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Research Article

Captive Snakes from Brazil as Carriers of Multidrug-Resistant Enterococci

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Abstract

Brazil has one of the most diverse herpetofauna and snakebites are an important health issue. The oral cavity of snakes harbored a wide range of bacteria. Enterococci have been isolated from animals, however, few studies have taken in snakes. In this sense, the present study aimed to evaluate *Enterococcus* spp. and their virulence attributes including antimicrobial resistance in oral cavities of healthy snake species in Brazil. Oral swabs from wild and

captive snakes were screened for enterococci distribution, antimicrobial susceptibility, resistance and virulence genes, and CRISPRs elements by PCR. Overall, 116 enterococci were detected and *Enterococcus faecalis* was dominant in all snake species, followed by *E. faecium*, *E. avium*, and *E. hirae*. Interestingly, no resistant enterococci were detected in wild snakes. In contrast, captive snakes were found to be carriers of resistant strains, including resistance to erythromycin, rifampicin, norfloxacin, ciprofloxacin, and tetracycline. *Enterococcus faecium* (50%) and *E. faecalis* (15.78%) isolates were multi-drug-resistant. Erythromycin resistance genes, the *msr*C and *erm*B, were detected in 13.33% and 6.67% of the isolates, respectively. The *tet*M (70%), *tet*L (30%) and *tet*S (10%) genes were detected in the tetracycline-resistant strains.

Among the virulence genes, *gel*E was the most frequent in all strains. CRISPR1-cas, orphan CRISPR2, and CRISPR3-cas elements were present in 16.03%, 15.79%, and 18.31% of the isolates, respectively. No antibiotic resistance was associated with CRISPRs. In conclusion, resistant enterococci in captive snakes are the result of confinement, antibiotic therapy and human contact. Resistant bacteria in captive snakes provide crucial information about public health safety.

Keywords: Enterococci; Maldi-TOF; Antimicrobial resistance; Virulence genes; CRISPRs; Snakes

1. Introduction

Snakes play an important role in maintaining balance in the ecosystem. The snakes diet ranges from invertebrates to vertebrates; in wildlife they eat a wide variety of animals including snails, insects, fish, frogs, lizards, snakes, amphibians, birds, rodents, bats, primates, and eggs of lizards and birds [1, 2]. Snakes are reptiles belonging to the order Squamata and sub-order Serpente. There are more than 3,900 species of snakes found in the world [3]. In Brazil, the diversity of ophidians is approximately 405 species, distributed into ten families: Anomalepididae, Leptotyphlopidae, Typhlopidae, Aniliidae, Tropidophiidae, Boidae, Viperidae, Elapidae, Colubridae e Dipsadidae [2, 4, 5]. These species are found in all Brazilian biomes, and some are kept in captive conditions, like zoos and serpent scientific breeders for poison extraction and subsequent production of antivenom [2, 4-6].

Not all snakes are venomous, in fact, 600 species are venomous and only 200 can kill or significantly wound a human. Snakebite envenoming is a major public health issue in the developing world; clinical reports have revealed that snakebites are a neglected public in many countries, with major impacts in Africa, Asia and Latin America [7]. According to data from the Brazilian Ministry of Health, during the period of 2009-2013, 144,060 snakebites were recorded in Brazil (an average of 28,812 cases per year), with an average mortality of 119 per year [8]. The deaths are caused by poisoning, as well as the snake mouth is colonized by bacteria that can be transmitted to the bitten patient through the skin injury associated with the bite, and may cause secondary infection along with envenomation [9]. Interesting, clinically relevant bacterial species have been found in the oral microbiota and bite wounds from snakes worldwide [10-12]. Diverse studies have revealed a mixture of both aerobic and anaerobic bacterial species in the oral cavity of snakes [13-16]. Panda et al. [17]

identified Gram-negative and Gram-positive bacteria, including clinical pathogens such as *Bacillus* spp., *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* in Indian cobra (*Naja naja*).

