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Antimicrobial resistance and arsenic tolerance in *Enterococcus* spp. isolated from free-ranging *Leopardus geoffroyi* in Candiota, Pampa Biome, Brazil: sentinel indicators of environmental pollution

[Resistência a antimicrobianos e tolerância ao arsênio em *Enterococcus* spp. isolados de *Leopardus geoffroyi* de vida livre em Candiota, no bioma Pampa, Brasil: sentinelas da poluição ambiental]

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Abstract: The Pampa Biome harbors a high diversity of endemic species but has experienced substantial pressure from anthropogenic activities that threaten wildlife conservation, including Geoffroy's cat (*Leopardus geoffroyi*). Within this context, this study evaluated antimicrobial-resistant and heavy-metal-tolerant enterococci isolated from oral and rectal cavities of 14 free-ranging Geoffroy's cats (*L. geoffroyi*) from Seival–Candiota region in Brazilian Pampa as indicators of environmental quality. Isolation was conducted on selective media, species identification was performed using MALDI-TOF mass spectrometry, and antimicrobial susceptibility was assessed by disk diffusion against 12 antimicrobials used in human and veterinary medicine. Antimicrobial resistance genes (*msrC, ermB, tetM, and tetL*) and heavy-metal tolerance genes (*arsA_I, arsA_II, and tcrB*) were investigated by PCR. A total of 111 enterococcal isolates were recovered. *Enterococcus faecium* (37.8 %) and *Enterococcus faecalis* (29.7 %) predominated, showing distinct distribution between oral and rectal samples. Overall, 74.8 % of isolates were resistant to at least one antimicrobial, mainly rifampicin (41.4 %), erythromycin (34.2 %), and ciprofloxacin (26.1 %). No resistance to gentamicin or vancomycin was detected. Multidrug resistance occurred in 17 isolates (20.5 %). Resistance determinants (*msrC, ermB, tetM, and tetL*) and arsenic tolerance gene *arsA_I* were identified, whereas *arsA_II* and *tcrB* were not detected. Findings demonstrate the presence of multidrug-resistant and arsenic-tolerant enterococci in free-ranging Geoffroy's cats, supporting their role as sentinel species of environmental quality and evidencing the influence of anthropogenic activities on animal health.

Keywords: Anthropogenic impact; environmental bioindicators; wildlife conservation; one health.

Resumo: O bioma Pampa abriga uma notável diversidade de espécies endêmicas, mas vem sofrendo intensos impactos decorrentes de atividades antrópicas, que ameaçam a conservação de espécies silvestres, tais como o gato-do-mato-grande (*Leopardus geoffroyi*). Nesse contexto, este estudo avaliou a resistência a antimicrobianos e a tolerância a metais pesados em enterococos isolados das cavidades oral e retal de 14 gatos-do-mato-grande (*L. geoffroyi*) de vida livre da região do Seival/ Candiota, no bioma Pampa, como bioindicadores da qualidade ambiental. O isolamento foi realizado em meios seletivos, a identificação das espécies foi feita por MALDI-TOF, e a suscetibilidade foi determinada pelo método de disco-difusão frente a 12 antimicrobianos de uso clínico e veterinário. Genes de resistência a antimicrobianos (*msrC, ermB, tetM e tetL*) e de tolerância a metais pesados (*arsA_I, arsA_II e tcrB*) foram investigados por PCR. Um total de 111 isolados de enterococos foi recuperado. *Enterococcus* faecium (37,8 %) e *Enterococcus faecalis* (29,7 %) predominaram, apresentando distribuição distinta entre amostras orais e retais. No geral, 74,8 % dos isolados apresentaram resistência a pelo menos um antimicrobiano, principalmente à rifampicina (41,4 %), à eritromicina (34,2 %) e à ciprofloxacina (26,1 %). Não foi detectada resistência à gentamicina nem à vancomicina. A resistência a múltiplos antimicrobianos foi observada em 17 isolados (20,5 %). Determinantes de resistência (*msrC, ermB, tetM e tetL*) e o gene de tolerância ao arsênio (*arsA_I*) foram identificados, enquanto *arsA_II* e *tcrB* não foram detectados. Em conclusão, a presença de enterococos resistentes a múltiplos antimicrobianos e tolerantes ao arsênio em gatos-do-mato-grande de vida livre reforça seu uso como bioindicadores da qualidade ambiental e evidencia o impacto das atividades antrópicas na saúde animal.

Palavras-chave: Impactos antropogênicos; bioindicadores ambientais; conservação da vida silvestre; saúde única.

