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A multidisciplinary approach to analyze the antimicrobial resistance in natural ecosystems

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ABSTRACT

Antimicrobial Resistance (AMR) poses a global threat to both human health and environmental well-being. Our study delved into Costa Rican wildlife reserves, uncovering a substantial human impact on these ecosystems and underscoring the imperative to pinpoint AMR hotspots. Embracing a One Health perspective, we advocated for a comprehensive landscape analysis that intricately intertwined geographic, climatic, forest, and human factors. This study illuminated the link between laboratory results and observed patterns of antimicrobial use, thereby paving the way for sustainable solutions. Our innovative methodology involved deploying open-ended questions to explore antimicrobial usage across livestock activities, contributing to establishing a comprehensive methodology. Non-invasive sampling in wildlife emerged as a critical aspect, shedding light on areas contaminated by AMR. Feline species, positioned at the apex of the food chain, acted as sentinels for environmental health due to heightened exposure to improperly disposed waste. Regarding laboratory findings, each sample revealed the presence of at least one antimicrobial resistance gene (ARG). Notably, genes encoding resistance to tetracyclines dominated (94.9%), followed by beta-lactams (75.6%), sulfonamides (53.8%), aminoglycosides (51.3%), quinolones (44.9%), phenicols (25.6%), and macrolides (20.5%). Genes encoding polymyxins were not detected. Moreover, 66% of samples carried a multi-resistant microbiome, with 15% exhibiting resistance to three antimicrobial families and 51% to four. The absence of a correlation between forest coverage and ARG presence underscored the profound human impact on wildlife reserves, surpassing previous estimations. This environmental pressure could potentially modify microbiomes and resistomes in unknown ways. As not all antimicrobial families encoding ARGs were utilized by farmers, our next step involved evaluating other human activities to identify the primary sources of contamination. This comprehensive study contributed crucial insights into the intricate dynamics of AMR in natural ecosystems, paving the way for targeted interventions and sustainable coexistence.

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1. Introduction

Antimicrobial resistance (AMR) presents a global threat due to the widespread use of pharmaceuticals in human activities (O'Neill, 2014; Qiao et al., 2018; Tzoc et al., 2004; Ventola, 2015; WHO, 2014). However, its implications for natural ecosystems, especially protected areas, remain poorly understood (Katakweba et al., 2015; Katakweba and Olsen, 2021; Rolland et al., 1985; Sacristán et al., 2020). Studies have indicated a correlation between the prevalence of antibiotic resistance in wild bacteria and human activity levels (Cevidanes et al., 2020; Katakweba et al., 2015; Katakweba and Olsen, 2021; Rolland et al., 1985; Sacristán et al., 2020).

Protected areas face escalating human encroachment, demanding efforts to mitigate deforestation and understand wildlife vulnerability (Eklund and Cabeza, 2017; Katakweba et al., 2015; Katakweba and Olsen, 2021; Skurnik et al., 2006). While wild animals are not conventionally exposed to pharmaceuticals, their movement across diverse environments can facilitate contact with resistant bacteria and antimicrobial resistance genes (ARGs) (Larsson and Flach, 2022; Torres et al., 2020). The ecological impact of AMR is frequently underestimated, emphasizing the need for a transdisciplinary approach to assess the intricate linkages between antimicrobial use, ARGs, and geographical/land use characteristics (Vargas-Villalobos et al., 2024).

A method for investigating the presence of AMR in the environment passively and non-invasively involves the collection of fecal samples. These not only enable the determination of ARGs presence but also facilitate identification at taxonomic levels, both at the species and individual levels (Zemanova, 2019). Genetic studies, such as analyzing fecal samples for bacterial DNA, effectively quantify bacteria and antimicrobial resistance (Jiang et al., 2013; Marti and Balcazar, 2013).

A pilot study conducted in Costa Rica successfully employed genetic analysis to quantify antibiotic resistance genes (ARGs) in the feces of jaguars and pumas, as well as in the rivers where these animals source their water (Angulo et al., 2023; Vargas-Villalobos et al., 2024). Notably, in the jaguars and pumas study, *tetQ* and *tetY* emerged as the most prevalent genes. Another investigation, focusing on Andean foxes in Chile, identified 18 out of 22 ARGs, with *tetQ* being notably prevalent. Furthermore, a study encompassing the guigna throughout its distribution range in Chile found that all individuals tested positive for at least one ARG, with *tetQ* and *tetW* being prevalent, and 43% exhibiting multi-resistance profiles. These collective findings underscore the widespread occurrence of ARGs in wildlife, emphasizing the critical need for ongoing monitoring of antibiotic resistance in diverse ecosystems (Angulo et al., 2023; Cevidanes et al., 2020; Sacristán et al., 2020).

Analyzing the presence and spread of AMR in natural ecosystems is crucial for effective conservation and public health measures. Studying ARGs in sentinel species offers unique insights into the impact of human activities on antimicrobial resistance in wildlife. Understanding the factors influencing AMR dynamics, including antimicrobial use patterns, landscape features, and the interconnectedness of human, animal, and environmental health, is essential for designing targeted interventions and mitigation strategies.

The objective of this study is to propose a strategy for analyzing the presence of ARGs in sentinel species, through a multidisciplinary methodology. This approach incorporates data on antimicrobial use in human activities, particularly livestock production, and considers prominent landscape characteristics. By understanding this relationship, this study aims to provide insights into the impact of human activities on antimicrobial resistance in tropical wildlife from a One Health view.

2. Materials and methods

2.1. Study area

The study area consisted of national parks, natural reserves, and the surrounding of two conservation areas in Costa Rica. These regions are distinguished by elevated human activities, encompassing the utilization of agrochemicals and fertilizers, alongside instances of water pollution and alterations in river courses (Jong, 2001; Wo-Ching et al., 2001). Notably, pharmaceuticals and Antibiotic Resistance Genes (ARGs) have been recently identified in surface rivers within these areas (Vargas-Villalobos et al., 2024).

The Central Conservation Area (CCAr) includes the Braulio Carrillo and Quetzales National Parks, which feature high mountains, dense forests, and numerous rivers (Chassot et al., 2009; SINAC, 2019). The Braulio Carrillo National Park has varying elevations, from 35 m.a.s.l. to 2906 m.a.s.l., with temperatures ranging from 3 °C to 24 °C and annual precipitation between 2500 mm^3 and 5734 mm^3. The Quetzales National Park ranges from 1240 m.a.s.l. to 3190 m.a.s.l., with temperatures between 5 °C and 14 °C and an average annual rainfall of 2648 mm^3 (SINAC, 2019; Zúñiga-Ortiz, 2014).

The Tortuguero National Park is in the Tortuguero Conservation Area (ACTo), characterized by multiple rivers, swamps, lowland forests, and hill forests (Castillo et al., 2000; Chassot, 2006; Ling, 2002; Polidoro et al., 2009; SINAC, 2019). Its altitudinal range extends from sea level to 311 m.a.s.l. (ACTO, 2009). The region has a humid tropical forest with an average annual precipitation of 6000 mm^3 and temperatures ranging from 25 °C to 30 °C (SINAC, 2019).