Enterococcus spp. are facultative anaerobic bacteria, belonging to the Phylum Firmicutes. Currently, the genus is composed of more than 50 species [18], with *E. faecalis* predominant in the gastrointestinal tract of humans and other mammals, followed by *E. faecium*, *E. hirae*, *E. durans*, *E. casseliflavus*, *E. gallinarum*, and *E. mundtii* [19]. These genera are also found in oral cavity and urinary tract of humans and other animals. They can also be found in different environments such as soil, water, sewage and plants [18]. However, enterococci are also important opportunistic pathogens for humans due to virulence factors and antibiotic resistance [20]. They represent the second most common cause of hospital-acquired infections, particularly affecting the urinary tract, wounds, and soft tissues. Researches have shown that enterococci species were isolated from human wound infections caused by dogs, cats, bears, and snake bites [15-21]. *Enterococcus* spp. were the most common pathogens isolated in infected bite wounds and oral microbiota of *Naja atra* in Taiwan [12]. Huang et al. [11], investigating bacterial infection associated with snakebites in central Taiwan, identified *Enterococcus* spp. as one of the most common pathogens. Chen et al. [10], analyzing snakebite from Northern Taiwan medical center, identified the *Enterococcus* spp. as the most frequently pathogens in the wound. In Brazil, group D streptococci (enterococci) were isolated in the abscesses at the site of *Bothrops* spp. bite [22].

Due to their remarkable ability to adapt to environmental conditions and ubiquity, enterococci have been used as sentinel organisms for tracking trends in resistance to antimicrobials [23]. Resistant enterococci have been isolated from captive and wild animals worldwide [24-30] and rare studies regarding snakes [15-21]. This could be justified by the difficulty to manipulate these animals, and also observing them in the wild environment since they make unseen movements in fields and forests [31]. Despite Brazil having one of the most diverse herpetofauna, studies evaluating bacteria in snakes' oral cavities are scarce, and most of them are associated with abscesses caused by bites of snakes [32-34]. This is the first study to report enterococci in the oral cavity of captive and wild snakes of several species in Brazil. We evaluated the antimicrobial susceptibility and virulence determinants of enterococci isolated from oral cavities of snake species in Brazil. The study intends to address if the snakes can be a reservoir of antibiotic-resistant enterococci that can spread through people and animals, contributing with information for public health safety.

2. Materials and Methods

2.1 Oral snakes samples collection

Fourteen oral swab samples were collected from wild and captive snake species (Table 1). Seven wild snakes were captured in the Pacotuba National Forest (FLONA- Pacotuba; 20º45'9.71"S, 41º17'21.27"W) – Espírito Santo state, and Caparaó National Park (20° 25'10"S, 41°48'54") – Serra do Caparaó, in the border between the states of Espírito Santo and Minas Gerais, southeastern Brazil. Sampling technique the active search (visual encounter survey protocol),

between March and May 2019, were used. Six different wild snakes species were captured: *Thamnodynastes strigatus*, *Leptophis ahaetulla*, *Pseudablabes patagoniesis*, *Oxyrhopus petolarius*, *Erythrolamprus poecilogyrus*, and *Bothrops jararaca*. After collection, the wild snakes were returned to nature.

Captive snakes (n = 7), belong to serpent scientific breeder of the Museum of Natural Sciences of the Rio Grande do Sul State Department of Environment and Infrastructure (MCN), Porto Alegre, Brazil, were handled using a snake hook, and the sampling were collected in January and May 2019 (Figure 1). To avoid adding a source of stress for the healthy snakes, the samples were collected during the routine procedures of the breeding facility, which follows all the international standards of animal welfare and biosecurity. Six different captive snake species were selected: *Philodryas olfersii*, *E. poecilogyrus*, *Oxyrhopus rhombifer*, *T. strigatus*, *Bothrops diporus* and *B. jararaca*.

Oral swabs were stored in Stuart transport medium (Oxoid™) and transported to the laboratory for microbiological analyses. The sampling was performed following regulations established by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), System Authorization and Information on Biodiversity (SISBIO) n° 300675 and n° 52838.

Table 1: Description of wild (FLONA de Pacotuba and Caparaó) and captive (MCN) snakes that oral samples were collected.