1. Introduction

The Pampa Biome represents valuable natural and cultural heritage because of its unique biodiversity and ecosystem services. Covering approximately 700,000 km², this grassland formation extends across southern Brazil, Argentina, and Uruguay [1]. In Brazil, the biome occurs exclusively in Rio Grande do Sul State, occupying about 193,836 km², which corresponds to 69 % of the state's territory and 2.3 % of the national territory [2]. The Pampa holds high environmental relevance, as it supports a rich diversity of endemic fauna, including more than 500 bird species and over 100 terrestrial mammal species [3]. Despite its importance for biodiversity conservation, the biome has experienced substantial loss of native vegetation over the years. Estimates indicate that original vegetation cover declined from 41.32 % in 2002 to 36.03 % in 2008 [4]. Accelerated landscape degradation, mainly driven by agricultural expansion (soybean and rice cultivation) and industrial development (mining and pulp production), has resulted in marked reductions in native fauna and flora.

Within this context, impacts of anthropogenic activities on Pampa ecosystems have been widely documented and monitored [5], reinforcing the urgency of conservation initiatives. The Pampa's Felines Project was therefore established to evaluate the effects of human pressures on wild felids, operating in collaboration with Geoffroy's Cat Working Group, an international research and conservation network [6]. Project activities are concentrated in Brazilian Pampa, where Geoffroy's cat (*Leopardus geoffroyi*) represents one of the flagship species. Although globally classified as Least Concern [7], this species is considered Vulnerable at national [8] and regional levels [9]. Major threats include habitat fragmentation, which increases exposure to human-modified landscapes and heightens risks of road mortality, retaliatory hunting, and pathogen transmission from domestic animals [10–12].

Beyond direct conservation threats, detection of subtle environmental impacts on host microbiota represents an additional critical dimension. In this sense, gut microbial communities may reflect host physiological status and environmental pressures, thereby functioning as indicators of ecosystem disturbance and anthropogenic influence [13,14]. Among microorganisms used as bioindicators, genus *Enterococcus* has received particular attention [15–21]. *Enterococcus* species are considered commensal inhabitants of gastrointestinal tract, also being found in the genitourinary tract and genitourinary tracts and oral cavity of humans and animals, but are also widely distributed in environmental matrices such as soil, water, and food [22–28]. Molecular and phylogenetic evidence indicates that the genus comprises more than 90 species; among these, *E. faecalis, E. faecium, E. hirae, E. durans, E. casseliflavus, E. gallinarum*, and *E. mundtii* are frequently detected in the animal gastrointestinal microbiota [29]. In recent years, increasing attention has been directed toward characterization of enterococcal diversity, antimicrobial resistance profiles, and heavy-metal tolerance in isolates obtained from wildlife. These studies have emphasized the potential of enterococci as sentinel organisms for monitoring animal and ecosystem health in potentially contaminated environments [23–28,30–33].

Moreover, despite the ecological importance of Pampa ecosystems and conservation concerns surrounding species such as Geoffroy's cat (*L. geoffroyi*), studies characterizing intestinal and oral microbiota from an environmental bioindicator perspective remain limited. Therefore, understanding this interface is essential for elucidating the consequences of human pressures and for supporting conservation strategies aligned with One Health principles. Based on the above, the present study aimed to characterize antimicrobial susceptibility and heavy-metal tolerance in *Enterococcus* spp. isolated from oral and rectal cavities of free-ranging Geoffroy's cats captured in the Seival region, in the municipality of Candiota, within the Brazilian Pampa. Ultimately, it also seeks to explore their applicability as bioindicators of environmental quality in natural habitats occupied by this species.

2. Material and methods

2.1 Study site – the Brazilian Pampa biome: municipality of Candiota

The municipality of Candiota is in southern Rio Grande do Sul State, approximately 387 km from the capital, Porto Alegre. It covers a total area of 933.628 km² and has an estimated population of 10,710 inhabitants, corresponding to a population density of 11.47 inhabitants per km² [34]. Of this area, about 73,234.754 hectares are used for agricultural activities, representing 78.4 % of the municipal territory. Local economic activity is strongly influenced by thermoelectric power generation, supported by extensive coal reserves in underlying geological formations. The municipality currently hosts three major mining and thermoelectric enterprises, in addition to cement production facilities associated with one of the largest global industrial conglomerates in this sector. These activities position Candiota as a strategic energy production hub at national level. Livestock production and crop farming also contribute substantially to local economy, particularly soybean and rice cultivation [35].

2.2 Sampling and collection of biological material from *L. geoffroyi*

Fourteen free-ranging Geoffroy's cats (*L. geoffroyi*) were captured between June 2022 and February 2023 in the Seival region, a district of the municipality of Candiota, Rio Grande do Sul State, Brazil (Supplementary Table 1). Capture procedures were conducted within framework of the Pampa Felines Project and followed methodological protocols described by Tirelli et al [12], as well as guidelines for handling wild mammals proposed by Sikes et al [36]

Capture and sampling activities were authorized by Chico Mendes Institute for Biodiversity Conservation (ICMBio) under license ICMBio/SISBIO-81869-2 for strictly scientific purposes. All animal procedures were previously reviewed and approved by Animal Use Ethics Committee of the Federal University of Rio Grande do Sul (CEUA-UFRGS), according to protocol CEUA/UFRGS-42867.