These diverse ecological areas provide a suitable setting for studying the presence of antimicrobial resistance in wildlife and its potential relationship with human activities and environmental factors (Fig. 1).

2.2. Wild felid scat samples

Convenience samples were collected opportunistically through non-invasive methods (without capturing the animals) (Soto-Fournier, 2014, p.) between 2011 and 2016, with most samples having valid geographic coordinates recorded. The scat sampling was aimed at obtaining especially wild felid species. When possible, the field collector recorded species identification by size or proximity to scrapes and tracks. In some cases, a trained dog was used to localize samples. Samples were dried using silica beads, stored at room temperature or frozen at −20 °C, and analyzed according to the Costa Rican regulations (permits R-005-2015-OT-CONAGEBIO, R-018-2015-OT-CONAGEBIO, R-021-2015-OxT-CONAGEBIO, R-005-2017-OT-CONAGEBIO, R-CM-UNA-003-2020-OT-CONAGEBIO, and R-CM-UNA-004-2020-OT-CONAGEBIO).

2.3. Species identification

DNA was extracted directly from all fecal scats with the QIAamp DNA Stool Mini Kit, with modifications (Chaves et al., 2010). All DNA samples were extracted at the Genetics Conservation Laboratory in the School of Biology at the University of Costa Rica. Species identification was conducted at: 1) the National Center for Biotechnological Innovations (CENIBiot), 2) the Center for Conservation Genetics and Global Felid Genetics Program, at the Sackler Institute for Comparative Genomics at the American Museum of Natural History (AMNH), New York, USA, or 3) Macrogen Inc, in Korea. Species-specific primers were used to amplify regions of three mitochondrial genes: cytochrome *b* (H15149, Farrell-R (Farrell et al., 2000; Kocher et al., 1989)), 12S (L1085, H1259 (Kitano et al., 2007),), 16S (L2513, H2714 (Kitano et al., 2007),). Sequences were edited using Sequencher, version 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA) and Geneious Pro, version 6.1.5. (Biomatters Ltd., Auckland, New Zealand). Moreover, they were aligned to an in-house reference database compiled for carnivore species. Sequence similarity among species was assessed by constructing a

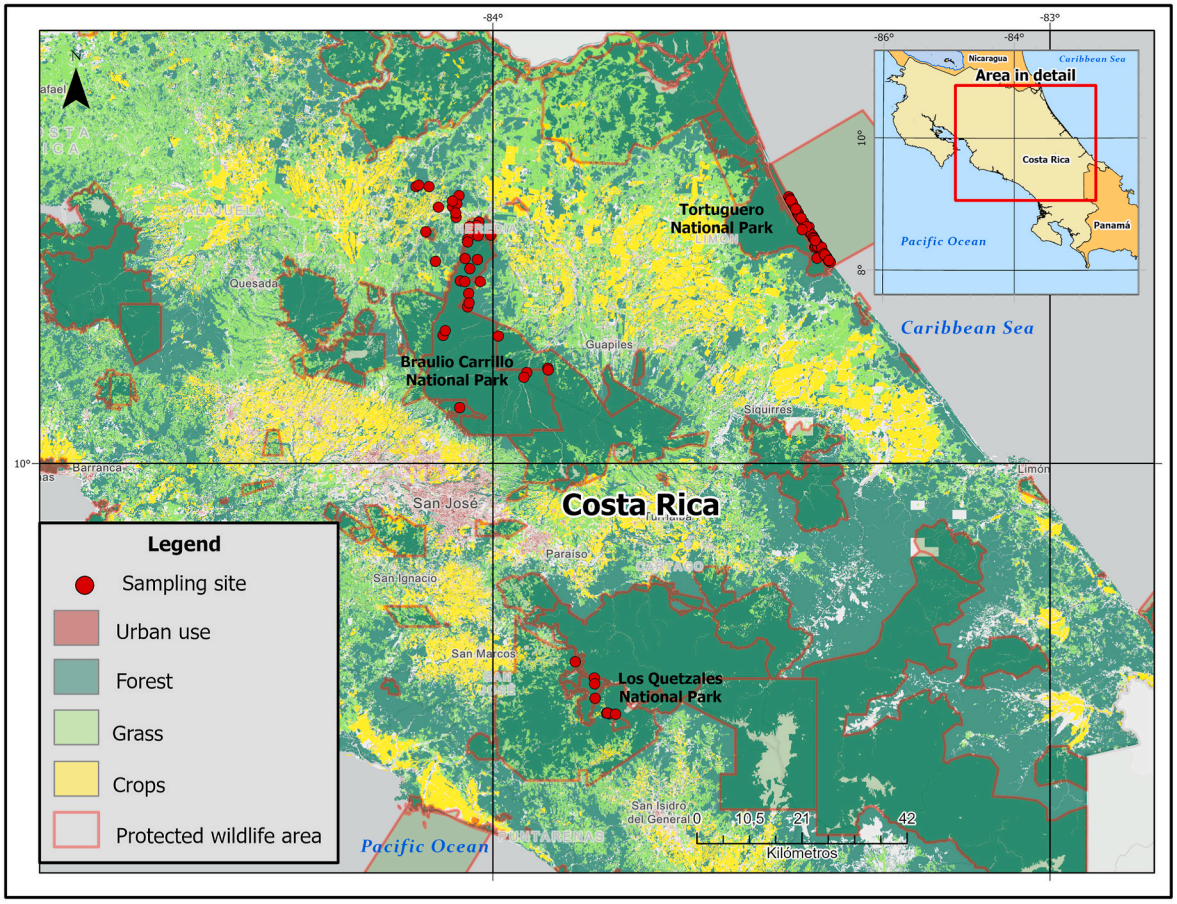

Fig. 1. Characteristics of land use where fecal samples from wild felids were collected.

phylogenetic tree using the neighbor-joining method to infer the origin of the samples. Fragments were sequenced in both directions.

For individual identification, 12 domestic cat-specific microsatellite primers were used (Menotti-Raymond et al., 1999), and primers were grouped into four multiplex reactions based on fluorescent tags and amplicon size. PCR reactions were carried out in different conditions according to the multiplex.

2.4. Characterization of antimicrobial uses through interviews

Open-ended inquiries were conducted with farmers residing in proximity to Tortuguero National Park and Los Quetzales National Park during September, October, and November 2019. These interactions took place directly on their farms or at local bovine auctions (see supplementary data). The selection of participants was based on convenience, considering their affiliation with Panthera and Nai Conservation, non-governmental organizations dedicated to nature conservation. Owing to the challenges posed by the Covid-19 pandemic, subsequent visits to other regions and engagement in additional activities were rendered unfeasible.

This activity was carried out to obtain information about the common practices of small-scale farmers regarding the use of pharmaceutical products, with a particular focus on antibiotics. The questionnaire covered various aspects, including information about their animals (species, quantity, production type), specifics of pharmaceuticals used (names, application methods, reasons for use), dosage, storage practices, their proficiency in using these products, and details regarding the location and frequency of purchase.