Habitat	Species (common name)	Family	N[1]	Collection	Diet
Wildlife/ FLONA	*Bothrops jararaca* (jararaca)	Viperidae	01	05/03/2019	Frogs, rodents [35]
	Erythrolamprus poecilogyrus (Goldbauch-Buntnatter)	Dipsadidae	01	05/03/2019	Frogs, fish, lizards and rodents [36]
	Leptophis ahaetulla (parrot snake)	Colubridae	01	04/24/2019	Frogs and lizards [37]
	Oxyrhopus petolarius (false-coral)	Dipsadidae	01	03/20/2019	Lizards, rodents and bird eggs [38]
	Pseudablabes patagoniensis (Patagonia green racer**)**	Colubridae	02	05/03/2019	Amphibians, frogs, birds, lizards, mammals, fish and snakes [35]
Wildlife/ Caparaó	*Thamnodynastes strigatus* (coastal house snake)	Dipsadidae	01	04/24/2019	Frogs, lizards and mammals [39]
Captive/ MCN	*Bothrops diporus* (jararaca-pintada)	Viperidae	02	01/13/2019	Wistar rats [40]
	Bothrops jararaca (jararaca)	Viperidae	01	01/13/2019	Wistar rats [40]
	Erythrolamprus poecilogyrus (Goldbauch-Buntnatter)	Dipsadidae	01	05/24/2019	Fish [40]
	Oxyrhopus rhombifer (Amazon false coral snake)	Dipsadidae	01	05/19/2019	Wistar rats [6]
	Philodryas olfersii (South American green racer)	Colubridae	01	01/13/2019	Wistar rats [40]
	Thamnodynastes strigatus (coastal house snake)	Dipsadidae	01	01/20/2019	Wistar rats [40]

1. N: number of animals

Figure 1: Oral sample collected from captive *Bothrops diporus* of the Museum of Natural Sciences of the Rio Grande do Sul State Department of Environment and Infrastructure (MCN), Porto Alegre, Brazil. Photo: Juliana Morais da Silva Heck.

2.2 Isolation and identification of enterococci from the oral cavities of captive and wild snakes

Oral swabs were pre-processed according to Prichula et al. [27]. Twenty colony-forming units were randomly selected from each sample. Phenotypic criteria, such as size/volume, shape, color, Gram staining, catalase production, capacity to growth at 45 °C and bile aesculine reaction, were used to separate the enterococci group and the non-enterococcal strains [41].

Selected pure colonies were stored in a stock solution of skin milk 10% (Difco, Sparks, MD, USA) and 10% glycerol (Neon Comercial Ltda, São Paulo, SP, BR) at -20 °C. Collected bacteria were identified by matrix-assisted laser ionization and desorption technique (MALDI-TOF) applied to *Enterococcus*, according to Sauget et al. [42]. MALDI-TOF analysis was performed using a LT Bruker microflex mass spectrometer (Bruker Daltonik GmbH) and spectra were automatically identified using BrukerBioTyper ™ 1.1 software.

Strains not identified by MALDI-TOF were submitted to species-specific PCR assay. Total DNA extraction was carried out by a physical-chemical method [43],

with a total volume of 25 µL, containing: 100 ng of DNA template, 1X PCR buffer (10 mM Tris–HCl [pH 9.0], (Invitrogen, Carlsbad, CA, USA), 1.5 mM of $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 200 µM of dNTPs (Ludwig Biotecnologia), 0.4 µM of each primer (Invitrogen, Carlsbad, CA, USA), 1.0 U of *Taq* polymerase (Invitrogen®). PCR conditions for all amplification reactions were as follows: initial denaturation at 94 °C for 5 min.; followed by 35 cycles of denaturation at 94 °C for 1 min.; the appropriate annealing temperature for each species (as listed in Supplementary Table 1) for 1 min.; extension at 72 °C for 1 min.; and final extension at 72 °C for 5 min.

2.3 Antibiotic resistance profiles of enterococci strain isolated from oral samples of snakes

All strains were screened for antibiotic susceptibility by Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute [44]. Eleven antibiotics commonly used in clinical and veterinary medicine were evaluated: ampicillin 10 µg (AMP), ciprofloxacin 5 µg (CIP), chloramphenicol 30 µg (CHL), erythromycin 15 µg (ERI), gentamicin 120 µg (GEN), nitrofurantoin 300 µg (NIT), norfloxacin 10 µg (NOR), rifampicin 5 µg (RIF), streptomycin 300 µg (EST), tetracycline 30 µg (TET) and vancomycin 30 µg (VAN). Minimum inhibitory concentration (MIC) of vancomycin was determined by broth microdilution and interpretation of the results was performed following CLSI guidelines [45]. *Staphylococcus aureus* ATCC 25923 and *E. faecalis* ATCC 29212 strains were used as quality control of disks. Isolates that showed a resistance profile to one, two, and three or more classes of antimicrobials were classified as: single-resistant (SR), double-resistant (DR), and multidrug-resistant (MDR), respectively [46]. Intermediate-resistant strains were grouped in the resistant strains.