Oral and rectal swab samples were collected from each individual, immediately placed in Stuart transport medium, and transported under refrigerated conditions (4 °C) to Wildlife Microbiology Laboratory at Institute of Basic Health Sciences, UFRGS. Samples were maintained under refrigeration until microbiological analyses were performed.

2.3 Isolation and phenotypic characterization of *Enterococcus* spp.

For selective isolation of bacteria belonging to genus *Enterococcus*, oral cavity (OC) and rectal cavity (RC) swabs were immersed in tubes containing 3 mL of Azide Dextrose Broth (HiMedia®, India) and incubated at 37 °C for 24 h. Subsequently, serial decimal dilutions were prepared in sterile saline solution (0.85 % NaCl). Aliquots of 100 µL from 10^{-4} and 10^{-5} dilutions were spread onto Brain Heart Infusion (BHI) agar supplemented with 4.5 % NaCl using surface-spreading technique and incubated at 37 °C for 24 h. After incubation, six colony-forming units (CFU) were randomly selected and subcultured onto Bile Esculin agar plates, followed by incubation under same conditions. Colonies showing esculin hydrolysis, evidenced by medium darkening, were subsequently subcultured onto BHI agar and incubated at 37 °C for 24 h for further analyses. Selected cultures were subjected to phenotypic characterization based on morphophysiological tests, including Gram staining and catalase activity assay, for presumptive identification of *Enterococcus* spp. Isolates exhibiting phenotypic characteristics associated with the genus were stored in cryotubes containing 1 mL of preservation medium composed of 10% skim milk and 10% glycerol at −20 °C for subsequent molecular identification at the species level [24, 27].

2.4 Identification of *Enterococcus* species

Species identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonik GmbH), following procedures described by Sauget et al [37] To differentiate *E. gallinarum* and *E. casseliflavus*, species characterized by low-level intrinsic resistance to vancomycin, two complementary biochemical assays were conducted as previously reported by Cartwright et al [38]. For pigment production assessment, a colony from each isolate was collected with a sterile swab and examined for yellow pigmentation, which is indicative of *E. casseliflavus*. Motility testing was additionally performed by inoculating a straight stab into semi-solid Brain Heart Infusion (BHI) agar culture to approximately two-thirds of the medium depth, followed by incubation at 37 °C for 24 h. Under these conditions, *E. casseliflavus* typically exhibits motility, whereas *E. mundtii* shows limited or absent motility, thereby enabling reliable differentiation between these species.

2.5 Determination of antimicrobial susceptibility profile

Antimicrobial susceptibility of *Enterococcus* isolates was assessed using the disk diffusion method on Mueller–Hinton agar plates, following the protocol standardized Kirby–Bauer et al [39] For each isolate, a bacterial suspension was prepared in 5 mL of sterile saline solution (0.85 % NaCl), and turbidity was adjusted to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL).

Standardized inoculum were uniformly spread onto Mueller–Hinton agar plates (Acumedia®, Neogen, Michigan, USA) using sterile swabs. Antimicrobial-impregnated disks were subsequently applied to agar medium surface, and the plates were incubated at 37 °C for 24 h.

After incubation, inhibition zone diameters were measured in millimeters and interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria[40], Isolates were categorized as susceptible (S), intermediate (I), or resistant (R). Tested antimicrobials included ampicillin (10 µg), vancomycin (30 µg), rifampicin (5 µg), tetracycline (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), linezolid (10 µg), erythromycin (15 µg), gentamicin (120 µg), and streptomycin (300 µg), in accordance with CLSI 2021 recommendations.

For analytical purposes, isolates classified as intermediate or resistant were grouped into a single resistant category. Resistance phenotypes were defined as single-drug resistance (SR), double-drug resistance (DR), or multidrug resistance (MDR) when isolates exhibited resistance to one, two, or three or more antimicrobial classes, respectively [41].

2.6 Genomic DNA extraction and detection of genes associated with antimicrobial and heavy-metal resistance

Genomic DNA from *Enterococcus* isolates was extracted by chemical lysis following protocol described by Bell et al [42]. Extracted DNA was stored at −20 °C until use in polymerase chain reaction (PCR) assays for detection of genes associated with antimicrobial resistance and heavy-metal tolerance. PCR conditions were based on previously standardized parameters, as showed in Table 1.