Moreover, the farmers' understanding of antimicrobial drugs and antimicrobial resistance was assessed. This involved inquiries about the treatments they administered for specific conditions such as diarrhea and mastitis, as well as their approach when their animals suffered from wounds or inflammation. Additional topics included their knowledge about animal diseases, waste management practices, and the handling of excreta, milk, and by-products. Furthermore, participants were asked about the disposal of waste containing potential drug residues, treatments of wastewater, and measures taken to safeguard water basins and springs. In instances where permission was granted, photographs were taken of pharmaceuticals, cabinets, and facilities.

2.5. Antimicrobial resistance gene quantification

The 16S rRNA genes were amplified in each DNA sample to detect the bacteria's presence, following the methodology proposed elsewhere (Doi and Arakawa, 2007). A valid sample was that whose 10-fold dilution showed a cycle threshold (CT) of less than 30. The 21 ARGs analyzed were selected as representatives of eight antibiotic groups: tetracyclines (*tetA*, *tetB*, *tetY*, *tetK*, *tetM*, *tetQ*, *tetS*, *tetW*), sulfonamides (*sulI*, *sulII*), aminoglycosides (*str*, *aadA*), chloramphenicols (*catI*, *catII*), macrolides (*erm*(B), *erm*(F)), quinolones (*qnrS*, *qnrB*), beta-lactams (bla_{TEM}, *mecA*), and polymyxins (*mcr-1*). The primers are described in Table S1.

The detection/non-detection of ARGs by PCR is not precisely equivalent to its presence/absence because an ARG may be present below the detection limit, which is influenced mainly by the nature of the matrix, particularly the presence of PCR inhibitors and nontarget DNA (Storteboom et al., 2010). Therefore, the number of ARG copies was approximated using the following unique formula (Sacristán et al., 2020):

$$\log_{10}(\text{percentageof an ARG}) = 2 + 0.33 * (ct_{16S\ rRNA} - ct_{ARG}),$$

where $ct_{16S\ rRNA}$ is the cycle threshold for bacterial determination, ct_{ARG} is the cycle threshold for each gene, and the value 0.33 is the mean slope for all the genes tested. The results were expressed in $\log_{10}$ of the hypothetical percentage of bacteria that each gene presented for the ARGs percentage load, with 10^{-7} being the minimum detectable value. Because the exact date when the felines defecated the samples is unknown, this formula allows a relative quantification based on the number of bacteria present.

Samples were also classified into a 'multi-resistant microbiome' if they presented at least three ARGs encoding resistance to at least three different classes of antimicrobials, and a 'non-multiresistant microbiome' following the classification proposed by Blanco-Peña et al. (2017) (Blanco-Peña et al., 2017).

2.6. Geographical analyses

2.6.1. Land use representation

The spatial distribution of ARGs was represented by the ArcGIS Pro-2.9.3 geographic information system analysis software, with the following layers: forest cover, rivers, roads, pastures, crops, and urban use (Ortiz and Soto, 2014). Most layers were obtained as a restitution product using ortho images produced between 2015 and 2018 by the National Geographic Institute of Costa Rica, made available through the National Territorial Information System (Instituto Geográfico Nacional, 2021). Data related to livestock production (bovine, swine, or avian) were generously provided by the National Animal Health Service (SENASA) based on a census carried out in 2014, including the net number of animals in each farm at that time.

The presence of human settlements, livestock farms (including the net number of cattle, pigs, and poultry), roads, crops, rivers, forests, pastures, and urban use was identified in a theoretical home range. A circular area of approximately 25 km^2 and 7 km in diameter was built around each sample with valid geographic coordinates, considering previous reports for jaguars (Núñez et al., 2020; Quigley et al., 2017; Schaller and Crawshaw, 1980). We applied this measurement to all samples as information for other species was not found. We then summarized all human activities and landscape features using GIS. Finally, the relationship between the normalized concentration of ARGs and human activities was examined based on the information generated.

2.6.2. Remaining vegetation index

In cases where valid geographic coordinates were available, each theoretical home range's remaining vegetation index (RVI) was expressed as the total percentage of forest cover. It was obtained as the product of the restitution carried out with orthoimages (Instituto Geográfico Nacional, 2021). Then, the regions were categorized using the following formula (Table 1).

2.7. Statistical analysis

A heatmap with the highest ARGs' relative concentrations was elaborated for each area. For visualization effects and due to the amplitude of the values (maximum value: 11.82/minimum value: 0.0008), values were transformed to a scale from 1 to 0, with one being the highest detectable and 0.0008 the minimum. This procedure allowed for the representation of values lower than 0.1, which were representative but because of values greater than 1, were not visualized in the heatmap.

A principal component analysis (PCA) was used to reduce the database's dimensionality and select the ARGs with the greatest weight in the separation by species and conservation area. Using the eigenvalues, we obtained the proportion of variance explained by each component. Then, with the main ARGs (according to the eigenvalues), we performed a PERMANOVA analysis with 999 permutations to determine if there were differences in gene concentrations between species and conservation areas.

A Spearman correlation analysis was performed for every conservation area to determine the relationship between the ARGs. A cluster was performed using Jaccar's index to visualize the dissimilarity between genes encoding resistance to antimicrobial families according to their absence or presence in each area. A canonical correspondence analysis (CCA) was conducted to analyze the relationships between resistance genes and geospatial and livestock productivity variables (e.g., percentage of livestock). All these analyses were performed through R and the packages ggplot2 (Wickham, 2016), FactoMineR (Lê et al., 2008), and Vegan (Oksanen et al., 2022).

Table 1
Categories of the remaining vegetation index.

Categories		RVI
NT	Not or scarcely transformed, highly sustainable	RVI ≥70%
PT	Partially transformed, medium sustainability	50%≤RVI<70%
MT	Moderately transformed, medium-low sustainability	30%≤RVI<50%
HT	Highly transformed, low sustainability	10%≤RVI<30%
CT	Completely transformed	RVI< 10%

3. Results

3.1. Species identification

In total, 78 scats were analyzed, provided from 34 jaguars,19 pumas, six ocelots (*Leopardus pardalis)*, two oncillas (*L. tigrina*), and 17 *Leopardus* sp. (Table S2).

3.2. Antimicrobial use analysis

This study encompassed 17 interviews with small livestock producers near two national parks in Costa Rica, focusing on bovine and trout production. The majority were engaged in bovine production, with antibiotic use varying between regions; tetracyclines were prominent in Tortuguero (42.9%), while beta-lactams prevailed near Los Quetzales (50%). Limited veterinary visits were reported, and farmers often treated animals without prescriptions. Predation on livestock and pets by wild animals was common (50%). Farmers exhibited varied knowledge about antibiotics, dewormers, and antimicrobial resistance. Medication storage practices varied, with 24% having drugs without labels, 12% with expired products, and 35% with containers in poor condition. Waste management practices included leaving droppings on the ground (65%) and varied methods for animal corpse disposal. A majority treated sewage (76%), but few conducted water (12%) and soil quality studies (12%). Watershed protection measures were lacking in most farms (82%).