2.4 Detection of virulence, resistance-associated genes and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) in enterococci by PCR

The presence of virulence genes, such as *ace* (adhesin to collagen of *E. faecalis*), *cyl*A (cytolysin) and *gel*E (gelatinase) was determined in all enterococcal isolates. On the other hand, only erythromycin- and tetracycline- resistance phenotypes were examined for the presence of macrolide (*erm*B and *msr*C) and tetracycline (*tet*L, *tet*M and *tet*S) resistance genes, respectively. PCR reactions followed the protocol described by Santestevan et al. [28]. Primers are described in Supplementary Table 1, with the appropriate annealing temperatures.

The presence of Type II CRISPRs elements (CRISPR1-cas, CRISPR2-orfan, and CRISPR3-cas) were investigated by PCR in all enterococcal samples. Primers for CRISPRs genes reported by Palmer and Gilmore [45] were used in PCR reactions. The primers and annealing temperatures used are listed in Supplementary Table 1. The PCR was performed as described by Huescas et al. [47].

3. Results

3.1 Enterococci species in the oral cavities of captive and wild snakes species from Brazil

A total of 116 enterococci (64 from wild and 52 from captive snakes) were recovered from 13 oral samples of snakes belonging to the species including *T. strigatus*, *L. ahaetulla*, *P. patagoniesis*, *O. rhombifer*,

O. petolarius, *P. olfersii*, *B. diporus and B. jararaca*. Only in one sample of captive snake belonging to *E. poecilogyrus* species was not detected enterococci.

As result, among the 116 *Enterococcus* spp. recovered, the most frequently isolated species were *E. faecalis* (78.45%), followed by *E. faecium* (12.07%), *E. avium* (6.03%), and *E. hirae* (3.45%).

Differences in the distribution of enterococci species were detected amongst the two groups of snakes, as shown in Figure 2. Among the 64 enterococci isolates from wild snakes, the species *E. faecalis* (82.81%; n = 53), *E. avium* (10.93%; n = 7), and *E. hirae* (6.25%; n = 4) were identified. On the other hand, 52 enterococci were isolated from captive snakes belonging to *E. faecalis* (73.07%; n = 38) and *E. faecium* (26.92%; n = 14).

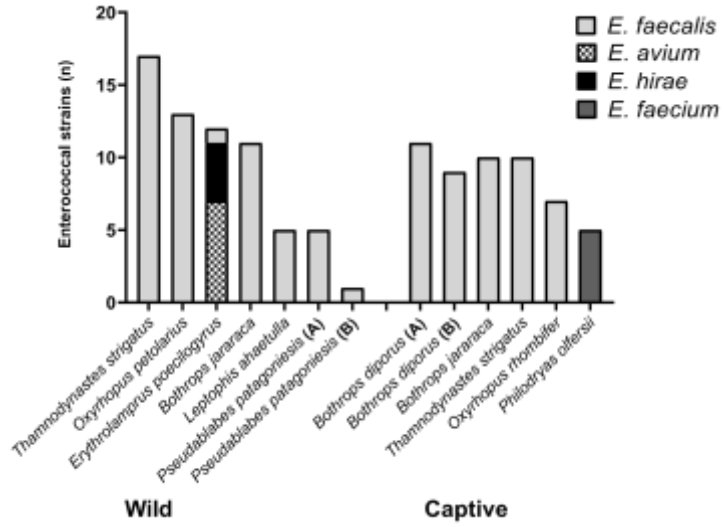

Figure 2: Distribution of *Enterococcus* species between wild and captive snake species.

3.2 Resistance profile in enterococci from wild and captive snakes

The enterococci isolated from wild snakes were susceptible to all antimicrobial agents tested. In contrast, of the 52 strains isolated from captive snakes, 45 (86.53%) were resistant to at least one antimicrobial agent tested. Strains showed resistance to erythromycin (57.69%), rifampicin (50%), ciprofloxacin/norfloxacin (30.77%), tetracycline (19.23%), nitrofurantoin (13.46%), and chloramphenicol (5.77%).

The percentages of DR and MDR strains isolated were 32.69% and 25%, respectively (Table 2). Of the 13 MDR strains, six (15.78%) were *E. faecalis* and seven (50%) were *E. faecium*. Interesting, one *E. faecalis* isolated from captive *B. diporus* showed resistance to six different antimicrobials tested (norfloxacin; chloramphenicol; erythromycin; nitrofurantoin; rifampicin; tetracycline) (Table 3).

