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Dysbiosis and Enterobacteriaceae-associated accumulation of antimicrobial resistance in the gut bacterial reservoir of captive sloth bear (Melursus ursinus)

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Abstract

Captivity-associated conditions modulate the gut microbiota in wild animals as they are given several medications including antibiotics, and are exposed to various anthropogenic stressors throughout their captive lives. Our understanding of how these factors modify the gut microbiome, its resistome and associated mobilome is nascent at the very best. In this study, we analysed metagenomic data from 215 captive sloth bears from across India to describe their gut microbiota in association with age, gender, duration in captivity, health status, body condition, etc. Overall, we found low microbial diversity and evenness across sampled locations, along with dominance of select taxa like *Streptococcus*, *Escherichia*, *Klebsiella*,*Sarcina* and *Clostridium*. On the contrary, we observed a very diverse resistome, and almost all bears consistently showed high levels of multi-drug resistance, even when not treated recently with antibiotics. Abundances of WHO-priority pathogens such as *Escherichia and Shigella* positively correlated to higher antibiotic resistance gene (ARG) abundances and richness. We further observed that ARGs which confer multi-drug resistance mostly via efflux pump pathways, were associated with plasmids and other mobile genetic elements, increasing the risk of resistance dissemination. Metagenome-assembled genomes (MAGs) of Enterobacteriaceae species such as *Escherichia spp. and Klebseilla spp*. showed a disproportionately high accumulation of ARGs, further underscoring their virulence. In summary, our study highlights the importance of metagenomic studies to understand the effects of captivity on the gut health and accumulation of antimicrobial resistance in endangered animals.

Introduction

The mammalian gut microbiome modulates and influences a range of host functions and processes like digestion, immune response, neuropsychological functions and even reproduction (Flint et al., 2012; Antwis et al., 2018; Nicolai et al., 2019; Sittipo et al., 2022). The dynamicism and plasticity of the gut microbiota help animals adapt to and evolve in diverse environmental conditions (Alberdi et al., 2016; Huang et al., 2022). Captivity, however, is one extreme environment that can drastically and, many times, irrevocably change the gut microbiome, physiology and health of wild animals (Mckenzie et al., 2017). Several studies report reduced or altered microbial diversity in captive animals coinciding with consistently less varied diet than their wild counterparts (Roth et al., 2019; Das et al., 2022). There is also evidence of change in composition and abundance of bacterial taxa with strong signals of domestication and urbanization (Reese et al., 2021), and enrichment of human-associated microorganisms (Alberdi et al., 2021; Diaz and Reese, 2021). In a study on threatened equids, Zhou et al. (2022) reported high levels of potentially pathogenic bacteria and biomarkers of physical or psychological disease.

The gut, we now understand, serves as a very important reservoir wherein probiotic, commensal and pathogenic organisms reside, interact with and influence each other as well as the host. At this juncture, the use of antibiotics to treat infections gains prominence, as indiscriminate and improper use of broad-spectrum drugs is capable of erasing beneficial bacterial strains while enriching pathogenic strains with **a**ntibiotic **r**esistant **g**enes (ARGs) (Rochegüe et al., 2021). Accumulation of ARGs leads to **a**nti**m**icrobial

resistance (AMR), a growing global threat recognized for its ability to render standard treatments ineffective against infectious microorganisms (WHO, 2014). Presently, AMR is of utmost concern globally and is declared as a top priority threat by the World Health Organisation (WHO, 2023). Extensive research investigates the status of AMR in various microbial niches in humans (Penders et al., 2013; Ahmed et al., 2024), and likewise in animals in the food sector and its associated industries (Jacobsen et al., 2023) with outcomes aligned to anthropocentric needs. There are however very limited investigations on AMR in wild animals (Akwongo et al., 2025; Smoglica et al., 2025). A few studies solely consider wildlife as a key reservoir or disseminator of AMR (Lee et al., 2022; Laborda et al., 2022; Doyle et al., 2025), instead of the more likely opposing paradigm that human interactions and interventions inadvertently lead to the propagation of AMR in wildlife (Mourkas et al., 2024). This aspect is particularly important in captive vulnerable and endangered animal populations, where there is a general increase in antibiotic usage to manage health conditions. Closed, and at many times unhygienic, quarters and routine administration of antibiotics create a situation conducive to the development and spread of resistant bacteria. Animals in zoos live in an environment that is greatly different from their natural habitats. They are introduced to therapeutics as prophylactic treatments and routine veterinary care. Moreover feed, like poultry, milk and eggs, provided in zoos and captive facilities may be priorly treated with antibiotics to ensure their quality and safety, thereby unintentionally introducing excessive antimicrobials into the animal's system.

Sloth bear (*Melursus ursinus*), belongs to Ursidae family and is endemic to the Indian subcontinent (Bargali et al., 2020). It is classified as 'Vulnerable' in the IUCN Red List of Threatened Species (Dharaiya et al., 2016), and is listed in Schedule 1 of the Indian Wildlife Protection Act (1972). Contributors to population decline include habitat loss and fragmentation, poaching and trade in body parts, and conflict. Historically in India, sloth bear cubs were captured, tortured and made to live in harsh conditions as part of the "dancing bear" tradition, where they endured severe physical and psychological abuse (Seshamani & Satyanarayan, 1997; D'Cruze et al., 2011). Long-term collaborative efforts between the Government of India and NGOs like Wildlife SOS ensured that these animals were successfully rescued and rehabilitated, reintegrating them into more humane environments that prioritize their welfare and conservation. AMR now poses an additional layer of risk to their health, potentially reducing the efficacy of treatments to infections. Given the vulnerable status of sloth bears, understanding their gut microbial composition, the AMR burden they carry and the role of management practices of captive facilities is essential for effective *ex-situ* conservation strategies.

Given a lack of empirical data on gut microbiome and AMR in sloth bear, we first aim to assess the gut microbiome of captive animals housed at various zoos and rescue centers across India, and investigate its burden of antimicrobial resistance genes and associated signatures. We further aim to understand the extent to which anthropogenic disruptions, like antimicrobial resistance (AMR), have infiltrated animal populations in the context of One Health, and how much of this impact now reflects persistently in the animals' microbiota. We finally intend to infer whether the resistance patterns align with veterinary treatment histories, diet and husbandry practices, and other key drivers of AMR accumulation.

Materials and Methods

Study area and sample collection

We collected 231 sloth bear samples from 14 zoos and rescue centers across India (Fig. 1) between November 2021 to September 2022. A large number of these animals (n = 187) are at Wildlife SOS rescue centers at Agra-Bear Rescue Facility, Bengaluru-Bannerghatta Bear Rescue and Rehabilitation Center, and Bhopal-Van Vihar Bear Rescue Facility. Animals housed in Wildlife SOS centers have a prior history of starvation, malnourishment and extensive abuse as they were beaten, de-clawed, had their nasal septum pierced to string ropes through it and chained up constantly. Post-rescue, these bears now live in large open spaces with enrichment paraphernalia, and readily available veterinary facilities.

We collected fecal samples within 15 mins of defecation, after moving the subject animal to another enclosure with the help of animal keepers. Samples were collected with sterile spatula and forceps into fresh 50ml microfuge tubes. As a precautionary measure, we used gloves and masks during sample collection. Sample tubes were labelled with details such as sample ID, date of collection, location, age and gender of the animal, and stored in a cold box (Zedblox Actipod™) at -10°C before transferring to -80°C in the laboratory for further analysis. Additionally, we also recorded the history, feeding and medication details, duration in captivity, and health status of each animal (Table S1).

DNA isolation and sequencing

We isolated DNA from all samples using Nucleospin DNA Stool kit (Machery-Nagel, Germany) adhering to the company's protocol and only increasing incubation time to get a better DNA yield. To minimize contamination, we aliquoted and carried out isolation in separate biosafety cabinets. Quality of isolated DNA samples were checked spectrophotometrically. DNA was stored at -20°C until further analysis. For each sample, 100ng of DNA was fragmented and tagged with adapters using bead-linked transposomes (BLT). Post tagmentation, cleanup was performed and the tagmented DNA was amplified through a 5-cycle PCR program to add index adapters. We constructed metagenomic sequencing libraries using Illumina DNA Prep Kit (Illumina Inc., USA), according to the manufacturer's protocols. Final library quality, concentration and size were determined with Qubit 4 (ThermoFisher Scientific, USA) and TapeStation (Agilent Technologies, USA). We sequenced the libraries on NovaSeq 6000 (Illumina Inc., USA) with 2x150 chemistry. Two hundred and fifteen samples out of 231 passed QC and were used for subsequent analysis.