3.3. Antibiotic resistance genes detection

All samples were positive for at least one ARG. ARGs encoding resistance to tetracyclines was found in 74 samples (94.9%), with *tetS* being the most frequent (82.8%) and *tetB* the least common (0%). Beta-lactams were also found in high percentages (75.6%), followed by sulfonamides (53.8%), aminoglycosides (51.3%), quinolones (44.9%), phenicols (25.6%), and macrolides (20.5%). Only genes encoding polymyxins were not detected (Table 2). The sample with the highest number of ARGs (n = 13) was a jaguar from the Tortuguero National Park, ACTo. Among the felids positive to 12 ARGs, one was classified as jaguar and one as *Leopardus* sp., both from ACTo, as well as one puma from Braulio Carrillo. In line with this, the animals with fewer ARGs (1) were a jaguar from Tortuguero and a puma and *Leopardus* sp. from Los Quetzales (Fig. 2 and 3; Fig. S1).

According to the PCA, it was determined that ARGs with the most significant weight were *erm*(F) (more than 60%), followed by *sulII* (close

Table 2
The number of copies of antimicrobial resistance genes (ARGs) determined in fecal samples of jaguars (*Panthera onca*), pumas (*Puma concolor*), ocelots (*Leopardus pardalis*), and oncillas (*L. tigrinus*) in Costa Rica during 2011–2016.

Gen	*Panthera onca* (n = 34)				*Puma concolor* (n = 19)				*Leopardus pardalis* (n = 6)				*Leopardus tigrinus* (n = 2)				*Leopardus* sp. (n = 17)				TOTAL	
	Min	Max	Positives		Min	Max	Positives		Min	Max	Positives		Min	Max	Positives		Min	Max	Positives		Positives	
			#	%			#	%			#	%			#	%			#	%	#	%
tetA	−5.5	−2.0	10	29.4%	−6.8	−1.5	5	26.3%	−1.1	−0.6	2	33.3%	n.d	n.d	0	0.0%	−3.9	−3.5	2	11.8%	19	24.4%
tetB	n.d.	n.d	0	0%	n.d.	n.d	0	0.0%	n.d.	n.d	0	0.0%	n.d	n.d	0	0.0%	n.d.	n.d	0	0.0%	0	0.0%
tetY	−6.3	−1.2	11	32.4%	−5.1	−2.8	3	15.8%	−3.0		1	16.7%	−2.0		1	50.0%	−4.5	−1.5	3	17.6%	19	24.4%
tetK	−6.9	−1.8	20	58.8%	−6.7	0.0	12	63.2%	−3.3	−0.7	5	83.3%	−2.8		1	50.0%	−4.9	−1.5	5	29.4%	43	55.1%
tetM	−7.2	−1.1	11	32.4%	−4.4	−2.0	4	21.1%	−3.6		1	16.7%	n.d	n.d	0	0.0%	−5.5	−3.8	3	17.6%	19	24.4%
tetQ	−6.6	−3.8	4	11.8%	−3.1	−3.0	2	10.5%	n.d.	n.d	0	0.0%	n.d	n.d	0	0.0%	−3.3	−2.9	2	11.8%	8	10.3%
tetS	−7.7	−0.3	27	79.4%	−7.1	−1.7	15	78.9%	−4.0	−1.5	5	83.3%	−1.1		1	50.0%	−5.5	−1.7	16	94.1%	64	82.1%
tetW	−6.6	−2.0	11	32.4%	−6.5	−1.0	4	21.1%	n.d.	n.d	0	0.0%	n.d	n.d	0	0.0%	−4.4		1	5.9%	16	20.5%
sulI	−5.4	−3.2	5	14.7%	−5.7	−2.0	3	15.8%	−2.6		1	16.7%	n.d	n.d	0	0.0%	n.d.	n.d	0	0.0%	9	11.5%
sulII	−4.9	−1.2	23	67.6%	−4.7	0.8	10	52.6%	−2.6	−0.3	4	66.7%	−0.1		1	50.0%	−4.9	−4.3	2	11.8%	40	51.3%
str	−7.9	−2.6	12	35.3%	−5.7	−2.0	7	36.8%	−1.8	−1.3	2	33.3%	−2.8		1	50.0%	−4.9	−2.7	2	11.8%	24	30.8%
aadA	−5.8	−2.6	14	41.2%	−5.6	−1.9	8	42.1%	−3.7	−2.4	4	66.7%	n.d	n.d	0	0.0%	−4.1	−1.7	3	17.6%	29	37.2%
catI	−7.8	−2.5	5	14.7%	−4.1	−2.5	3	15.8%	−2.2		1	16.7%	n.d	n.d	0	0.0%	−2.7		1	5.9%	10	12.8%
catII	−5.3	−3.0	9	26.5%	−6.5	−2.7	5	26.3%	n.d.	n.d	0	0.0%	n.d	n.d	0	0.0%	n.d.	n.d	0	0.0%	14	17.9%
***erm*(B)**	−6.0	−4.0	4	11.8%	−5.5	−2.6	2	10.5%	−2.4		1	16.7%	n.d	n.d	0	0.0%	n.d.	n.d	0	0.0%	7	9.0%
***erm*(F)**	−6.5	−0.8	8	23.5%	−6.5	1.1	3	15.8%	n.d.	n.d	0	0.0%	n.d	n.d	0	0.0%	−0.7		1	5.9%	12	15.4%
qnrB	−6.3	−0.3	19	55.9%	−5.0	1.0	12	63.2%	−2.7	−1.8	2	33.3%	n.d	n.d	0	0.0%	−4.3	−3.5	2	11.8%	35	44.9%
qnrS	n.d	n.d	0	0.0%	n.d.	n.d	0	0.0%	n.d.	n.d	0	0.0%	n.d	n.d	0	0.0%	n.d.	n.d	0	0.0%	0	0.0%
***bla*$_{TEM}$**	−6.6	−0.2	26	76.5%	−6.2	0.6	13	68.4%	−2.8	0.7	6	100%	−1.6		1	50.0%	−4.9	−1.1	10	58.8%	56	71.8%
mecA	−7.7	−5.9	3	8.8%	−6.7		1	5.3%	n.d.	n.d	0	0.0%	n.d	n.d	0	0.0%	−3.2		1	5.9%	5	6.4%
mcr-1	ND	ND	0	0.0%	n.d.	n.d	0	0.0%	n.d.	n.d	0	0.0%	n.d	n.d	0	0.0%	n.d.	n.d	0	0.0%	0	0.0%

to 22%), *bla*$_{TEM}$ (close to 10%), *tetK* and *qrn*B (5% each) (Fig. S2). In addition, it was observed that all feline species were in contact with ARGs regardless of the conservation area (Fig. S3). Through a PERMANOVA test, we found differences in the percentages registered of these five ARGs according to the species and the conservation area (PERMANOVA felines: df = 4; F = 0.322; $p = 0.00$; conservation area: df = 2; F = 0.361; $p = 0.00$). There were also differences in the concentrations recorded between *L. pardalis* and *P. concolor* (Table S4). Furthermore, there were differences in percentages between Los Quetzales with Braulio Carrillo and Tortuguero but not between Braulio Carrillo and Tortuguero (Table S5).