Table 2: Antimicrobial resistance profiles among enterococci isolated from oral samples of captivity snakes.

Strains (*n*)	**Number (%) of resistant strains[1]**						**Profiles[2]**		
	CIP/NOR	**CHL**	**ERY**	**NIT**	**RIF**	**TET**	**SR**	**DR**	**MDR**
E. faecalis (38)	11 (28.95)	3 (7.89)	21 (55.26)	6 (15.79)	18 (47.37)	6 (15.79)	11 (28.94)	16 (42.10)	6 (15.78)
E. faecium (14)	5 (35.71)	0	9 (64.29)	1 (7.14)	8 (54.14)	4 (28.57)	3 (21.42)	1 (7.14)	7 (50)
Total (52)	16 (30.77)	3 (5.77)	30 (57.69)	7 (13.46)	26 (50)	10 (19.23)	14 (26.92)	17 (32.69)	13 (25)

[1]Antimicrobials: CIP/NOR, ciprofloxacin/norfloxacin; CHL, chloramphenicol; ERY, erythromycin, NIT, nitrofurantoin; RIF, rifampicin, TET, tetracycline.

[2]Profiles: SR, single-resistant; DR, double-resistant; MDR, multidrug-resistant.

Table 3: Antimicrobial resistance phenotypic profile of *Enterococcus* spp. isolated from oral samples of captive snakes.

		Number of resistant enterococci by snake species					
Profile[1]	**Antimicrobials[2]**	**Species**	**B.d[3]**	**B.j[4]**	**O.r.[5]**	**P.o[6]**	**T.s[7]**
SR	RIF	*E. faecalis*		3	1		
		E. faecium	1				
	TET	*E. faecalis*					
	ERY	*E. faecalis*			1		3
		E. faecium				1	
	NIT	*E. faecium*				1	
	NOR-CIP	*E. faecalis*		1			2
DR	ERY/RIF	*E. faecalis*	7				
	CLO/NOR	*E. faecalis*					1
	ERI/NIT	*E. faecalis*		1			1
	CLO/ERY	*E. faecalis*	1				
	ERY/NOR	*E. faecalis*			1		
	RIF/NIT	*E. faecalis*			1		
	RIF/NOR	*E. faecalis*		1			1
	RIF/TET	*E. faecalis*		1			
	TET/ERY	*E. faecium*	1				
MDR	TET/RIF/ERI	*E. faecium*	2				
	RIF/ERY/CIP-NOR	*E. faecium*	4				
		E. faecalis	2	1			1
	TET/RIF/ERY/NOR	*E. faecium*	1				
	TET/RIF/ERI/NIT	*E. faecalis*					1
	TET/RIF/CLO/ERY/NOR/NIT	*E. faecalis*	1				

1. SR: single-resistant; DR: double-resistant; MDR: multidrug-resistant. 2. Antimicrobials: ERY, erythromycin; CIP, ciprofloxacin; NOR, norfloxacin; RIF, rifampicin; NIT, nitrofurantoin; CHL, chloramphenicol; TET, tetracycline. 3. B.d: *Bothrops diporus* (jararaca-pintada); 4. B.j: *Bothrops jararaca* (jararaca); 5. O.r: *Oxyrhopus rhombifer* (Amazon false coral snake); 6. P.o: *Philodryas olfersii* (South American green racer) and 7: T.s.: *Thamnodynastes strigatus* (coastal house snake).

3.3 Occurrence of resistance and virulence-associated genes and Clustered Regularly Inter-spaced Short Palindromic Repeats (CRISPRs) in enterococci

The frequency of erythromycin-resistant strains (n = 30) positive for the *erm*B and *msr*C genes were 6.67%

(n = 2) and 13.33% (n = 4), respectively (Supplementary Table 2). Among the 10 tetracycline-resistant enterococci, seven (70%) were positive to *tet*M gene, three (30%) to *tet*L gene, and one (10%) to *tet*S gene (Supplementary Table 2).

Virulence genes were detected among all enterococci species. The *gel*E was the most frequent (59.48%; n = 69), followed by *ace* (57.76%; n = 67), and *cyl*A (1.72%; n = 2). The *gel*E gene presented a higher percentage in wild snakes, while ace and *cyl*A genes showed a similar frequency between the snakes Supplementary Table 3).