Detection of antimicrobial resistance genes was performed in isolates phenotypically resistant to erythromycin and tetracycline. These antimicrobial agents are widely used in human and veterinary medicine, and resistance mechanisms in enterococci are frequently associated with mobile genetic elements. PCR was performed on these strains to detect the erythromycin resistance genes *ermB* and *msrC*, as well as tetracycline resistance genes *tetL* and *tetM*. *Enterococcus* spp. strain 485 and *E. faecium* CM5-2 were used as positive controls for *ermB* and *msrC*, respectively. *E. faecium* CM11-3 and CM5-6 served as positive controls for *tetL* and *tetM* detection.

To investigate genes associated with heavy-metal tolerance, all isolates were screened for presence of *arsA_I* and *arsA_II*, which participate in arsenic resistance, and for *tcrB*, a determinant linked to copper resistance. *E. faecalis* ATCC 29212 was used as positive control for *arsA_I* and *tcrB*, whereas *Listeria monocytogenes* ALD11249.1 was employed as positive control for *arsA_II* detection [21].

Table 1. The oligonucleotide primers and the PCR parameters used to detect genes associated with arsenic, copper, erythromycin, and tetracycline in *Enterococcus* spp.

Primer	Primer sequence (5′–3′)	MW[1] (bp)	AT[2]	Reference
Arsenic				
arsA_I_F	GGCAAT YGCCGCAGCAAT TGA	643	58ºC	Rebelo et al [21]
arsA_I_R	TCCAGAAGCAGAGAAGT			
arsA_II_F	GTAGAAGGT TTAGTTGTCGCC ATGTAAG	728	62ºC	Rebelo et al [21]
arsA_II_R	TGRGGAAAT TCT TTT GGT			
Copper				
tcrB_F	CATCACGGTAGCTTTAAGGAGATTTTC	663	56ºC	Hasman et al [43]
tcrB_R	ATAGAGGACTCCGCCACCATTG			
Erythromycin				
ermB_F	GAAAAGGTACTCAACCAAATA	574	52ºC	Sutcliffe et al [44]
ermB_R	AGTAAC GGTACTTAAATTGTTTAC			
msrC_F	AAGGAATCCTTCTCTCTCCG	343	52ºC	Werner et al [45]
msrC_R	GTAAACAAAATCGTTCCC G			
Tetraclyne				
tetL_F	ACTCGTAATGGTGTAGTTGC	625	58ºC	Frazzon et al [46]
tetL_R	TGTAACTCCGATGTTTAACACG			
tetM_F	GTTAAATAGTGTTCTTGGAG	657	52ºC	Aerestrup et al [47]
tetM_R	CTAAGATATGGCTCTAACAA			

1: MW: molecular weight in base pairs; 2: AT: annealing temperature.

3. Results and discussion

To evaluate potential effects of anthropogenic activities on populations of free-ranging Geoffroy's cats (*L. geoffroyi*) captured in the Seival region, municipality of Candiota, within Pampa Biome, enterococci isolated from oral and rectal cavities were used as bioindicators.

3.1 Distribution of *Enterococcus* species in oral and rectal cavities of *L. geoffroyi*

In this study, a total of 111 *Enterococcus* spp. isolates were recovered from oral (OC) and rectal (RC) samples obtained from 14 free-ranging Geoffroy's cats captured in the Seival region between June 2022 and February 2023. From each sample, an average of three to four colonies showing morphology consistent with genus *Enterococcus* was selected for further characterization. Of total isolates, 50 (45 %) originated from oral swabs and 61 (55 %) from rectal swabs (Supplementary Table 2).

Among identified species, *E. faecium* (37.8 %; n = 42) and *E. faecalis* (29.7 %; n = 33) predominated, followed by *E. hirae* (13.5 %; n = 15), *E. casseliflavus* (9.9 %; n = 11), *E. durans* (7.2 %; n = 8), and *E. mundtii* (1.8 %; n = 2). Species distribution varied according to anatomical sampling site. Oral samples were mainly composed of *E. faecalis* and *E. faecium*, whereas rectal samples showed greater species diversity, including higher frequency of *E. hirae* and exclusive detection of *E. mundtii* (Figure 1).