The correlation analysis showed an intra- and inter-family relationship in the data. In ACTo, ARGs encoding resistance to tetracyclines can be significantly correlated. For example, *tetM* had a stronger positive correlation with *tetS* and *tetW*; also, *tetS* with *tetW*. Regarding inter families, *tetY* was correlated with *mecA* and *qnrB* with *bla*$_{TEM}$ (Fig. 4). A particular case was obtained in Braulio Carrillo National Park and its surroundings, where all ARGs were significantly correlated to each other, except *tetA*, which was unrelated to any other ARG (Fig. 5). In Quetzales National Park and nearby, *tetY* had a stronger positive correlation with *tetM* and *tetQ*, and with *tetM* with *tetQ*. In addition, *tetS* was correlated with *sulII*, and *mecA* with *tetY, tetM, and tetQ*. The genes *str* and *bla*$_{TEM}$ were present on a minor but significant scale (Fig. 6).

Regarding the relationship between genes encoding resistance to antibiotic classes, four groups were created in a cluster (Fig. S4). Tetracyclines and beta-lactams showed the most remarkable similarity (approximately 80%), followed by sulfonamides and aminoglycosides. The latter is also associated with quinolones by almost 50%. A relationship exists between resistance to tetracyclines, beta-lactams, quinolones, sulfonamides, and aminoglycosides (close to 60%). The ratio of all seven classes (including phenicols and macrolides) is less than 10%. In addition, 66% of the samples carried a multi-resistant microbiome, with 15% positive for three antimicrobials classes, and 51% for four classes.

3.4. Geographical analyses

The RVI was calculated for 62 samples: 15 from Los Quetzales (n = 23), 21 from Braulio Carrillo (n = 21), and 26 from Tortuguero (n = 34). All home ranges from Los Quetzales and Tortuguero classified as "not or scarcely transformed, highly sustainable". Concurrently, most from Braulio Carrillo (15 out of 21) were "not or scarcely transformed, highly sustainable" and six were "partially transformed, medium sustainability".

Based on the CCA test, *qnrB* was more representative when porcine productivity was present. Meanwhile, *bla*$_{TEM}$, *erm*(F), *sulII*, and *cat*ll were more frequent when cattle and chicken productivity increased and when higher densities of rivers and roads were present. Other genes, such as *mecA* and *sulI*, did not seem related to the geospatial variables analyzed (Fig. S5).

4. Discussion

In our prior work, we identified antimicrobial resistance genes (ARGs) in the feces of jaguars and pumas, suggesting the potential influence of human activities on tropical ecosystems using wild felids as ARG sentinels (Angulo et al., 2023). While our sample size was limited, it hinted at the need for a One Health interdisciplinary analysis. Livestock production, essential for which is antimicrobial use, particularly among small-scale producers, lacks professional guidance in Costa Rica, leading to the widespread use of antibiotics like tetracyclines, beta-lactams, and aminoglycosides, as observed here and corroborated by earlier national research (Tortós et al., 2006). The absence of veterinary oversight contributes to misdiagnoses and inappropriate therapies (Scott, 2013), fostering poor record-keeping and suboptimal storage conditions.

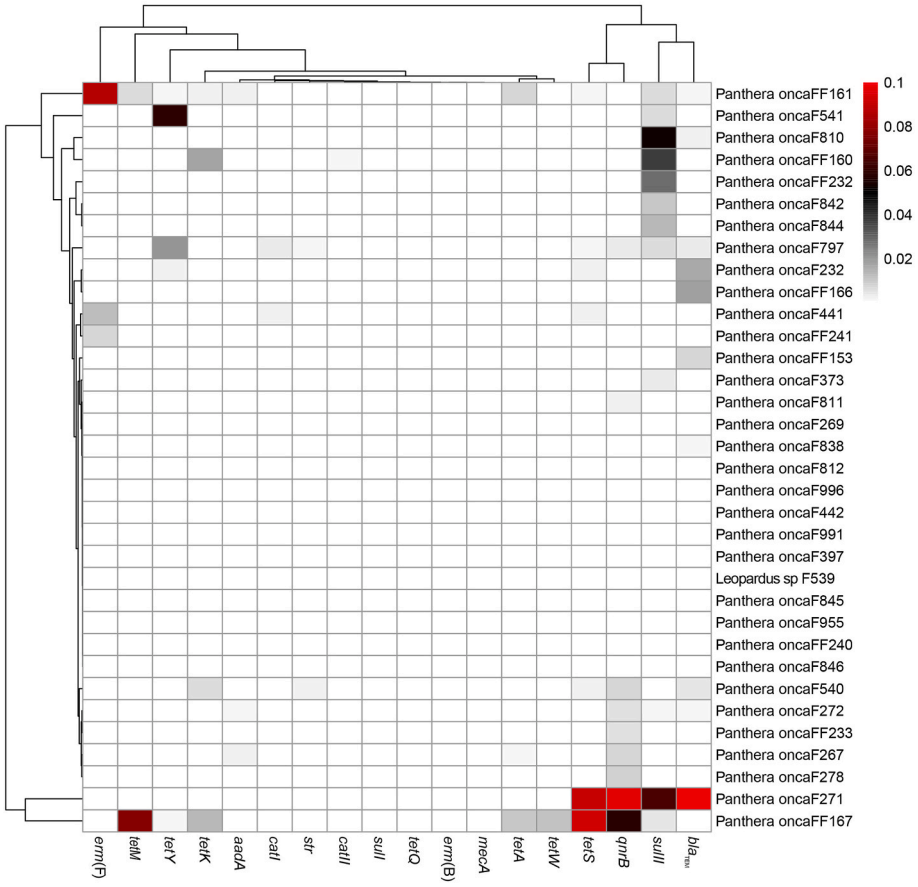

Fig. 2. Heat map representing the percentage load of antimicrobial resistance genes (ARGs) in fecal samples of wild felids (*Panthera onca* and *Leopardus* sp.) nearby the Tortuguero National Park (ACTo), Costa Rica. The scale indicates the relative concentration of each gene. The X-axis cluster indicates the degree of association among ARGs, while the Y-axis cluster shows the association among felid individuals.

Farmers, guided by pharmaceutical companies or neighbors, often misuse antibiotics due to limited understanding, confusing them with dewormers or other medications. Our interviews exposed regional disparities in antibiotic use, challenging assumptions about ARGs solely originating from livestock activities. Notably, quinolones, particularly *qnrB*, were detected in significant amounts, highlighting diverse antibiotic usage patterns. This underscores the need for comprehensive data from various governmental departments beyond livestock, including human medicine and agriculture. Certain agricultural practices, such as placing calves near forests or using contaminated residues, contribute to ARG dissemination. To mitigate antimicrobial resistance, especially in low disease frequency regions, strategies improving farm animal welfare and reducing antibiotic release via excreta, urine, or milk are imperative (Berendsen et al., 2018; Liu et al., 2016).