Bioinformatics analyses

The sequence data obtained was converted and demultiplexed from BCL to FASTQ format with bcl2fastq conversion software, and further quality checked for Phred score, per base N content, sequence length distribution, over-represented sequences and adapter content using FastQC v0.12.1 (Andrews, 2010) and

MultiQC v1.17 (Ewels et al., 2016). We noticed contamination with Nextera transposase adapter, and therefore filtered the reads for adapter contamination using Trimmomatic v0.39 (Bolger et al., 2014) with the following parameters: ILLUMINACLIP: NexteraPE-PE.fa:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:10:20 MINLEN:75. Post trimming, reads were re-checked to ensure removal of adapter sequences. Cleaned reads were filtered for host reads using Host Contamination Removal Tool (HoCoRT) v1.2.2 (Rumbavicius et al., 2023) by referring to the genome sequence of the closest living sister species, the Malayan sun bear (GenBank Accession ID: GCA_028533245.1), as reference genome of sloth bear is presently not available. Cleaned reads were also checked for their coverage using Nonpareil v3.5.5 (Rodriguez-R & Konstantinidis, 2014). Reads were assembled into contigs using MEGAHIT v1.2.9 (Li et al., 2015) with the option: –k-min 21 –k-max 141 –k-step 20. We then mapped the reads to the index built with assembled contigs to check for overall assembly rate using Bowtie2 v2.5.2 (Langmead & Salzberg, 2012). The mapped SAM files from Bowtie2 were converted to BAM format using SAMtools v1.18 (Li et al., 2009), and were coordinate sorted for further analysis.

Taxonomic Classification

Cleaned and filtered reads were classified using PlusPFP database (October 2025 release), which contains Standard (RefSeq archaea, bacteria, viral, plasmid, human, UniVec_Core) plus RefSeq protozoa, fungi and plant, as referenced by Kraken2 v2.1.2 (Wood & Salzberg, 2014). Further, the tool Bracken v2.9 (Lu et al., 2017) was employed to re-assign unclassified reads to various Operational Taxonomic Units (OTUs) using the Kraken report and database to obtain final estimated read counts. Taxonkit v0.20.0 (Shen & Ren, 2021) was used to filter only for bacterial OTUs at various taxonomic levels. Read counts of Bracken were normalised using the Npc_standardization.R script with a target coverage of 0.95 (Aldeguer-Riquelme et al., 2025).

Identification of antimicrobial resistance genes and mobile genetic elements

We analysed the assembled contigs with Resistance Gene Identifier (RGI) v6.0.3 which uses the **C**omprehensive **A**ntibiotic **R**esistance **D**atabase (CARD) as reference (Alcock et al., 2023) to identify ARGs. We filtered ARGs with more than 98% identity for further analysis. RGI output for all samples was saved as BED files and was version-sorted. The sorted BED and BAM files were used to calculate coverage of the contigs with ARGs using BEDtools coverage command v2.31.1 (Quinlan & Hall, 2010). ARG abundance was calculated based on RPKM or Reads per kilo base per million mapped reads (Zhang et al., 2022)

$$RPKM = \frac{ReadsMapped}{\frac{Genelength}{1000} \times \frac{TotalReads}{1{,}000{,}000}}$$

Wherein, ReadsMapped corresponds to coverage value of the contig stretch where an ARG was detected, GeneLength corresponds to length of ARG, and TotalReads corresponds to total number of bacterial reads at the genus level per sample. While the CARD database associates each gene with an extensive list of individual drug classes, we streamlined these for analysis by designating groups with ≥ 3 drug classes as “multi-drug resistant” groups - MDR-1, MDR-2, and so on (Table S2).

We identified mobile genetic elements (MGEs) in the form of plasmids and pro-viral elements using geNomad v1.8.1 (Camargo et al., 2024). Plasmid-annotated contigs were investigated for presence of ARGs identified previously with RGI, to establish co-occurrence of ARGs with MGEs such as integrons and transposons using BacAnt v3.4.0 (Hua et al., 2021).

Construction of Metagenome-Assembled Genomes (MAGs) and identification of key drivers of AMR

In order to understand the contribution of the most abundant bacterial genera towards antimicrobial resistance in the gut environment, we extracted reads of the top 5 genera viz. *Streptococcus*, *Escherichia*, *Clostridium*, *Sarcina* and *Klebsiella*, that constituted nearly 75% of the average abundance across all locations and constructed MAGs. Reads were extracted using the extract_kraken_reads.py script from Kraken tools repository (Lu et al., 2022), and assembled into contigs at individual sample-level using MEGAHIT with the parameters --kmin 21 --kstep 20 --kmax 141. These contigs were then pooled for each location and a single concatenated FASTA file was generated per location with SemiBin v2.2.1 concatenate_fasta command (Pan et al., 2022). MAGs were then binned using SemiBin multi_easy_bin mode for locations with multiple samples and with single_easy_bin mode for locations with only one sample. Individual sample contig files were mapped to concatenated location-wise FASTA files to generate BAM files that were used in these modes to generate sample-wise bins for all locations. This method ensured lesser chimeric assemblies since contigs were assembled at sample level, but also higher coverage since binning was at location-level, thus obtaining the best out of the sample and co-assembly models.

We assessed the quality of assemblies with checkM2 v1.1.0 utilizing default machine learning models trained on GTDB release 202 and UniRef100-KO DIAMOND database v1.1.0 (Chklovski et al., 2023). Only high quality MAGs (completeness > 90% and contamination < 5%) based on GSC standards of MIMAG (Bowers et al., 2017) were filtered and annotated using GTDB-Tk v2.6.1 classify_wf workflow (Jain et al., 2018; Chaumeil et al., 2022). Genomes were assigned taxonomy based on GTDB reference database release r226 (Parks et al., 2022). Annotated high-quality MAGs were then de-replicated using dRep v3.6.2 dereplicate function (Olm et al., 2017) with --S_algorithm skani parameter (Shaw & Yu, 2023). The dereplicated, high-quality, annotated MAGs were assessed for presence of ARGs using RGI tool, while integrons and transposons were identified using BacAnt tool. Coverage of the ARGs identified in bins, across all samples, was calculated with bedtools multicov function. Coverage values for all 215 samples were normalised by gene length and total sequencing depth using RPKM method. We then averaged the RPKM values for the ARGs by mean for all samples that belong to the same location.

Visualizations and Statistical Analyses

Results were visualized using RStudio v4.3.3 and RStudio server v4.4.1 (R Core Team, 2023). We obtained mean read counts of all the identified taxa across all samples at each location. We further filtered the most abundant genera at each location to obtain a unique list of 22 genera and calculated their relative abundances as 'fraction total reads' classified to OTU by total number of reads. Similarly, the RPKM abundance matrix was also used to identify top AMR gene families and drug classes based on their overall prevalence in all the 14 locations.

From this point onwards data from only five captive locations - Nehru Zoological Park (NZP), Hyderabad (n = 10), Sri Chamarajendra Zoological Gardens (SCZG), Mysuru (n = 11), Wildlife SOS, Bhopal (WSOS-BH, n = 15), Wildlife SOS, Bannerghatta (WSOS-BNP, n = 59), and Wildlife SOS, Agra (WSOS-A, n = 97) were considered for further analysis, as all other locations had very few individuals. Shannon and other alpha diversity indices were derived using estimate_richness function in phyloseq package. Within this group, we again analysed alpha diversities for the three Wildlife SOS centers with additional covariables like antibiotics and deworming medications given in the three months prior to sample collection, history of hepatic or kidney disorders, body score index, and seropositivity for tuberculosis. One-way ANOVA, followed by a post hoc correction for multiple comparisons using Tukey's HSD (honestly significant difference) test, was performed to check the significance of our results. Dunn's post hoc test was applied after Kruskal–Wallis, and p-values were adjusted using Benjamini–Hochberg method. ANOVA pairs with adjusted p-value less than 0.05 were considered significant.