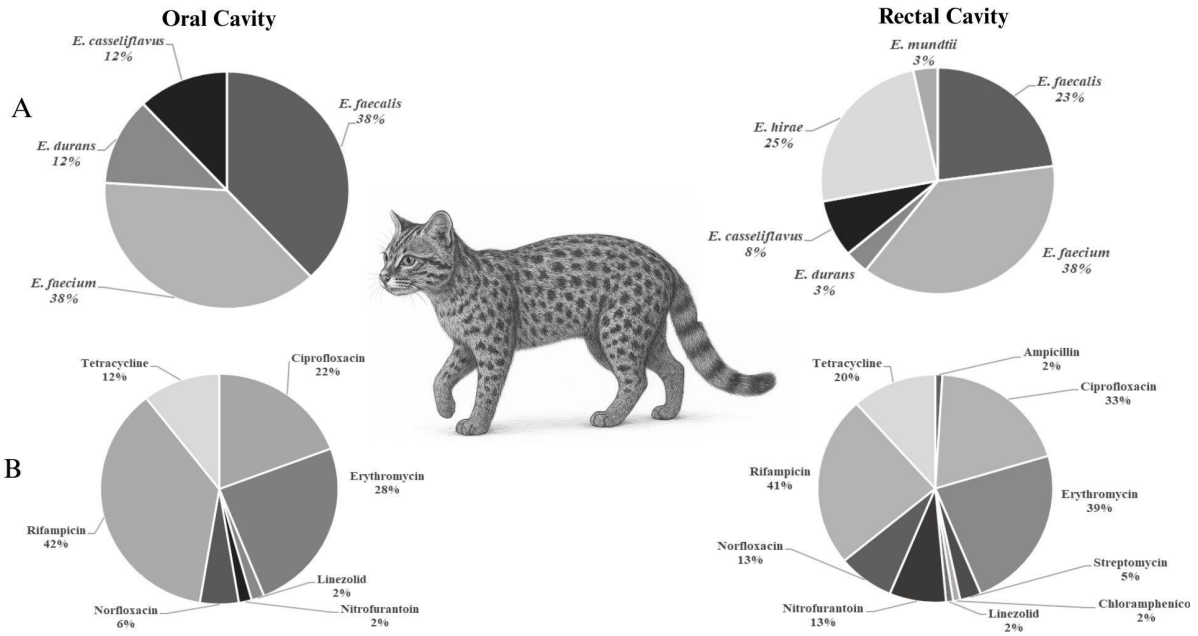

Figure 1. Distribution of *Enterococcus* spp. species. (a) and antimicrobial resistance profile (b) in oral and rectal samples from free-ranging Geoffroy's cats (*Leopardus geoffroyi*) captured in the Seival region, municipality of Candiota, Pampa Biome, Brazil, between June 2022 and February 2023.

Diversity of enterococcal species identified in this study appears to reflect the dietary habits of this felid, which primarily preys on small mammals and small birds [48–52]. High prevalence of *E. faecalis* may be associated with rodent consumption, as Lauková et al [53] reported occurrence rates of approximately 70 % for this species in the intestinal microbiota of wild rodents. Similarly, the occurrence of *E. faecalis*, *E. faecium*, *E. mundtii, E. durans*, and *E. hirae* may be related to avian prey intake, since these species are frequently reported in gut microbiota of wild birds [54] and broiler chickens [55]. Overall, enterococcal profile observed in free-ranging Geoffroy's cats suggests that dietary ecology plays a significant role in shaping colonization patterns of oral and intestinal microbiota.

3.2 Resistance profile of enterococcal isolates from *L. geoffroyi*

Among 111 isolates evaluated for susceptibility to tested antimicrobials, 83 (74.8 %) exhibited resistance to at least one agent, as detailed in Table 2. Highest resistance frequencies were observed for rifampicin (41.4 %), erythromycin (34.2 %), and ciprofloxacin (26.1 %). Lower resistance rates were recorded for tetracycline (13.5 %), norfloxacin (9.9 %), nitrofurantoin (4.5 %), streptomycin (2.7 %), linezolid (1.8 %), ampicillin (0.9 %), and chloramphenicol (0.9 %). No isolate showed resistance to gentamicin or vancomycin.

Table 2. Number (%) of antimicrobial resistant *Enterococcus* spp. isolated from oral and rectal cavities of free-ranging Geoffroy's cats captured (*Leopardus geoffroyi*) in the Seival region, municipality of Candiota, Pampa Biome, Brazil, between June 2022 and February 2023.

	Number (%) of antimicrobial resistant enterococcal species						
	Species (n)						
Antimicrobial	*E. faecium* (n=42)	*E. faecalis* (n=33)	*E. casseliflavus* (n=11)	*E. hirae* (n=15)	*E. durans* (n=8)	*E. mundtii* (n=2)	Total (n=111)
Ampicillin	0	0	0	0	1 (12.5)	0	1 (0.9)
Ciprofloxacin	22 (52.4)	1 (3.03)	1 (9.1)	2 (13.3)	3 (37.5)	0	29 (26.1)
Chloramphenicol	1 (2.3)	0	0	0	0	0	1 (0.9)
Erythromycin	17 (40.5)	12 (36.4)	4 (36.4)	2 (13.3)	2 (25)	1 (50)	38 (34.2)
Streptomycin	2 (4.6)	0	0	0	1	0	3 (2.7)
Nitrofurantoin	1 (2.3)	0	0	1 (6.7)	3	0	5 (4.5)
Norfloxacin	9 (21.4)	0	0	1 (6.7)	1	0	11 (9.9)
Rifampicin	19 (45.2)	13 (39.4)	6 (54.5)	4 (26.6)	4 (50)	0	46 (41.4)
Tetracycline	11 (26.1)	1 (3.0)	2 (18.2)	0	1 (12.5)	0	15 (13.5)
Linezolid	1 (2.3)	1 (3.0)	0	0	0	0	2 (1.8)