Similar ARG patterns in wild animals globally emphasize the interconnectedness of ecosystems. Representative ARGs for crucial human antibiotic classes were identified, suggesting their transfer to agricultural land or surface waters through various routes. The last one was recently determinate in the same study regions (Vargas-Villalobos et al., 2024). In bodies of water contaminated with antibiotics, bacteria can easily increase their AMR, either through mutations induced by environmental pressure or through horizontal exchange of ARGs among different bacterial species. This phenomenon is observed more intensely in temperate waters where bacteria maintain their activity (Murray et al., 2021). On the other hand, wastewater treatment plants are known to be critical points in the development of AMR (Guo et al., 2017). A recent study conducted in China demonstrated that the presence of ARGs is higher in municipal wastewater than in hospital wastewater (Zhang et al., 2021). Soil contamination with ARGs can also occur in several pathways, such as the excretions of livestock undergoing antibiotic therapy on the farm soil, the use of manure as fertilizer in agricultural lands, irrigation of crops with contaminated wastewater, and the leaching of antibiotic particles, as well as resistant bacteria or ARGs, into other regions (Kumar and Pal, 2018). A study conducted in 2023 has confirmed the presence of ARGs in clouds, as they become part of the water cycle, providing another pathway for the transmission of ARGs between regions and even continents. Thus, rainfall can become another source of soil and water contamination by ARGs without an apparent connection to antibiotic consumption in the corresponding regions (Rossi et al., 2023). The actual level of antibiotic-resistant bacteria and ARGs in soil is alarming and poses new challenges for soil remediation and conservation (Li et al., 2023). Both soil, grass, and water pollution with ARGs and the acquisition of resistant bacteria by wildlife accessing farms, mainly rodents and passerines, facilitate the entry of ARGs into the trophic chain in the ecosystem, ensuring their maintenance and circulation among a multitude of wildlife species (Eckert et al., 2018).

Tetracyclines, among the most widely used antibiotic families, are prevalent in Costa Rican human and veterinary medicine and agriculture (Rodriguez et al., 2006; Tortós et al., 2006). Our study aligns with previous reports of tetracycline resistance genes (*tet* genes) being here highly prevalent (94.9%) (Angulo, 2017; Blanco-Peña et al., 2017; Cevidanes et al., 2020; Ewbank et al., 2021; Sacristán et al., 2020). This persistence in the environment is substantiated by the increased likelihood of wild animals in proximity to food animal agriculture carrying *E. coli* isolates with tetracycline resistance (Berendsen et al., 2018; Kozak et al., 2009). The lack of *tet* genes in some jaguars from the ACTo region

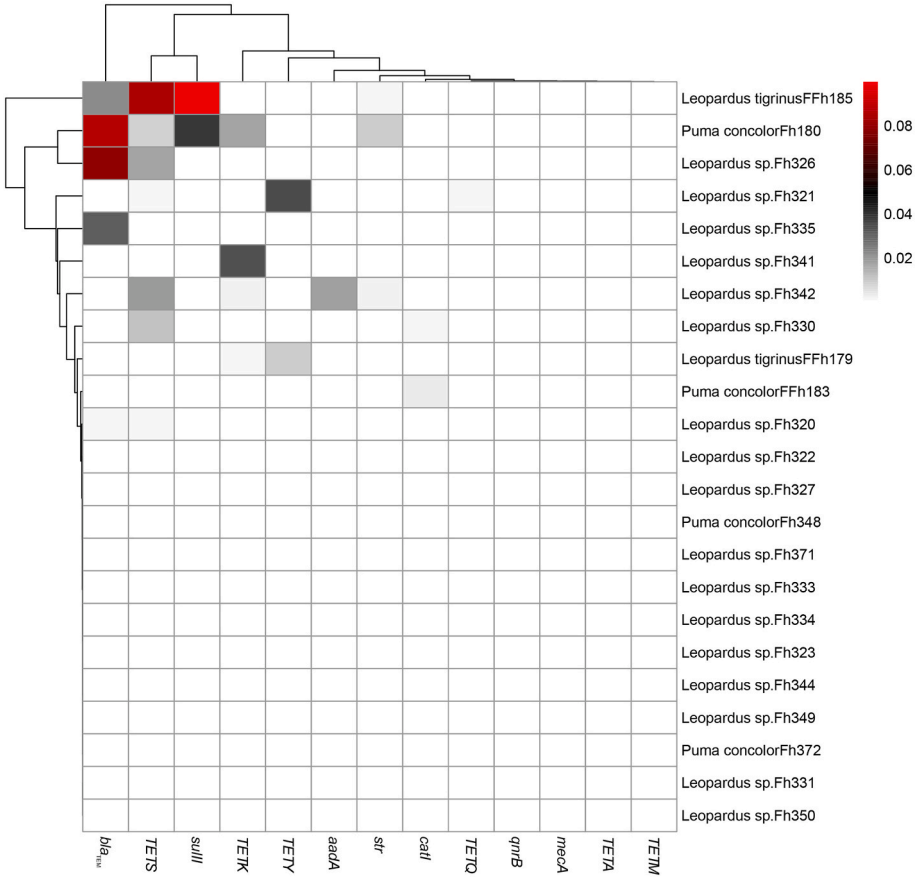

Fig. 3. Heat map representing the percentage load of antimicrobial resistance genes (ARGs) in fecal samples of wild felids (*Puma concolor*, *Leopardus tigrinus*, and *Leopardus* sp.) nearby Los Quetzales National Park, Costa Rica. The scale indicates the relative concentration of each gene. The X-axis cluster indicates the degree of association among ARGs, while the Y-axis cluster shows the association among felid individuals.

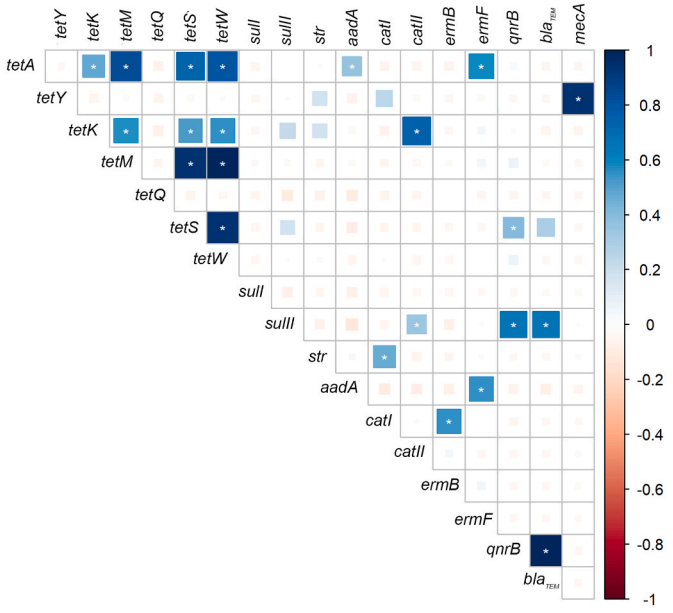

Fig. 4. Antimicrobial resistance genes (ARGs) correlation in fecal samples of wild felids collected in the Tortuguero National Park and surroundings, Costa Rica. The scale indicates the correlation values. *Significant correlation.

