGCGTGTCTATGATGTTCAC	-		
	tet(Q)	AGAATCTGCTGTTTGCCAGTG	+	169	Jiang et al. 2013
		CGGAGTGTCAATGATATTGCA	-		
	tet(S)	GAAAGCTTACTATACAGTAGC	+	169	Jiang et al. 2013
		AGGAGTATCTACAATATTTAC	-		
	tet(W)	GAGAGCCTGCTATATGCCAGC	+	168	Jiang et al. 2013
		GGGCGTATCCACAATGTTAAC	-		
Sulfonamides	*sul*I	CGCACCGGAAACATCGCTGCAC	+	163	Jiang et al. 2013
		TGAAGTTCCGCCGCAAGGCTCG	-		
	*sul*II	TCCGGTGGAGGCCGGTATCTGG	+	191	Jiang et al. 2013
		CGGGAATGCCATCTGCCTTGAG	-		
Aminoglycosides	*str*	AATGAGTTTTGGAGTGTCTCAACGTA	+	147	Wang et al. 2014
		AATCAAAACCCCTATTAAAGCCAAT	-		
	*aad*A	GCAGCGCAATGACATTCTTG	+	282	Devarajan et al., 2016
Phenicols		ATCCTTCGGCGCGATTTTG	-		
	*cat*I	GGTGATATGGGATAGTGTT	+	349	Jiang et al. 2013
		CCATCACATACTGCATGATG	-		
	*cat*II	GATTGACCTGAATACCTGGAA	+	567	Jiang et al. 2013

Macrolides	*erm*(B)	CCATCACATACTGCATGATG	-	364	Chen et al., 2007
		GATACCGTTTACGAAATTGG	+		
	erm(F)	GAATCGAGACTTGAGTGTGC	-	309	Chen et al., 2007
		CGACACAGCTTTGGTTGAAC	+		
Quinolones	*qnr*B	GGACCTACCTCATAGACAAG	-	263	Cummings et al., 2011
		GGMATHGAAATTCGCCACTG	+		
	*qnr*S	TTYGCBGYYCGCCAGTCGAA	-	118	Marti and Balcázar, 2013
		GACGTGCTAACTTGCGTGAT	+		
Betalactams	$bla_{\text{CTX-M}}$	TGGCATTGTTGGAAACTTG	-	591	Edelstein et al. 2004
		TTTGCGAT GTGCAGTACCAGTAA	+		
	bla_{TEM}	CGATATCGTTGGTGGTGCCATA	-	425	Devarajan et al., 2016
		AAAGATGCTGAAGATCA	+		
	*mec*A	TTTGGTATGGCTTCATTC	-	99	Francois et al. 2003
		CATTGATCGCAACGTTCAATTT	+		
Polymyxins	*mcr*-1	TGGTCTTTCTGCATTCCTGGA	-	218	Nieto-Claudin et al., 2019
		TGATACGACCATGCTCCAAA	+		
	16S rRNA	GCCACCACAGGCAGTAAAAT	-	352	Jiang et al. 2013
		ATGGCTGTCGTCAGCT	+		
		ACGGGCGGTGTGTAC	-		

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