Enterococci isolated from rectal samples showed greater frequency and complexity of resistance compared with those isolated oral samples. In isolates from oral samples, rifampicin displayed highest resistance rate (42 %), followed by erythromycin (28 %) and ciprofloxacin (22 %). In rectal isolates, resistance frequencies were higher for rifampicin (41 %), erythromycin (39 %), and ciprofloxacin (33 %). Tetracycline resistance occurred at moderate levels in both anatomical sites but was more frequent in rectal isolates (20 % vs. 12 %). Resistance to norfloxacin, linezolid, and nitrofurantoin remained relatively low in both groups, although slightly higher in rectal isolates (Figure 1b). Species such as *E. faecium, E. faecalis, E. durans*, and *E. casseliflavus* contributed differentially to these resistance patterns, indicating that rectal microbiota harbored enterococci with higher resistance frequency and broader diversity of multidrug-resistant phenotypes than oral microbiota (Supplementary Tables 3 and 4).

These findings demonstrate that enterococci isolated from oral and rectal cavities of free-ranging Geoffroy's cats exhibit resistance to antimicrobials widely used in human clinical practice and veterinary medicine, particularly ciprofloxacin, erythromycin, rifampicin, and tetracycline [56–58]. The presence of resistant strains in wildlife populations not subjected to antimicrobial treatment suggests indirect acquisition through diet or environmental exposure.

Therefore, detection of resistant enterococci in free-ranging Geoffroy's cats samples likely reflect contact with anthropogenic pressures within their habitats. The animals studied occur in areas exposed to such activities, including livestock production that may release antimicrobial residues into soil and water bodies, promoting selection and dissemination of resistant bacteria. These observations highlight the influence of human-modified environments on wildlife microbiota and reinforce the value of enterococci as sentinel indicators of environmental quality within integrated One Health surveillance frameworks [58-60].

Among the 83 resistant isolates, 45 (54.2 %) exhibited single-drug resistance (SR), 21 (25.3 %) double-drug resistance (DR), and 17 (20.5 %) multidrug resistance (MDR). Rectal isolates showed higher proportion of MDR phenotypes compared with oral isolates (Figure 2). The presence of MDR enterococci may be associated with ecological overlap between wildlife and human activities, as wild felids and canids often share anthropized environments. Previous

studies have reported MDR enterococci in rectal swabs of wild cats from the Pampa Biome and in clinical infections of domestic cats hospitalized in southern Brazil [27, 56]. These findings reinforce concerns regarding the selection and dissemination of multidrug-resistant bacteria among human and animal populations.

Influence of human activity on occurrence of resistant *Enterococcus* spp. in wildlife has become increasingly evident, highlighting association between anthropogenic pressure and dissemination of resistance mechanisms. Heck et al [28] evaluated enterococci isolated from free-ranging and captive snakes and reported that animals without human contact were susceptible to all antimicrobials tested. In contrast, isolates obtained from captive snakes exhibited resistance to multiple antimicrobial classes. Similarly, another study reported higher frequency of resistant strains in samples from free-ranging capuchin monkeys (*Sapajus nigritus*) frequently exposed to human activity, suggesting that anthropized environments may favor selection and spread of these microorganisms [23].