We plotted Principal Coordinates Analysis (PCoA) plots for beta diversity among the locations and for each of the above variables using Bray-Curtis dissimilarity matrices with vegdist and pco functions of vegan and ecodist packages in RStudio. PERMANOVA (**Per**mutational **M**ultivariate **An**alysis **o**f **Va**riance) was performed on these distance matrices using adonis function of vegan package with 999 permutations, and a p-value < 0.05 was considered significant.

In order to understand the effect of covariables at different locations, taxonomic diversity with gaussian distribution was modelled as a dependent variable to a Linear Mixed Model (LMM) in lme4 package in RStudio. Location of animal was taken as a random effect, and variables like health status, age and sex of the animal were taken as fixed effects. To understand the relationship between gut microbiome diversity and ARG presence and abundance, we conducted correlation tests between Shannon indices of microbial diversity with both, total number of ARGs and sum total ARG abundance per sample. Spearman's correlation was chosen over Pearson's as it is a non-parametric, rank-based test and does not assume a linear relationship or normally distributed data. Significance was assessed at p-value < 0.05. We derived Spearman's correlation coefficient (ρ, rho) which ranges from − 1 to 1, and indicates the strength and direction of the monotonic relationship between ranked diversity and AMR abundance. Further, a distance correlation test was performed to assess both linear and non-linear associations between variables, with significance evaluated at $p < 0.05$. We also assessed the correlation of individual bacterial genus abundance with ARG abundance and numbers, for genera that are present in at least

50% of all the samples, and reported significant genera (p-value < 0.05). All p-values from Spearman correlation tests were corrected for multiple testing using Benjamini–Hochberg false discovery rate. Additionally, to understand the effect of prolonged time in captivity on gut microbial diversity, number of ARGs and total ARG abundance in an animal, we extended the Spearman rank correlation test between these variables for 65 individuals for which we had information on when they were captured from the wild, and their duration in captivity (in years).

ARGs co-occurring with MGEs were visualized as heatmaps, generated using Heatmap functions in RStudio, with the relative abundance of ARGs as the color scale.

Cohort description

Out of 231 sloth bear samples collected, 16 samples failed at NGS QC, and hence, data from only 215 sequenced samples were used for further analysis. Out of these, a total of 171 samples were from the three Wildlife SOS centers, viz. 59 from Wildlife SOS, Bannerghatta; 15 from Wildlife SOS, Bhopal; and 97 from Wildlife SOS, Agra. Amongst the remaining 44 samples, 11 and 10 samples were from Sri Chamarajendra Zoological Gardens, Mysuru and Nehru Zoological Park, Hyderabad respectively; 4 samples each were from Tiger and Lion Safari, Thevarakoppa and Van Vihar National Park, Bhopal; 3 samples each were from Etawah Safari Park, Etawah; Kakatiya Zoological Park, Warangal; and Bannerghatta Biological Park and Rescue Center, Bengaluru. The smallest groups were 2 samples each from Kanpur Zoological Park, Kanpur and Gorewada Rescue Center, Nagpur, and one sample each from Alipore Zoological Gardens, Kolkata and Arignar Anna Zoological Park, Chennai (Fig. 1). All animals were further classified based on gender, age, health status, antibiotics given in the three months prior to sample collection, and duration in captivity at each location (Table S1). Animals were grouped as young (0–3 years), adult (3–15 years) and old (15 + years) based on available literature on sexual maturity and reproduction in this species. Animals were considered healthy unless they were off-feed for multiple days and lethargic, there were visible external wounds or if there was any history of disease and prolonged treatment. In case of the three Wildlife SOS centers, we had additional information on deworming medications given in the three months prior to sample collection, history of hepatic or kidney disorders, body score index, and seropositivity for tuberculosis. Each animal at Wildlife SOS centers was scored based on body condition and weight (1 = skinny; 2 = thin; 3 = ideal; 4 = overweight; 5 = obese).

Results

The 215 sequenced sloth bear samples yielded an average of ~ 17.5 million forward and reverse reads each, per sample. Nearly 92% of forward and reverse reads survived after trimming to remove Nextera transposase adapter contamination. On an average, we lost roughly 5% of the sequence data in each sample due to host contamination. We observed approximately 85% alignment rate in all samples, and average N50 value was 2706 bp indicating a moderate to good assembly (Table S3). We identified a total of 2342 unique OTUs and 367 unique ARGs across 14 locations.

Gut microbiota composition and diversity

Presence of pathogenic or opportunistic bacteria

The most abundant phyla across all locations were Bacillota, Bacteroidota, Pseudomonadota and Actinomycetota, and made up for more than 98% of all bacterial phyla found in the gut microbiome of captive sloth bears (Fig. 2A). Five opportunistically pathogenic genera - *Streptococcus*, *Sarcina*, *Escherichia*, *Clostridium* and *Klebsiella*, were the most abundant genera identified across all locations, and together they comprised nearly 75% of all the classified reads. Relative abundances of top 22 genera across all 14 locations are depicted in Fig. 2B. It was interesting to note that animals from Wildlife SOS centers showed a very similar composition of major genera, possibly reflecting similar diet plans and medication schedules at these centers. We also detected viral and fungal reads in multiple samples across locations. We identified higher proportions of tailed-bacteriophages belonging to the Caudoviricetes family in animals from Tiger and Lion Safari, Thevarakoppa and Wildlife SOS, Bhopal.

Lower gut microbiome diversity in captive sloth bear

We calculated alpha and beta diversities at the genus level to identify between-sample and between-location differences in microbial diversities. Median alpha diversity, as indicated by Shannon index, for five main locations was between 2.5 to 1.5 indicating low gut bacterial diversity in all animals (Fig. 2C). We noticed a slightly higher median Shannon index value (> 2) for the three Wildlife SOS centers compared to the other two groups – NZP and SCZG (Fig. 2C). Other diversity metrics such as Observed species (Richness), Berger-Parker Index (Dominance), Inverse Simpson and Simpson (Diversity), Pielou's Evenness (Distribution) and Nonpareil N_d value indicate higher dominance and lesser evenness for these 5 locations (Fig. S1).

One-way ANOVA of the Shannon index values showed that NZP and WSOS-BNP, and NZP and WSOS-A pairs are significantly different. Age, sex, and health status of animals did not significantly influence the gut microbial diversity. Dunn's post hoc test after Kruskal–Wallis showed that NZP diversity differed significantly from that of all three WSOS centers. Subsequently we fit a Linear Mixed Model (LMM) to understand the influence of multiple factors (location, age, sex and health status) on observed alpha diversities. LMM showed that nearly 20% of the variance can be explained by location. Age of the animal has a weak effect on diversity, with older animals having lesser diversity than younger individuals. Gender and health status of the animal seemed to have almost no effect on diversity. Summary of these observations is represented in Fig. S2.

PCoA plots of beta-diversity based on Bray-Curtis dissimilarity matrix did show clustering of samples, but not based on any of the variables used in the study (Fig. 2D & Fig. S3). Scatter plot with 95% confidence ellipses showed high overlap and there is no significant difference between all five locations. The same was substantiated by PERMANOVA test with 999 permutations, with a very poor R-squared value for location (R^2 = 0.03313) although the p-value was significant (p = 0.019), indicating a significant variation between groups which cannot be explained by location alone. Additional analysis on the effects

of other covariates in Wildlife SOS locations such as antibiotics given or seropositivity for tuberculosis etc. did not show significant effects on gut bacterial diversity (Fig. S4).