CRISPR1-cas, orphan-CRISPR2, and CRISPR3-cas elements were positive in 16.03%, 15.79%, and 18.31% of the strains, respectively (Table 4). The orphan-CRISPR2 was detected at a low frequency in enterococci strains collected from captive snakes and CRISPR3-cas in wild snakes. CRISPR1-cas was found in similar frequency among the strains. No antibiotic resistance was associated with CRISPRs elements.

Table 4: Number (%) of CRISPRs elements identified in enterococci isolated from oral samples of wild and captive snakes.

		Number (%) CRISPRs elements		
Habitat	**Species (n)**	**I**	**II**	**III**
Captive	*E. faecalis* (38)	2 (5.26)	2 (5.26)	11 (2.94)
	E. faecium (14)	8 (57.14)	0	3 (21.42)
	Subtotal (52)	10 (19.23)	2 (3.84)	14 (26.92)
Wildlife	*E. avium* (7)	0	0	0
	E. hirae (4)	1 (25)	0	0
	E. faecalis (53)	6 (11.32)	16 (30.18)	5 (9.43)
	Subtotal (64)	7(10.93)	16 (25)	5 (7.81)
	Total (116)	17(14.65)	18 (15.51)	19 (16.37)

4. Discussion

4.1 Enterococci species occurrence and distribution in oral cavities of captive and wild Brazilian snake species

In this study, we detected the enterococci genus, bacteria of clinical relevance known as multidrug-resistant nosocomial pathogens, in snake species from Brazil. A few studies have previously examined the oral microorganisms from captive and wild Brazilian snake species [33, 34, 48]. Fonseca et al. [33] detected the presence of diverse bacterial, including clinical pathogens such as coagulase-negative staphylococci, *Bulkolderia* sp., *Moraxella* sp., *Proteus* sp., *S. aureus*, and *Yersinia enterocolitica* in oral samples of several captive snakes species. Jorge et al. [34] detected the presence of group D streptococci (*Enterococcus* spp.)

in oral samples of *B. jararaca*. Currently, in relation to wild snakes, there is only one study that isolated *Pseudomonas aeruginosa* and *Proteus vulgaris* from oral samples of *Crotalus durissus terrificus* snakes in Brazil [48].

Enterococcus faecalis was the most common enterococcal species detected in oral samples of captive and wild snakes in this study. The results observed here are in agreement with the literature, Padhi et al. [13] identified *E. faecalis* as the most frequent enterococci species in the oral cavity of free-living vipers (*Echis carinatus*) in Orissa, India. Plentz et al. [49] collected 46 samples from boid snake species and also identified *E. faecalis* as one of the most frequent species in oral and traqueal samples of *Python bivittatus*. A microbiological study carried out by Gatti et al. [50] in Argentina analyzed the oral cavity of free-living *B. alternatus*, *B. neuwiedi*, *B. ammodytoides*, *B. jararaca* and *B. jararacussu* and found 37 bacterial strains; among them, six were *E. faecalis* and one *Enterococcus* sp. The other enterococci species isolated here have already been found in samples of amphibians, reptiles, mammals, and birds [18, 27, 28, 30].

The diet of snakes ranges from invertebrates to vertebrates, and varies widely among species, some being generalist and preying on a wide variety of prey categories, while others are highly specialized [1, 2, 36]. There is a distinct difference between the snakes diet of captive and wild snakes. One of the greatest differences is the availability of food variety or lack of it. Whereas in the wild they have high dietary diversity, in captivity they are fed with a low dietary diversity composed of small rodents (Wistar rats) or fish. These differences in the diet may have contributed to the distribution of enterococci species among the snakes evaluated in this study.

4.2 Multidrug-resistant enterococci in captive snakes and absence of resistant strains in wild snakes

The antimicrobial susceptibility profile showed that only captive snakes revealed resistant enterococci colonizing the oral cavity. The absence of resistant enterococci in samples from wild snakes may be associated with two factors in the wildlife: (i) the snakes can go without eating for about six months, thus reducing exposure to microorganisms; and (ii) the snakes try to avoid human contacts, being less exposed to impacts of anthropogenic activities. Our findings were consistent with other studies that evaluated the antimicrobial susceptibility of bacteria isolated from the oral cavity of wild snakes [51-53]. Shaikh et al. [51] also observed that Gram-positive and Gram-negative bacteria isolated from venomous snakes, in India, were susceptible to antimicrobials. Artavia-León et al. [52] found that the vast majority of wild snake isolates in Costa Rica showed antibiotic susceptible microorganisms. A recent study with presumed *Naja* spp. bites in Vietnam found large amounts of susceptible *E. faecalis* strains isolated from local wounds [53].