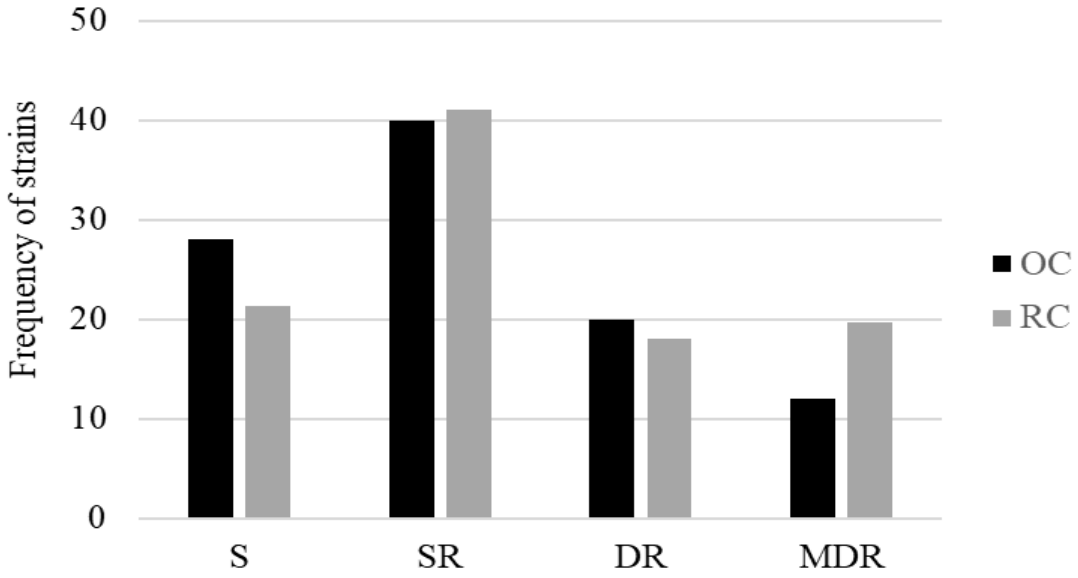

Figure 2. Antimicrobial-resistance profiles of enterococci isolated from oral cavity (OC) and rectal cavity (RC) samples of free-ranging Geoffroy's cats (*Leopardus geoffroyi*) captured in the Seival region, municipality of Candiota, Pampa Biome, Brazil, between June 2022 and February 2023. S, susceptible; SR, single-drug resistance; DR, double-drug resistance; MDR, multidrug resistance.

3.3 Detection of genes associated with resistance to tetracycline (*tetL* and *tetM*) and erythromycin (*msrC* and *ermB*), and tolerance to arsenic (*arsA_I* and *arsA_II*) and copper (*tcrB*)

After antimicrobial susceptibility testing of 111 enterococcal isolates, strains recovered from the same host frequently exhibited similar susceptibility patterns, suggesting possible clonal relatedness. To avoid overestimation in the analyses of antimicrobial resistance and heavy-metal tolerance genes, 81 isolates displaying distinct phenotypic profiles were selected, including 41 from rectal cavity (RC) and 40 from oral cavity (OC).

For detection of resistance determinants, 30 erythromycin-resistant and 18 tetracycline-resistant isolates were subjected to conventional PCR targeting *ermB* and *msrC* (erythromycin resistance), and *tetL* and *tetM* (tetracycline resistance) (Table 3). Among the erythromycin-resistant isolates, 13 presented resistance genes, of which 12 (40.0 %) were positive for *msrC*, and one isolate (3.3 %) carried both *ermB* and *msrC* genes. Of the *msrC*-positive isolates, 12 (92.3 %) were identified as *E. faecium* and one (7.7 %) as *E. durans*. The isolate harboring both genes was

identified as *E. faecium* and originated from oral cavity. The presence of *msrC* gene in enterococci from wildlife has been previously reported [23,2] and the literature describes this determinant as species-associated with *E. faecium* [61], confirming the hypothesis raised in this study, in which 12 samples positive for the gene were from the species. Conversely, 17 erythromycin-resistant isolates (56.6 %) showed no amplification for the genes investigated here, suggesting involvement of additional resistance determinants not evaluated in this study.

Among the 18 tetracycline-resistant isolates, 5 (27.7 %) were positive for *tetM*, one (5.5 %) for *tetL*, and 8 (44.4 %) harbored both genes (Table 3). The *tetM* gene was most frequently detected in *E. faecium* (n = 9), followed by *E. durans* (n = 2), *E. faecalis* (n = 1), and *E. casseliflavus* (n = 1), whereas *tetL* was identified in *E. faecium* (n = 5), *E. durans* (n = 3), and *E. casseliflavus* (n = 1). Four isolates were negative for targeted genes, indicating potential presence of alternative tetracycline resistance mechanisms in these strains.

Detection frequencies of *tetL* and *tetM* are consistent with previous investigations reporting these determinants in enterococci from wildlife populations, including study by Araújo [27]. The high prevalence of *tet* resistance genes may be explained by their frequent association with plasmids and mobile or conjugative genetic elements, which facilitate horizontal transfer among bacterial populations [61, 62].

All isolates were additionally PCR-screened for *arsA*_I and *arsA*_II, genes associated with arsenic tolerance (Table 3). Thirty-six isolates (44.4 %) were positive for *arsA*_I, whereas none showed amplification of *arsA*_II. Screening for *tcrB*, a determinant linked to copper resistance, yielded negative results in all isolates.

The presence of *arsA*_I among enterococci recovered from free-ranging felids suggests that arsenic-tolerant microorganisms may be selected under environmental contamination pressures in this habitat. Mining activities and intensive agriculture, both prevalent in study region, represent potential sources of arsenic release into terrestrial and aquatic ecosystems [63]. Supporting this interpretation, Mocellin et al [33] reported association between presence of *arsA* genes in enterococci isolated from seabirds of Abrolhos Archipelago and elevated concentrations of arsenic and other metals (Fe, Mn, Cd, and Pb) detected in blood and/or plumage following Fundão dam disaster. These findings reinforce hypothesis that the occurrence of arsenic tolerance genes in environmental enterococci reflects selective pressures exerted by metal exposure through food webs and/or contaminated habitats.