Antibiotic resistance gene composition and diversity

Highly abundant multi-drug resistance genes

A wide array of ARGs were identified at each of the 14 locations. Total number of ARGs, unique ARGs (without duplicates), and unique to location ARGs are given in Table 1. Total number of ARGs detected was nearly proportional to sample size, and this can be understood intuitively as a higher detection of ARGs with larger sampling. While total number of unique ARGs in a location remained consistently between 53–80 for all groups with sample sizes < 10, it increased to 100–257 for groups with bigger sample sizes. Moreover, we see that the total number of ARGs did not stabilise after a certain sample size, and unique ARGs also kept increasing. Further, the total number of 'unique to location' ARGs increased proportionally with increase in sample size. Therefore, irrespective of location, we can conclude that approximately 50 ARGs could be identified in each captive sloth bear, and bigger the group, higher were the chances of finding new and unique ARGs. A total of 92 unique AMR gene families were identified across 14 locations. We have represented top 21 AMR gene families from these locations in Fig. 3A. The most abundant genes confer efflux-pump-based resistance to antibiotics, and they constitute nearly 50% of the resistance at all locations. Another note-worthy aspect is that the profiles of AMR gene families for Wildlife SOS locations remain similar, just as in the case of microbial diversity. Relative abundance of top 23 drug classes out of a total of 73, is shown in Fig. 3B. We observed that nearly 10 of the top 23 drug classes are MDR groups, all conferring resistance to a wide range of commercially available antibiotics. Wildlife SOS centers again showed very similar resistome profiles at the drug class level (Fig. 3B).

Table 1
Description of antibiotic resistance genes (ARGs) identified in captive sloth bear samples

Location	Total no. of Samples	Total no. of ARGs	Total no. unique ARGs, without duplicates	Total no. of ARGs unique to each location
AAZP	1	54	53	1
ZGA	1	55	53	1
GRC	2	63	47	1
KNZP	2	93	73	2
ELSP	3	117	68	1
BBP	3	144	87	1
KAZP	3	203	91	5
TLST	4	180	80	1
VVNP	4	123	80	4
NZP	10	532	109	6
SCZG	11	818	130	19
WSOS-BH	15	827	155	11
WSOS-BNP	59	3409	211	42
WSOS-A	97	3982	257	76

Uniformly high ARG diversities

Alpha diversity plot for all five locations – NZP, SCZG, WSOS-BH, WSOS-BNP and WSOS-A showed a very high median Shannon index value (> 3) indicating the presence of diverse and highly abundant ARGs in all animals (Fig. 3C). One-way ANOVA highlighted that two groups: WSOS-BNP – SCZG, and WSOS-A – WSOS-BNP, showed significant differences in their ARG diversities. Dunn's post hoc test applied after Kruskal–Wallis identified three significantly different pairs, viz, NZP - WSOS-A, SCZG - WSOS-A, and WSOS-A - WSOS-BNP. Interestingly we found no significant difference in ARG diversities in animals which were given or not given antibiotics in the past 3 months prior to sample collection (Fig. 3D). This is also true for microbial diversity (Fig. S5 A and B) indicating that absence of recent exposure to antibiotics does not seem to have any noticeable positive effect on gut bacterial or ARG diversities in these animals.

Similar to bacterial diversity, we performed a Linear Mixed Model (LMM) on the Shannon index values based on ARG abundance RPKM matrix to understand the influence of multiple factors on alpha diversity. Since the data were right skewed, log of Shannon index was modelled, after removing

singletons, while retaining random and fixed effects as stated previously. Location explained only about 10% of the variance, while age, sex and health status of the animal had almost no effect on observed AMR diversity (Fig. S6). In order to understand whether the high ARG diversity was only due to a few samples or characteristic of the entire group, we plotted beta diversity as a PCoA plot based on Bray-Curtis dissimilarity matrix (Fig. 4A). Although the scatter plot did not show any significant clustering, it had interestingly taken an inverted horse-shoe shape (Fig. 4B), also known as Guttman effect (Morton et al., 2017). Samples from all five locations were evenly dispersed and the 95% confidence ellipses overlapped, indicating that high ARG abundance is in fact a common feature across all locations and that all these locations are uniformly diverse. PERMANOVA test showed a significant p-value, but poor R^2 value for location (R^2 = 0.10565), implying that ARG diversities did not cluster based on locations, and that the differences in ARG diversities found across locations is not strongly influenced by any of the studied factors (Fig. S6). Since we had a significant number of samples (n = 97) from WSOS-A, we analysed this group further to understand underlying causes of the inverted horse-shoe shape. While most of the ARGs exhibited multi-drug resistance and were not targeted to specific antibiotics, we did see a variation between the two arms of the inverted horseshoe. The right arm had a higher abundance of ARGs with efflux-pump-based resistance, while the left arm had a higher abundance of ARGs with resistance mechanisms involving target replacement or target alteration.

Spearman's correlation analysis revealed a weak but significant negative correlation between Shannon indices for microbial diversity and total number of ARGs (ρ = -0.31, p-value = 8.9e-0.6), and total ARG abundance (ρ = -0.2, p-value = 0.0051), indicating that animals with lower gut microbial diversity seem to be associated with higher richness and abundance of ARGs (Fig. 5A, Fig. S7). Distance correlation test, with 999 replicates, revealed a significant relationship between microbiome diversity and total number of ARGs (dCor = 0.2578183, p = 0.02) and ARG abundance (dCor = 0.39478, p-value = 0.001), indicating a moderately dependent non-monotonic or non-linear relationship. A high number of genera constituted the 'core microbiome' in captive sloth bears, as a low number of genera were filtered out at 20% or 30% prevalence. Hence, after filtering for 50% prevalence, i.e. genera present in at least 50% of the animals, we arrived at a list of 1973 out of 2342 genera. Out of these, 1464 genera which correlated significantly (padj-value < 0.05) with sum total of ARG abundances are depicted in Fig. 5B. Genera with highest positive correlation with AMR abundance are WHO-priority pathogens like *Escherichia* and *Shigella*. Only 39 genera had positive correlation coefficients, while the remaining 1425 genera had negative correlation coefficients (Table S4). In case of genera abundance against ARG richness in a sample, only 49 genera (13 negative and 36 positive) were found to be significantly linked. Majority of the genera contributing to highest numbers of ARGs were similar to the ones found in Fig. 5B. We did not find any significant effect of time spent in captivity on overall gut bacterial diversity, ARG count and total ARG abundance.

Prevalence of mobile genetic elements (MGEs) co-occurring with antibiotic resistant genes (ARGs)

ARG drug classes co-occurring with various MGEs are listed in Fig. 6. Our analysis revealed that 21 out of 22 multidrug resistant groups co-occur with various mobile genetic elements, further highlighting the

fact that not only is there a high prevalence of multi-drug resistant ARGs, but many of them also co-occur with plasmids or pro-viral elements. Nearly 20% (2178 out of 10600) of ARGs found across all locations were co-occurring with one or the other MGEs. Barring a few locations with low sample size, this trend was consistent for plasmids across all locations. In fact, we found more plasmids associated with ARGs of various drug classes in the 5 locations with higher sample sizes – NZP, SCZG, WSOS-BH, WSOS-BNP and WSOS-A (Fig. 6). Pro-viral element co-occurrence was also majorly noticed in these locations (Fig. 6), possibly implying that a larger sampling would be necessary for detecting such co-occurrence.

Across all locations, we observed that the most abundant plasmid-associated ARGs are *acrAB-tolC, marA and* mutated *E. coli SoxR/S* (Fig. 6). These ARGs confer resistance against multiple drugs. MDR-1, MDR-4 and fluoroquinolone resistance were predominant drug classes and were primarily enriched in SCZG, followed by NZP and Wildlife SOS centers. The above genes are mainly responsible for controlling the bacterium's response to oxidative stress, and also confers antibiotic resistance through efflux pump and target alteration (Kumawat et al., 2023). Animals in NZP showed the lowest gut bacterial diversity, and in parallel had a high abundance of plasmid-associated e*rmB* gene which confers resistance to macrolide, lincosamide, and streptogramin B (MLSB) antibiotics. We also observed a higher abundance of plasmids carrying carbapenem-resistant $bla_{OXA-232}$ in sloth bears at SCZG. Similar to plasmids, efflux-based *E.coli* genes (*emrE*) were mostly noted to be associated with pro-viral elements at the five locations with higher sample sizes (Fig. 6). In case of animals at WSOS-A, we also observed higher abundance of two genes *PmrF* and *ugd*, associated with pro-viral elements, which provide resistance to the last resort drug class, polymyxin (Fig. 6). Further analysis within plasmid regions revealed that the most abundant ARGs also frequently co-occur with additional MGEs like integrons and transposons. Highly abundant ARGs like *dfrA*, *aadA*, *ANT(3")-Ila*, *sul3* and *cmlA1* that were noted in plasmids (Fig. 6) at Van Vihar National Park (VVNP), Bhopal are present along with integron cassettes, further underlining their virulence. These genes confer resistance to several drug classes such as aminoglycoside, tetracycline, sulfonamide, quinolone, chloramphenicol and diaminopyrimidine antibiotics. A set of diaminopyrimidine and aminoglycoside drug class resistant ARGs were found along with integrons in animals at WSOS-BNP. Transposons were observed to co-occur with ARGs in animals at 8 out of the 14 locations. Individuals at three locations – VVNP, WSOS-BNP and WSOS-A in particular, had multiple genes that confer multi-drug resistance via efflux pumps and tetracycline resistance mediated by antibiotic target protection. The plasmid-associated e*rmB* gene, mentioned earlier in bears at NZP, was seen to be flanked by transposons. We also found the common plasmid-associated efflux pump gene, *acrAB-tolC*, co-occurring with transposons in sloth bears at VVNP (Fig. 6).