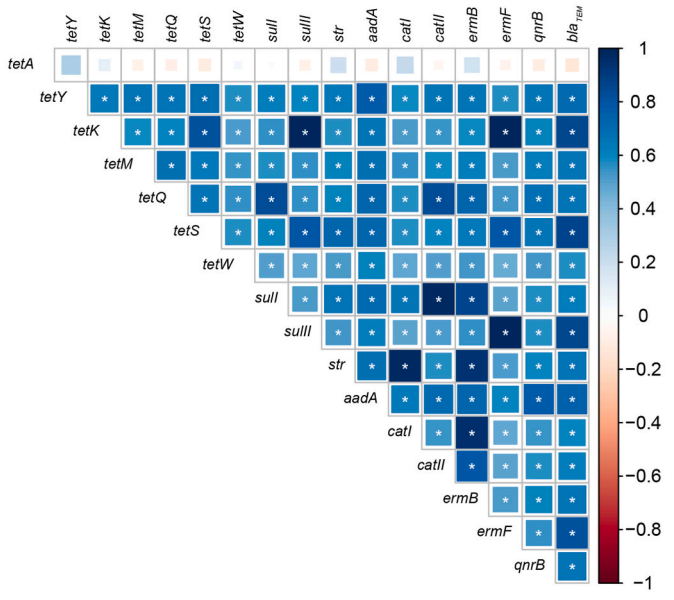

Fig. 5. Antimicrobial resistance genes (ARGs) correlation in fecal samples of wild felids collected in the Braulio Carrillo National Park and surroundings, Costa Rica. The scale indicates the correlation values. *Significant correlation.

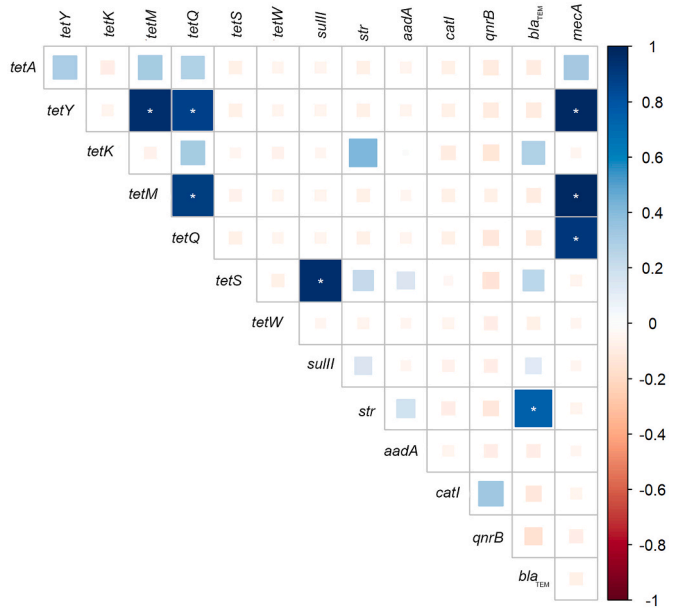

Fig. 6. Antimicrobial resistance genes (ARGs) correlation in fecal samples of wild felids collected in Los Quetzales National Park and surroundings, Costa Rica. The scale indicates the correlation values. *Significant correlation.

was surprising but inconclusive, potentially influenced by the region's characteristic floodings that limit contact with these genes or substances.

Beta-lactams, another extensively used antibiotic group, exhibit biologically active remnants in the environment, though they are less likely to persist substantially due to relative instability (Pikkemaat et al., 2016). The high prevalence of ARGs encoding resistance to beta-lactams (75.6%) in our study mirrors findings in wild felids in Chile (Sacristán et al., 2020). The detection of the *mecA* gene (8.8%) is significant, as it confers resistance to methicillin and cross-resistance to other beta-lactam antimicrobials, contributing to nosocomial infections in Latin America (Guzmán-Blanco et al., 2009).

Sulphonamide resistance genes (*sul* genes) showed a lower percentage than previous reports in jaguars and pumas but exceeded levels in pigeons from Costa Rican urban parks (Angulo et al., 2023; Blanco-Peña et al., 2017). However, the frequency of *sul* genes in different studies, despite using the same lab methodology, emphasizes the need for evaluating product use frequency in diverse regions (Cevidanes et al., 2020; Sacristán et al., 2020).

Resistance genes to aminoglycosides were found at high percentages (41.2% for *aadA* and 35.3% for *str*), possibly reflecting differences in antibiotic use between countries (Cevidanes et al., 2020; Sacristán et al., 2020). In this sense, it is important to consider that human, agriculture, and livestock activities in Costa Rica utilize aminoglycosides (Díaz-Madriz et al., 2020; Rodriguez et al., 2006; Rodríguez Sánchez, 2008; Tortós et al., 2006), which could be related with our results.

Wild felids in our study appeared less exposed to resistance genes to phenicols compared to other urban animals in Costa Rica (Blanco-Peña et al., 2017). The ban on chloramphenicol in livestock production may contribute to this discrepancy, though illegal use, or the employment of other phenicols cannot be ruled out (Poder Ejecutivo, 1997).

Resistance genes to macrolides, lincosamides, and streptogramins (*erm*(B) and *erm*(F) genes) were prevalent in our results, consistent with findings in Andean foxes in central Chile (Cevidanes et al., 2020). The detection of the *erm*(F) gene in higher proportions may be attributed to its broad bacterial host range (Chung et al., 1999).

Although the *qnrS* gene was previously found in jaguars (Angulo et al., 2023), it was not detected in our study, while *qnrB* was prevalent in almost half of the individuals. Some studies have reported that the two gene products may compete for binding to gyrase, but whether multiple *qnr* proteins have an additive effect on the minimum inhibitory concentrations is unclear (Cattoir et al., 2007; Hu et al., 2008, p. 4; Strahilevitz et al., 2009). Because we could not determine its use during the interviews, additional human activities should be evaluated, such as human medicine, agriculture, and other animal care (e.g., pets, avians, and porcine). However, some quinolones, such as enrofloxacin or ciprofloxacin, are considered critically important antimicrobials (CIAs), as they last as the best option for treating severe resistant infections in humans, especially for serious salmonellosis and colibacillosis treatment. CIAs classes of antimicrobials that meet two conditions: 1) they are the sole or one of the limited available therapies to treat severe infections in humans; and 2) they are used to treat infections in humans caused by zoonotic bacteria or bacteria with ARGs acquired from non-human sources (World Health Organization, 2019). The global increase of CIA-resistant bacteria in livestock and even wildlife is of concern, as it represents a threat to public health due the horizontal transfer ability of bacteria.