Table 3. Detection of genes conferring resistance to tetracycline, erythromycin, and arsenic in enterococci isolates obtained from oral and rectal cavities of free-ranging Geoffroy's cats (*Leopardus geoffroyi*) captured in the Seival region, municipality of Candiota, Pampa Biome, Brazil, between June 2022 and February 2023.

	Gene	Cavity	No. and species positive for the gene	Total no. of strains positive for the genes
Antimicrobial				
Erythromycin (30)	*msrC*	OC	4 *E. faecium*	4
		RC	7 *E. faecium*	8
			1 *E. durans*	
	erm B+msr C	OC	1 *E. faecium*	1
Tetracycline (18)	*tetL*	RC	1 *E. durans*	1
	tetM	OC	1 *E. faecalis*	1
		RC	4 *E. faecium*	4
	tetM +tetL	OC	1 *E. durans*	3
			2 *E. faecium*	
		RC	1 *E. casseliflavus*	5
			1 *E. durans*	
			3 *E. faecium*	
Heavy metals				
Arsenic (111)	*arsA_I*	OC	5 *E. casseliflavus*	22
			2 *E. durans*	
			4 *E. faecium*	
			11 *E. faecalis*	
		RC	2 *E. casseliflavus*	14
			1 *E. durans*	
			5 *E. faecium*	
			5 *E. faecalis*	
			1 *E. hirae*	

OC: oral cavity and RC: rectal cavity.

4. Conclusion

Antimicrobial resistant enterococci were isolated from oral and rectal cavities of free-ranging Geoffroy's cats (*Leopardus geoffroyi*) captured in the Seival region, municipality of Candiota, in the Pampa Biome, Rio Grande do Sul State, Brazil. These isolates harbored resistance determinants associated with tetracycline and erythromycin (*msrC, tetL*, and *tetM*), as well as arsenic tolerance gene *arsA*_I. The detection of these strains indicates exposure of wild felids to environments influenced by anthropogenic activities. Thus, this study reinforces the role of enterococci as environmental bioindicators, emphasizing their significance in understanding the interface among animal health, human health, and ecosystem conservation.

Supplementary material (available only in the online version: https://revistas.ufg.br/vet/article/view/84024)

Table S1. Biological and capture information for free-ranging Geoffroy's cats (*Leopardus geoffroyi*) included in the study

Table S2. Distribution of *Enterococcus* spp. among oral and rectal samples of free-ranging Geoffroy's cats captured in the Seival region, municipality of Candiota, Pampa Biome, Brazil, between June 2022 and February 2023

Table S3. Number (%) of antimicrobial resistant enterococci isolates recovered from oral cavity samples of free-ranging Geoffroy's cats captured in the Seival region, municipality of Candiota, Pampa Biome, Brazil, between June 2022 and February 2023

Table S4. Number (%) of antimicrobial resistant enterococci isolates recovered from rectal cavity samples of free-ranging Geoffroy's cats captured in the Seival region, municipality of Candiota, Pampa Biome, Brazil, between June 2022 and February 2023.

Conflict of interest statement

The authors declare no conflicts of interest.

Data availability statement

The complete dataset supporting the results of this study is available in the article and in the Supplementary Material section.

Author Contributions

Conceptualization: Toigo, A. L., Frazzon, A. P. G.; Data curation: Toigo, A. L., Cassol, M. G., Frazzon, A. P. G.; Formal analysis: Toigo, A. L., Cassol, M. G., Frazzon, A. P. G.; Funding acquisition: Frazzon, A. P. G.; Investigation: Toigo, A. L., Cassol, M. G., Frazzon, A. P. G.; Methodology: Frazzon, A. P. G.; Resources: Peters, F. B., Albano, A. P. N., Favarini, M. O.; Supervision: Frazzon, A. P. G.; Project administration: Prichula, J., Frazzon, A. P. G.; Visualization: Toigo, A. L., Cassol, M. G., Lozano, L. M. V., Frazzon, A. P. G.; Writing – original draft: Toigo, A. L., Lozano, L. M. V., Prichula, J., Frazzon, A. P. G.; Writing – review & editing: Peters, F. B., Albano, A. P. N., Favarini, M. O., Lozano, L. M. V., Prichula, J., Frazzon, A. P. G.

Generative AI Use Statement

During the preparation of this manuscript, the authors used ChatGPT to generate the image of the wildcat presented in Figure 1. After using this tool/service, the authors reviewed and edited the content as appropriate and assume full responsibility for the content of this publication.

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