Metagenome-Assembled Genomes (MAGs) and key drivers of AMR

Reads corresponding to the five most abundant genera viz. *Streptococcus* (Taxon ID: 1301), *Escherichia* (Taxon ID: 561), *Sarcina* (Taxon ID: 1266), *Clostridium* (Taxon ID: 1485) and *Klebsiella* (Tax ID: 570) were extracted from all 215 samples. We restricted this genome-resolved analysis to the above genera, as

they together accounted for ~ 75% of average community abundance across all locations, in order to focus our computational resources on the taxa most likely to drive the community-level resistome. We acknowledge that this targeted approach precludes genome reconstruction for rarer taxa and we do not rule out possible ARG hosts other than these genera. Contigs were assembled at sample-level and relevant assembly stats are summarised in Table S5. The 14 locations' pooled binning processes yielded a total of 1741 bins, out of which 442 high quality bins were identified. We noted high redundancy after assigning taxonomy to these 442 bins, and so we de-replicated the bins to obtain 23 high quality de-replicated bins. We resolved 21 out of the 23 bins up to species level, while we resolved all bins until genus level. We identified ARGs with greater than 98% identity in 7 out of the 23 bins, and their abundances are depicted in Fig. 7. We noticed that three Enterobacteriaceae species - *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella variicola*, harboured nearly 93% (65 of 70) of the ARGs identified, majority of which were multi-drug resistance efflux pump-based genes. These bacterial species are known to develop resistance to a wide range of drug classes (WHO, 2024). This disproportionate concentration of ARGs, supports our inference that Enterobacteriaceae species play a dominant role in the resistome of captive sloth bears.

Peptide drug class resistance gene was also noted in all the bacterial species except in *Streptococcus faecavium*, while exclusively in *Clostridium perfringens*. We identified macrolide and streptogramin drug class resistance genes in *Streptococcus faecavium*, tetracycline resistance gene in *Streprococcus parasuis*, and peptide and macrolide resistance genes in *Streptococcus agalactiaae*. *Klebsiella pneumoniae* was exclusively associated with MDR-8 and MDR-11, while *Klebsiella variicola* was found to be associated with MDR-20 and penem; penam drug classes (Fig. 7). ARGs in *Escherichia* and *Klebsiella* spp. were seen in high abundances in sloth bears at Kanpur Zoological Park, Kakatiya Zoological Park, Tiger and Lion Safari at Thevarakoppa, and Sri Chamarajendra Zoological Gardens (Fig. 7).

Discussion

Endangered and vulnerable species, and the microorganisms they harbour mainly in their gut, are subject to several environmental stressors like habitat loss, climate change and pollution, competition for resources and food, and hunting. Exponential growth and global movement of human populations, and forest fragmentation have brought wildlife in close proximity to humans and livestock, exposing it to environmental pollutants and antimicrobial drugs (Li et al., 2024; Mourkas et al., 2024). This situation is further aggravated in captivity where animals are treated for injuries and/ or disease, and are inadvertently fed antibiotic- and pesticide-treated food leading to dysbiosis of the gut microbiome and a buildup in antimicrobial resistance (Miller et al., 2024). To the best of our knowledge this is the first comprehensive metagenomic/ shotgun sequencing study on the gut microbiome of bears. Our study assessed captive sloth bears housed across an extensive, biodiverse landscape, wherein we derive crucial understandings on the possible effects of location, age of animal, and duration in captivity on gut microbial diversity and AMR status of the animal. Given that studies which focus on the extent and impact of AMR on wildlife health are negligible (Mitchell, 2023), our research offers valuable insights for the management and conservation of endangered species.

We observed that Bacillota, Bacteroidota, Pseudomonadota, and Actinomycetota were the most dominant bacterial phyla in all the sloth bears. This is similar to studies based on 16S rRNA sequencing in Andean bear (Borbón-García et al., 2017), black bear (Gillman et al., 2020), brown bear (Trujillo et al., 2022) and polar bear (Franz et al., 2022; Lu et al., 2024) irrespective of whether the subject was captive or wild. Strikingly we found *Streptococcus*, *Escherichia*, *Klebsiella, Sarcina* and *Clostridium* as the most abundant genera in all locations, some of which are designated by WHO as critical priority pathogens capable of developing resistance to antibiotics (WHO, 2024). Overall, we detected lower microbial diversity in all sloth bear samples from NZP, SCZG, WSOS-BH, WSOS-BNP and WSOS-A, although the diversity was higher in the Wildlife SOS centers than Hyderabad and Mysuru zoos, and it was higher in younger animals than older individuals. These findings are similar to those reported in several species of canids, primates and equids (Clayton et al., 2016; McKenzie et al., 2017), gaint panda (Guo et al., 2019) and brown kiwi (San Juan et al., 2021). Although we do not have samples from wild sloth bear for comparison, our data indicate that the microbial diversity gradually declines with increasing age of the animal. This is a very critical change in captive animals as higher gut bacterial diversity acts as an indicator of positive gut microbiota that would resist the growth of infectious agents and also acts as a barrier to AMR buildup (Spragge et al., 2023).

There are a rising number of studies globally on wildlife species as reservoirs, disseminators or sentinels of anthropogenic AMR contamination in the environment (reviewed extensively by Li et al., 2024). These studies generally focus on migratory birds and wild mammals which move close to or scavenge in human habitation or agriculture land. However, studies on prevalence or buildup of AMR in endangered species in captivity which could affect their health and survival are limited (Guo et al., 2019; Ramey & Ahlstrom 2020; Campbell et al., 2020; Wang et al., 2021; Cao et al., 2023; Zhai et al., 2024). We observed that the ARGs identified in captive sloth bears belong to various AMR gene families and antibiotic drug classes indicating diverse resistance to various antibiotics, as also highlighted by high ARG alpha diversity. The four most abundant AMR gene families confer resistance to more than six broad-spectrum antibiotics like fluoroquinolones, macrolides, cephalosporins, tetracycline and carbapenems. These ARGs confer resistance, mostly via efflux pump, across antibiotic classes, largely through ABC (ATP-binding cassette antibiotic efflux pump), MFS (Major facilitator superfamily antibiotic efflux pump), and RND (Resistance-nodulation-cell division antibiotic efflux pump) families.

Our study not only shows that ARG richness and diversity are very high in captive animals, but also that the total number of ARGs as well as ARGs unique to a location increase as we sample more individuals. Campbell et al. (2022) similarly reported high ARG abundance and richness in captive apes dominated by β-lactam and tetracycline resistance genes. More importantly, we observed that almost all captive sloth bears consistently have high levels of AMR even when they have not been treated with antibiotics in the recent past (≤ 3 months). While we do not have much information on reversibility of antibiotic resistance (Guo et al., 2024), our findings suggest that the gut bacteria of captive animals might be evolving in response to other stressors like environment and nutrition. The other possible explanation is the horizontal transfer of antibiotic resistance mediated by mobile genetic elements like plasmids (Baker et al., 2018; Liu et al., 2022). Interestingly, we saw the horseshoe or Guttman effect (Morton et al., 2017)

in the PCoA plot of AMR diversities. Although the scatter plots did not show any effect of location on AMR diversity and abundance, samples seem to separate along two sides of the horseshoe based on mechanism of antibiotic resistance and associated mobile genetic elements. A caveat here is that the history of antibiotic use was recorded only as a binary variable (treated versus untreated) without detailed information on drugs used, dosage, duration or cumulative lifetime exposure. A single short course versus repeated or chronic treatment can have very different ecological consequences for the resistome, and therefore this coarse variable might have masked the real effects of antibiotic exposure. Similarly, since the recovery trajectory of gut microbiota after antibiotic treatment can extend well beyond three months (Palleja et al., 2018), our sampling window may not have been long enough to capture the full dynamics of microbial diversity and ARG loss/regain.