Along with quinolones, third and higher generation cephalosporins, carbapenems, polymyxins and colistin are also considered CIAs for human medicine. Polymyxin resistance data in Central America are scarce, with only Nicaragua reporting high resistance in the '90s (Mayatepek et al., 1993). Despite low use in Costa Rica, continued monitoring of polymyxin resistance genes is crucial, given the country's proximity to Nicaragua.

Our study indicates wild felids' exposure to all ARGs, irrespective of the conservation area, with 66% carrying a multi-resistant microbiome. This prevalence surpasses rates reported in other Latin American studies using similar diagnostic methodologies (Cevidanes et al., 2020; Sacristán et al., 2020). However, correlations between each ARG presence varied by region, suggesting regional differences in human activities and substance use. For instance, in urban pigeons in Costa Rica, multi-resistance was associated with *sul* genes, *tetA*, and *qnrS* (Blanco-Peña et al., 2017). This regional variability underscores the need for a nuanced understanding of antibiotic resistance patterns, even within a relatively small country like Costa Rica, where land use significantly differs across regions.

Numerous contamination sources exist in different Costa Rican ecosystems, including pesticides (Córdoba Gamboa et al., 2020; Daly et al., 2007; Rainwater et al., 2007; Rämö et al., 2018), fertilizers (Arriagada et al., 2010; Reynolds-Vargas et al., 2006), and heavy metals (Guzmán and Jiménez, n.d.), alongside negative impacts from urbanization (Reynolds-Vargas et al., 2006), hunting (Arroyo-Arce et al., 2014), land use change, and tourism (Bolt et al., 2018; Broadbent et al., 2012). Despite this, little is known about the influence of emerging pollutants, such as antimicrobials and pharmaceuticals, on ARG presence in bacterial communities (Ramírez-Morales et al., 2021). The exclusive focus on intrahospital environments neglects the potential release of antimicrobial resistance into wastewater and other matrices. Runoff and pond sediments can contribute to ARG dissemination (Palhares et al., 2014; Santamaría et al., 2011; Vieira et al., 2010), and humans can serve as vectors for ARGs, as evidenced by their presence in food, shoe soles, and hands (Braykov et al., 2016; Corzo-Ariyama et al., 2019; Mattiello et al., 2015; Rodríguez et al., 2015). While antimicrobials of natural origin are responsible for maintaining a balanced microbiome, their presence in a naïve area or synthetic antimicrobials can drastically alter the microbiome and biofilm formation, thus disrupting the ecosystem (Pishchany and Kolter, 2020). Like animals undergoing treatment, antibiotic particles contaminating the environment can exist in their active form and interact with surrounding bacteria, thereby altering the microbiome of the environment (Bhalodi et al., 2019). For example, the presence of certain antimicrobial agents in manure leads to changes in the functional diversity of microorganisms, significantly reducing them and disrupting the soil ecosystem (Han et al., 2020). Moreover, antimicrobials at sublethal doses can modify the synthesis of some metabolites such as fatty acid or LuxC, an enzyme

involved in the bioluminescence of Vibrio and other bacteria (Pishchany and Kolter, 2020). Additionally, as mentioned earlier, the bioaccumulation capacity of antimicrobial molecules in plants ensures their passage into the food chain, potentially similarly affecting the microbiomes of wild species (Li et al., 2023). Moreover, some antimicrobials can alter the seed germination, root elongation and overall plant health at sub-inhibitory concentrations (Singer et al., 2016).

While our study comprehensively addressed various aspects, significant gaps persist. Limited information on antibiotic use in agriculture in Costa Rica necessitates further investigation (Jiménez et al., 2022). Understanding medication usage in all human activities, including veterinary medicine and agriculture, is crucial, given that antibiotic residue concentrations vary by use (Camotti Bastos et al., 2018). The management of human excreta and the contribution of rural hospitals or health services effluents to environmental contamination remain critical aspects that require analysis.

Future studies should also consider the role of heavy metals in driving antimicrobial resistance, especially in a country like Costa Rica, where the use of some heavy metals as vitamin supplements in livestock adds to the presence of volcanoes and potential illegal mining activities (Irfan et al., 2022). Besides, regularly updating data on landscape changes is essential for accurate analysis of retrospective data. Also, microplastics can play an important role on the selection and dissemination of AMR (Stevenson et al., 2024).

Despite the World Health Organization's (WHO) commitment to monitoring antimicrobial resistance globally (WHO, 2014), current available data are fragmented and lack representation. A more inclusive approach, considering environmental aspects, is necessary for identifying hotspots, dangerous human contamination sources, and effective mitigation strategies. Developing transdisciplinary methodologies within the One Health framework is essential for evaluating ARG presence. Such a comprehensive approach offers a better chance of accurately determining contamination routes and should be followed by the development of mitigation actions and educational campaigns for all populations, especially those with limited connectivity and economic resources.

5. Conclusion

- Our findings reveal that the human impact on Costa Rican wildlife reserves is more significant than previously estimated. However, the consequences of this impact on wild populations remain largely unknown.
- There is an urgent need to establish a comprehensive methodology for identifying AMR hotspots and understanding the corresponding routes of contamination. Achieving this requires a thorough investigation into the current use of antimicrobials across various human activities. In our study, we employed open-ended questions as an alternative method to gather this essential information directly in the field.
- The application of non-invasive sampling methods in wildlife proves invaluable for pinpointing areas highly contaminated with antimicrobial resistance and revealing the extent to which human activities affect wild landscapes. Given the broad range of movement and lifespan of feline species, their exposure to improperly disposed waste from human and animal antibiotic use may be heightened. Consequently, wild felids emerge as sentinels of environmental health, occupying the top of the food chain.
- To accurately identify antimicrobial resistance genes (ARGs) in the wild, it is imperative to adopt lab methodologies grounded in genetics, moving beyond traditional culture-based approaches. This shift is essential, considering that a significant portion of bacteria cannot be cultivated using standard procedures.
- Achieving a genuine One Health perspective also necessitates the incorporation of a comprehensive landscape analysis. This should encompass diverse geographic, climatic, forest, and human characteristics. Evaluation of these factors must be intricately linked to the results obtained from the lab study and the observed patterns of antimicrobial use in the region.

CRediT authorship contribution statement

Kinndle Blanco-Peña: Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Francisco Quesada-Alvarado:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Denis Salas-González:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Sandra Estrada-König:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition. **Roberto Salom-Pérez:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Stephanny Arroyo-Arce:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Adriana Villalobos-Araya:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Josué Rivera-Castillo:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Bárbara Martín-Maldonado:** Writing – original draft, Validation, Investigation, Data curation. **Daniel Corrales-Gutiérrez:** Writing – review & editing, Investigation, Conceptualization. **Valeria Gallardo-Castro:** Methodology, Investigation, Formal analysis. **Gustavo Gutiérrez-Espeleta:** Supervision, Project administration, Funding acquisition, Conceptualization. **Andrea Chaves:** Writing – review & editing, Project administration, Methodology. **Fernando Esperón:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Fabio Chaverri-Fonseca:** Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kinndle Blanco-Pena reports financial support was provided by Fondo de Educación Superior Estatal del Consejo Nacional de Rectores de Costa Rica (CONARE).

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.118549.

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