However, as evidenced in this work, captive snakes revealed multidrug-resistant enterococci colonizing the oral cavity. The occurrence of MDR strains has been associated with the proximity of animals to human activities, since enterococci are sentinel species [24, 54]. In the captive environment, feeding, use of antibiotics in a therapeutic manner, and human

contact may have a major impact on the resistance of enterococci from captivity snakes. Other studies have associated resistant-enterococci isolated from animals with the proximity of human activities and/or to the environmental resistance [25-28, 55-57]. Previous studies examining the oral microbiota of captive snakes found high incidences of antibiotic resistance traits [17, 58, 59]. In India, *N. naja* captured from various localities (households) of Odisha were found to be harbouring antibiotic-resistant bacteria [17].

As shown by Hejnar et al. [58], resistant *Steno-trophomonas maltophilia* strains were isolated from captive snakes. Besides, *Salmonella enteritidis* isolated from edible snakes showed resistance to most drugs, but susceptibility to tetracycline and amikacin [59].

The emergence of MDR clinical pathogens such as enterococci are well-recognized to be one of the most important current public health issues [60]. Broad spectrum antibiotics are usually prescribed following snakebite and wound infection after cobra bites worldwide. Prophylactic antibiotic administration in snake bitten patients is recommended to prevent secondary infections from animal bites, and according to international guidelines amoxicillin-clavulanate is recommended [61].

However, to avoid the selection of pathogenic bacteria resistant to drugs, studies have been showing that antibiotic administration in snake bitten patients should be considered only in those with severe local signs of envenomation, or empiric use in those having local or general signs of infection, regardless of the degree of envenoming [61].

4.3 Determinants of virulence and antibiotic resistance genes in enterococci isolated from wild and captive snakes from Brazil

Tetracycline and erythromycin are prescribed in veterinary medicine [62, 63]. The isolation of tetracycline and erythromycin-resistant enterococci in captive snakes can be related to the administration of antibiotics in these animals, as well as in rodents. In the present study, *tet*L, *tet*M and *tet*S genes were detected in tetracycline-resistant and *erm*B and *msr*C genes were present in erythromycin-resistant enterococci strains. The frequency of these genes detected in the present study is congruent with the results obtained in previous studies conducted on *Enterococcus* strains isolated from wild and captive animals [24, 27, 28, 54].

Genes likely important for colonization in many contexts, but also studied for coding virulence traits were revealed in this study. The *gel*E gene was detected in enterococci from samples of snakes of the both groups, although it was more prevalent in wild snakes while *ace* and *cyl*A genes had a similar prevalence in both groups. Our data corroborate other studies that recovered *E. faecalis* isolated from diverse origins over the past 100 years and showed a prevalence of the *gel*E and *ace* genes in genomes of clinical and environmental strains [26]. The presence of *ace* genes may be associated with the permanence of strains in the oral cavity of snakes, as it encodes an adhesion to collagen, aiding in the colonization and permanence of host cells. In contrast, the low frequency of the *cyl*A gene in the analyzed samples corroborates with recent studies that recovered enterococci for animals, such as mammals [30], reptiles [27], birds [26-27] and insects [25]. The

virulence genes in the snake strains analyzed in this study may demonstrate a symbiotic characteristic between strains and the host.

In clinical, MDR *E. faecium* and *E. faecalis* are associated with CRISPR defects [18, 45]. In this study, we observed that there was not a direct association between the absence of CRISPR–Cas and the presence of resistance in enterococci isolated from captive snakes. Therefore, further studies involving the analysis of the whole genome sequencing of these isolates might elucidate the genetic aspects of CRISPRs in enterococci strains isolated from captive and oral snake species in Brazil.

5. Conclusion

In conclusion, this work advances our understanding of the nature and ecology of enterococci in wild and captive snake species in Brazil. Our data showed that enterococci seem to be a natural member of the oral microbiota of these animals, although the presence of resistance traits in captive animals indicate that human contact and confinement may be important factors in the spread of resistant enterococci. Therefore, further studying monitoring the resistant strains on the oral cavity of these animals constitutes important for snakebite management to determine public health safety plans.