The gut bacterial diversity in captive sloth bear showed significant inverse correlation with both ARG abundance and richness. As mentioned earlier a rich microbial composition is crucial to prevent AMR buildup (Klümper et al., 2024), and this is true across diverse microbial niches (Mao et al., 2023; Klümper et al., 2024; Shayista et al., 2024). Abundances of pathogenic genera like *Shigella*, *Escherichia* and *Salmonella* showed highly significant positive correlations with ARG abundance and richness. We also see significant positive correlations with lesser studied enterobacteria like *Citrobacter* and *Kluyvera* which are fast emerging globally as organisms resistant to broad spectrum antibiotics (Yao et al., 2021; Qin et al., 2021; Monte et al., 2024). Our results indicate the possibility of a selectively thriving group of organisms or even the likely accumulation of resistant genes in microbiomes with reduced compositional diversity. This statement can be supported by the fact that the abundances of very few genera are elevated along with higher AMR abundance and richness, while the abundances of approximately 1400 genera are reduced indicating lowered microbial diversity. However, since our data are cross-sectional, the direction of this association cannot be established from our findings alone. It is plausible that low gut microbial diversity creates a niche that favours ARG-carrying taxa. It is equally possible that a high ARG burden — often carried by pathobionts and mobile genetic elements — actively suppresses commensal populations, thereby lowering diversity, as has been shown experimentally in humans following broad-spectrum antibiotic exposure (Palleja et al., 2018).

Multi-drug resistance mediated through efflux pump genes was found to be largely associated with a high abundance of plasmids at most of the captive locations, more so at the five locations - NZP, SCZG, WSOS-BH, WSOS-BNP AND WSOS-A – with higher numbers of sloth bears. A large number of plasmids were seen to be associated with *E. coli* mutations leading to resistance to several broad-spectrum antibiotics like tetracycline, ciprofloxacin, ceftazidime, carbapenems and other β-lactams, etc. thereby compromising the clinical utility of widely used drugs against Gram-negative bacteria. In their review, Castañeda-Barba et al. (2024) describe plasmids as a cornerstone in the dissemination of AMR between bacterial hosts in different environments. The emergence, transfer and persistence of plasmid-mediated resistance, even in the absence of selection pressures like active antibiotic treatment, are extremely complex phenomena which occur simultaneously at different biological levels. Although difficult to eliminate, careful and conscientious administration of antibiotics to animals will definitely restrict selection of plasmid-associated AMR. It is also important that these animals are housed in hygienic

conditions with good sanitation and nutrition. Buildup of plasmid-mediated resistance in pathogenic bacteria can be diluted and even prevented by maintaining high microbial diversity in the gut (Loftie-Eaton et al., 2021).

We saw a similar trend even in locations with fewer numbers of animals. For example, Van Vihar National Park in Bhopal had four sloth bears at the time of sample collection, all of which have been in captivity for 14 + years. We observed a build-up in *Sarcina* in these individuals, and also a higher abundance of plasmid- and MGE-associated ARGs primarily against diaminopyrimidines and aminoglycosides. Presence of diverse resistance genes in this single site could either mean intense and persistent antibiotic selection pressure or a highly permissive environment for microbial gene exchange. The latter is more likely as the average gut bacterial diversity is low in this location, combined with the presence of virulent MGEs. While the abundances of non-plasmid mobile genetic elements like pro-viral elements, integrons and transposons associated with ARGs were much lower than plasmid abundance in captive sloth bear gut, these too play important roles in horizontal gene transfer in bacterial communities and in developing antimicrobial resistance. Macrolides are broad spectrum antibiotics effective against a wide variety of Gram-positive bacteria, and we see efflux pump-based macrolide resistance genes associated to pro-viral elements in sloth bear. Horizontal transfer and spread of such ARGs can have serious consequences on health of captive animals.

We noted that a large majority of ARGs (93%) were localized within MAGs classified as *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella variicola*. These taxa are well-recognized members of Enterobacteriaceae and are frequently implicated as major reservoirs and disseminators of antimicrobial resistance across clinical and environmental settings (Penders et al., 2013; Woolhouse et al., 2015). Their dominance as ARG carriers in the sloth bear gut microbiota indicates that resistance is not randomly distributed across taxa but is concentrated within specific phylogenetic groups with high genomic plasticity. Importantly, these genera are characterized by their ability to acquire and disseminate resistance determinants via horizontal gene transfer, particularly through plasmids, integrons and transposons. This aligns strongly with our observations of widespread co-occurrence of ARGs with mobile genetic elements, including plasmid-associated efflux pump systems such as *acrAB-tolC* and regulatory genes like *marA*. The convergence of MAG-resolved taxonomy with mobilome analysis provides compelling evidence that ARG dissemination in sloth bear gut is driven by a network of highly adaptable bacterial hosts and mobile elements, rather than isolated genetic events (Partridge et al., 2018; Castañeda-Barba et al., 2024).

While Enterobacteriaceae are naturally present in mammalian gut microbiomes, their disproportionate contribution to the resistome in this study suggests an ecological imbalance. In relatively undisturbed systems, ARGs are typically more diffusely distributed and often associated with intrinsic resistance mechanisms. In contrast, the enrichment of clinically relevant, high-identity ARGs within *Escherichia* and *Klebsiella* genomes points toward anthropogenic selection pressures shaping the gut microbiome of captive sloth bears. Such pressures could arise from indirect antibiotic exposure through diet, environmental contamination, or historical veterinary interventions, even in the absence of recent

antibiotic administration (Martínez, 2009; Li et al., 2024). The location-specific patterns observed in ARG abundance further support this interpretation. Sites such as KNZP, KAZP, TLST and SCZG, which showed higher abundance of ARGs, also exhibited increased representation of Pseudomonadota (Proteobacteria), the phylum to which *Escherichia* and *Klebsiella* belong. Expansion of Proteobacteria is often considered a hallmark of gut dysbiosis and has been linked to inflammatory and stressed gut environments (Shin et al., 2015). In this context, the proliferation of these taxa may create a permissive ecological niche for ARG accumulation and exchange, reinforcing the negative association observed between microbial diversity and ARG burden. Although other genera such as *Clostridium* and *Streptococcus* were associated to specific resistance classes, their overall role in the resistome seems comparatively limited. This highlights a functional specialization within the microbiome, where a subset of taxa act as primary hubs of resistance. Concentration of ARGs within a few dominant and mobile taxa is particularly concerning as it increases the likelihood of rapid dissemination across microbial communities and potentially across hosts.

Taken together, the genome-resolved analysis strengthens our overall findings by demonstrating that antimicrobial resistance in captive sloth bears is structured and ecologically driven. The concentration of ARGs within highly mobile, clinically relevant taxa underscores the health and role of wildlife microbiomes within the broader One Health framework, linking environmental contamination, animal health, and potential public health risks. Given the disparity in resources across institution types, recommendations should be tiered: rescue centers with dedicated veterinary and laboratory infrastructure, like the Wildlife SOS centers in this study, are better positioned to pilot targeted microbiome-restoration interventions such as probiotic supplementation or fecal microbiota transplantation from low-ARG-burden donor animals, approaches shown to reduce resistance-gene carriage and restore microbial diversity in other host systems (Millan et al., 2016). In contrast, standard zoos with more limited diagnostic capacity would benefit most from regular probiotic supplementation, and low-cost routine environmental monitoring — periodic screening of enclosure soil, water, and feed for AMR and key indicator organisms (e.g., *E. coli*, *Klebsiella* spp.) — as a practical starting point for health surveillance (Doyle et al., 2025).

Conclusions

This study provides a comprehensive assessment of the gut microbiome, its resistome and mobilome of captive sloth bears across India. We demonstrate that captivity is associated with reduced microbial diversity and a consistently high burden of antimicrobial resistance genes, many of which confer multi-drug resistance through efflux-based mechanisms. A key finding of this study is the disproportionate localization of ARGs within members of Enterobacteriaceae, particularly *Escherichia coli* and *Klebsiella spp.*, as revealed through metagenome-assembled genomes. The strong association of these ARGs with plasmids, integrons and transposons highlights the central role of horizontal gene transfer in shaping the resistome. Importantly, the persistence of high ARG abundance irrespective of recent antibiotic exposure suggests that resistance determinants are now stably embedded within the gut microbial communities of captive animals. The observed negative relationship between microbial diversity and ARG burden,

coupled with the expansion of Proteobacteria, indicates a dysbiotic gut environment that favors the proliferation and maintenance of resistant taxa. These findings collectively point toward sustained anthropogenic influence on the microbiome of captive wildlife, likely mediated through long-term environmental exposure, diet and management practices. From a conservation and management perspective, our results emphasize the need to incorporate antimicrobial resistance surveillance into wildlife health monitoring programs. Strategies aimed at improving gut microbial diversity—through optimized diet, probiotic supplementation, reduced antibiotic usage, and improved sanitation may help mitigate the accumulation and spread of resistance. Within the One Health context, captive sloth bears emerge as important sentinel systems reflecting the environmental dissemination of antimicrobial resistance. Addressing AMR in such settings will not only improve animal health and welfare but also contribute to broader efforts in controlling the spread of resistance across ecological boundaries.

Declarations

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Author Contributions

VPP, VSK, ABS and PAR conceived and planned the study, and designed experiments. SN, NS and GK collected samples and metadata, and isolated DNA. SN prepared samples for sequencing. VPP and VSK performed bioinformatics analyses. AAS, SI, MVB, MKP and MAH provided logistic support and resources. ABS and PAR arranged for funds and managed the project. VPP, VSK, SN, ABS and PAR wrote the manuscript. All authors reviewed and edited the manuscript.

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Availability of data and materials

Sequence data analysed in this study have been deposited in the European Nucleotide Archive (ENA) with BioProject accession number PRJEB101775. The scripts used for analysis have been deposited in Zenodo at doi:10.5281/zenodo.20406887

Ethical Statement

Permissions to collect samples for this study were granted by Chief Wildlife Wardens of West Bengal (No.1703/WL/4R-31/2021, dated 01/09/2021), Maharashtra (No: Desk-22(8)/WL/Research/CR-37(21-22)/1344/21-21, Nagpur, dated 07/09/2021), Telangana (No.26803/2012/WL-2, dated 14/09/2021), Tamil Nadu (No.4822/2021/WL1, dated 28/01/2022), Madhya Pradesh (No./M.H.-II/ Research/2824, Bhopal, dated 13/04/2022), Karnataka (No.PCCF(WL)/E2/CR-37/2021-22, dated 17/05/2022), and Uttar Pradesh (No. 23-2-12(G), Lucknow, dated 20/05/2022). All sampling adhered strictly to ethical guidelines for non-invasive genetic research and complied with the Wildlife (Protection) Act, 1972, Government of India. No animal was captured or handled during data collection.

Clinical trial number

Not Applicable

Consent for publication

Not Applicable

Competing Interests

All authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Figures

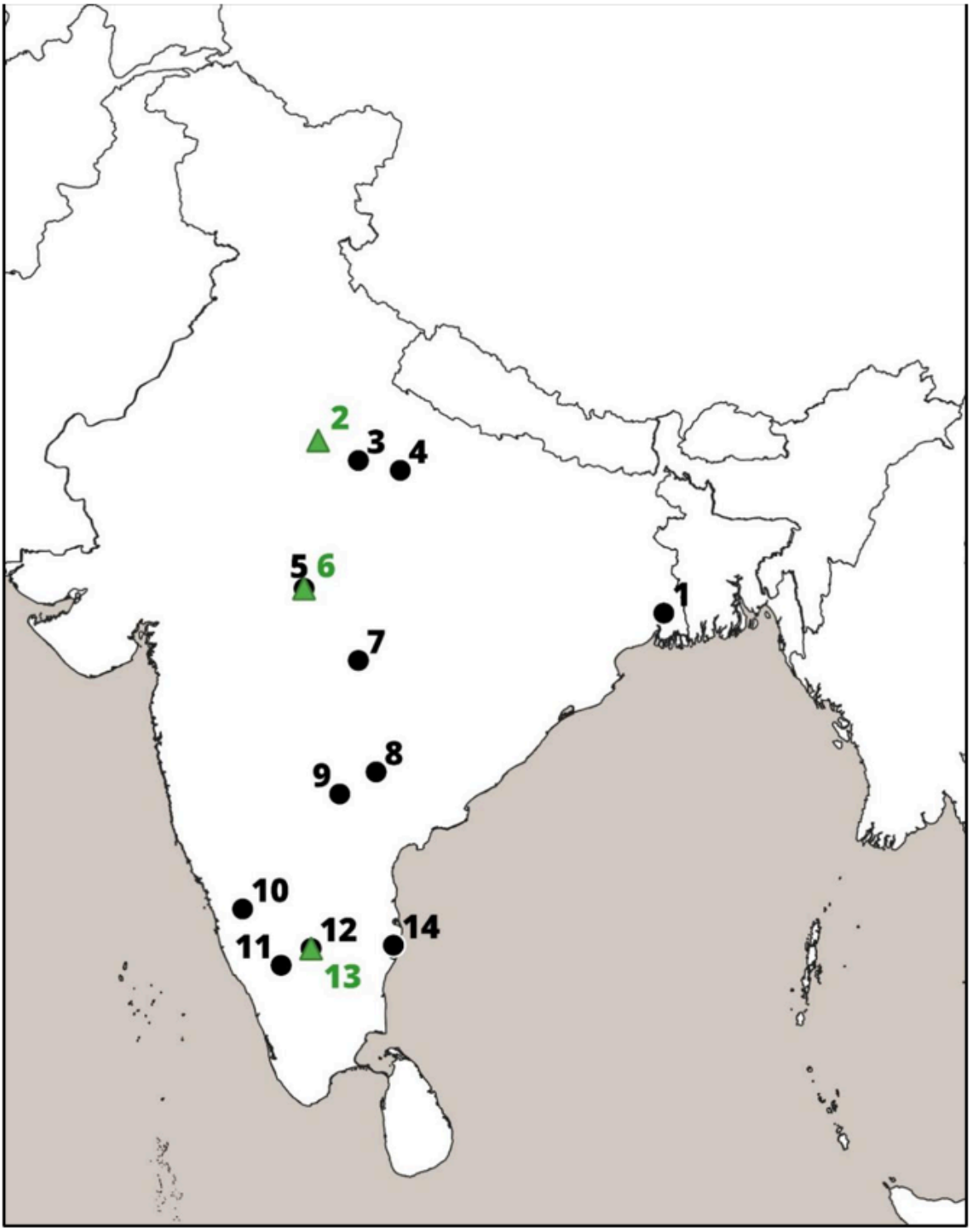


Figure 1

Locations of zoos and rescue centers in India where captive sloth bear samples were collected. 1 – Zoological Garden Alipore, Kolkata (ZGA); 2 – Wildlife SOS, Agra (WSOS-A); 3 – Etawah Safari Park (ESP); 4 – Kanpur Zoological Park (KNZP); 5 – Van Vihar National Park, Bhopal (VVNP); 6 – Wildlife SOS, Bhopal (WSOS-BH); 7 – Gorewada Rescue Center, Nagpur (GRC); 8 – Kakatiya Zoological Park, Warangal (KAZP); 9 – Nehru Zoological Park, Hyderabad (NZP); 10 – Tiger and Lion Safari, Thevarakoppa (TLST); 11 – Sri Chamarajendra Zoological Garden, Mysuru (SCZG); 12 – Bannerghatta Biological Park (BBP); 13 – Wildlife SOS, Bannerghatta National Park (WSOS-BNP); 14 – Arignar Anna Zoological Park, Chennai (AAZP). The three Wildlife SOS centers are highlighted in green as maximum number of samples were collected from these locations.