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Declaration of Competing Interest

None to be declared

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Supplementary Material

Supplementary Table 1: Primers used in the PCR reactions carried out for detection of enterococci species (*E. faecalis* and *E. faecium*), resistance (*erm*B, *msr*C, *tet*L, *tet*M, *tet*S), virulence (*ace*, *cyl*A, and *gel*E), and CRISPRs genes (*CRISPR*1, *CRISPR*2, and *CRISPR*3).

	Gene	Nucleotide sequence (5′-3′)	AT[1] (°C)	Size (bp[2])	Reference
E. faecalis	E16s-F	CCGAGTGCTTGCACTCAATTGG	66	136	[64]
	E16s-R	CTCTTATGCCATGCGGCATAAAC			
E. faecium	EM1A-F	TTGAGGCAGACCAGATTGACG	62	172	[65]
	EM1B-R	CGGAAGTGATGCTTCCTACTG			
Erythromycin	ermB_F	GAAAAGGTACTCAACCAAATA	52	547	[66]
	ermB_R	AGTAACGGTACTTAAATTGTTTAC			
	msrC 3	AAGGAATCCTTCTCTCTCCG	52	343	[67]
	msrC 4	GTAAACAAAATCGTTCCCG			
Tetracycline	tetL_F	ACTCGTAATGGTGTAGTTGC	58	625	[68]
	tetL_R	TGTAACTCCGATGTTTAACACG			
	tetM_F	GTTAAATAGTGTTCTTGGAG	52	657	[69]
	tetM_R	CTAAGATATGGCTCTAACAA			
	tetS_F	TGGAACGCCAGAGAGGTATT	58	720	[69]
	tetS_R	ACATAGACAAGCCGTTGACC			
Adhesion	ace1_F	AAAGTAGAATTAGATCACAC	57	320	[29]
	ace2_R	TCTATCACATTCGGTTGCG			
Cytolysine	cylA_TE17	TGGATGATAGTGATAGGAAGT	54	517	[70]
	cylA_TE18	TCTACAGTAAATCTTTCGTCA			
Gelatinase	gelE_TE9	ACCCCGTATCATTGGTTT	50	402	[71]
	gelE_TE10	ACGCATTGCTTTTCCATC			
CRISPRs	crispr1_F	CAGAAGACTATCAGTTGGTG	55	783	[52]
	crispr1_R	CCTTCTAAATCTTCTTCATAG			
	crispr2_F	CTGGCTCGCTGTTACAGCT	55	variable	[52]
	crispr2_R	CCAATGTTACAATATCAACCA			
	crispr3_F	GCTGAATCTGTGAAGTTACTC	50	258	[52]
	crispr3_R	CTGTTTTGTTCACCGTTGGAT			

[1]AT: annealing temperatures; [2]bp: base pair.

Supplementary Table 2: Distribution of erythromycin- and tetracycline-resistance genes in the enterococci isolated from oral samples of captivity snakes.

Specie	**Number (%) of strains positive for resistance genes**						
	Erythromycin			**Tetracycline**			
	R*	*msr*C	*erm*B	R*	*tet*L	*tet*M	*tet*S
E. faecalis	21	3 (14.29)	2 (9.52)	6	1 (16.67)	3 (50)	1 (16.67)
E. faecium	9	1 (11.11)	0	4	2 (50)	4(100)	0
Total	30	4 (13.33)	2 (6.67)	10	3 (30)	7 (70)	1 (10)

*R, number of resistant strains.

Supplementary Table 3: Number (%) of virulence genes among enterococci isolated from oral samples of wild and captive snakes.

Habitat	**Strains (*n*)**	**Number (%) of positive enterococci**		
		ace	***cyl*A**	***gel*E**
Wildlife	*E. avium* (7)	4 (57.14)	0	6 (85.71)
	E. hirae (4)	0	0	4 (100)
	E. faecalis (53)	32 (60.38)	1 (1.89)	42 (79.25)
	Subtotal (64)	36 (56.25)	1 (1.56)	52 (81.25)
Captive	*E. faecalis* (38)	21 (55.26)	1 (2.63)	13 (34.21)
	E. faecium (14)	10 (71.43)	0	4 (28.57)
	Subtotal (52)	31 (59.62)	1 (1.92)	17 (32.69)
	Total (116)	67 (57.76)	2 (1.72)	69 (59.48)