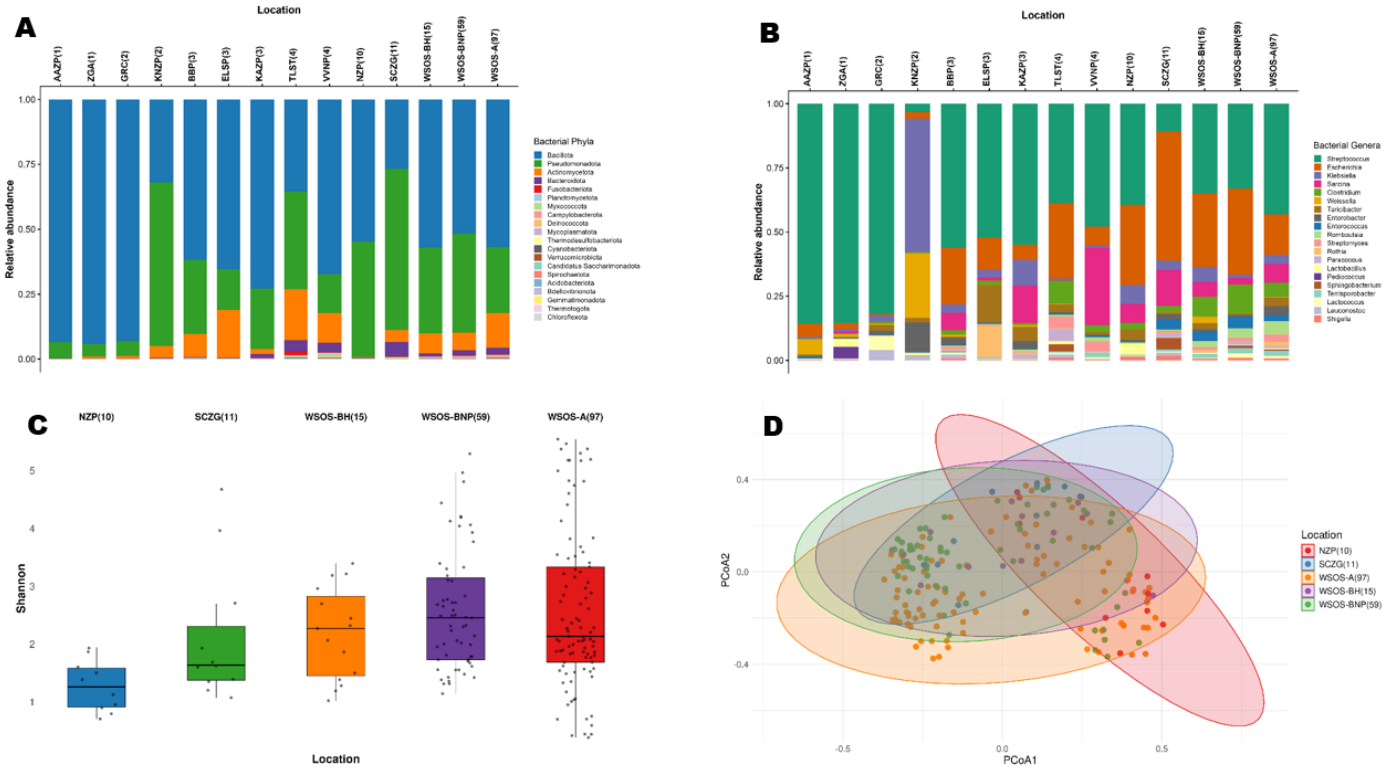


Figure 2

Bacterial community profiles at the phylum **(A)** and genus **(B)** levels in sloth bear samples from 14 captive locations in India. **(C)** Alpha diversity index and **(D)** PCoA of beta diversity based on Bray-Curtis dissimilarities for sloth bear samples from Nehru Zoological Park, Hyderabad (n=10), Sri Chamarajendra Zoological Gardens, Mysuru (n=11), Wildlife SOS, Bhopal (n=15), Wildlife SOS, Bannerghatta (n=59), and Wildlife SOS, Agra (n=97).

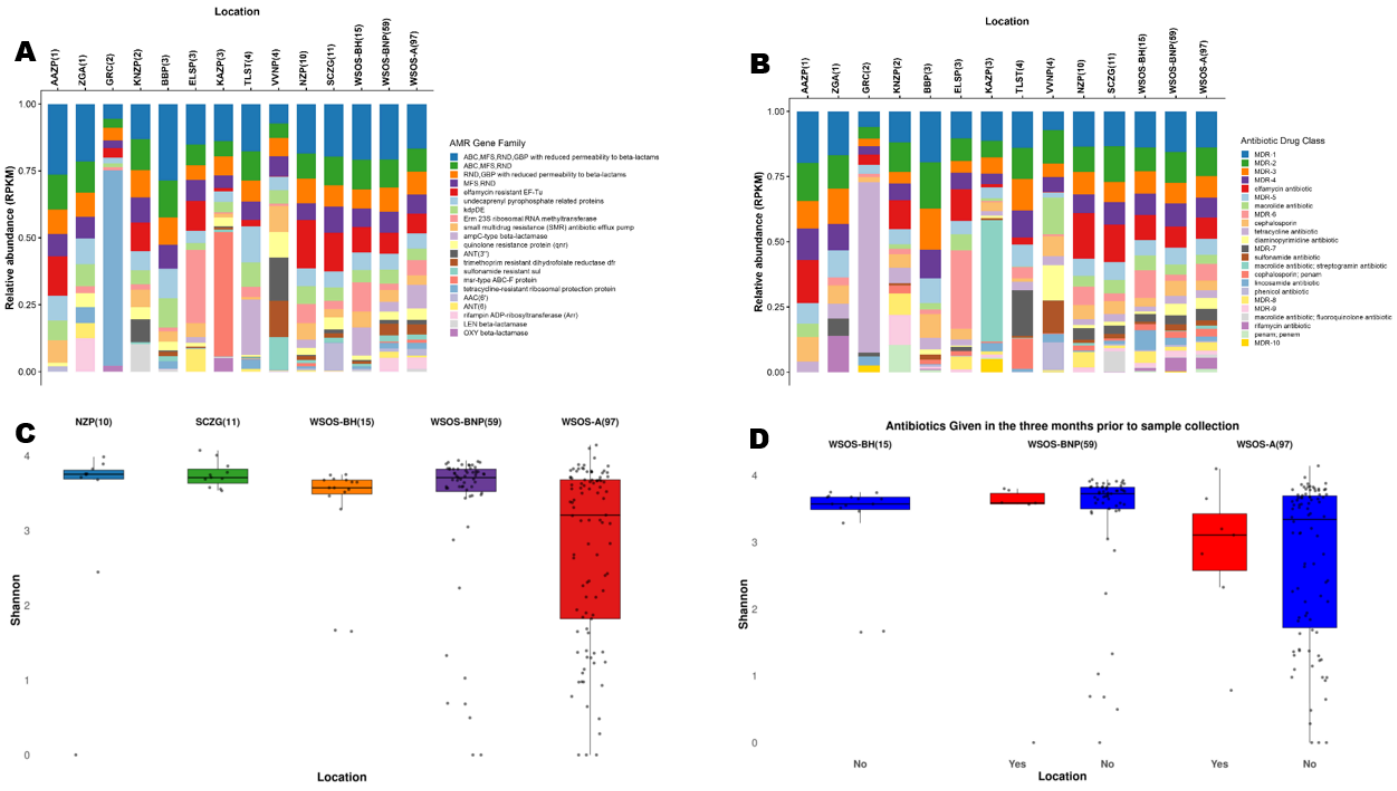


Figure 3

(A) Relative abundance of ARGs in reads per kilobase per million mapped reads (RPKM) grouped by AMR gene families, and **(B)** relative abundance of ARGs in RPKM stratified by antibiotic drug classes in sloth bear samples from 14 captive locations in India. **(C)** Alpha diversity index of ARGs in sloth bear samples from Nehru Zoological Park, Hyderabad (n=10), Sri Chamarajendra Zoological Gardens, Mysuru (n=11), Wildlife SOS, Bhopal (n=15), Wildlife SOS, Bannerghatta (n=59), and Wildlife SOS, Agra (n=97). **(D)** Alpha diversity index of ARGs in sloth bear treated (red) or not treated with antibiotics ≤ 3 months (blue) at the three Wildlife SOS centers.

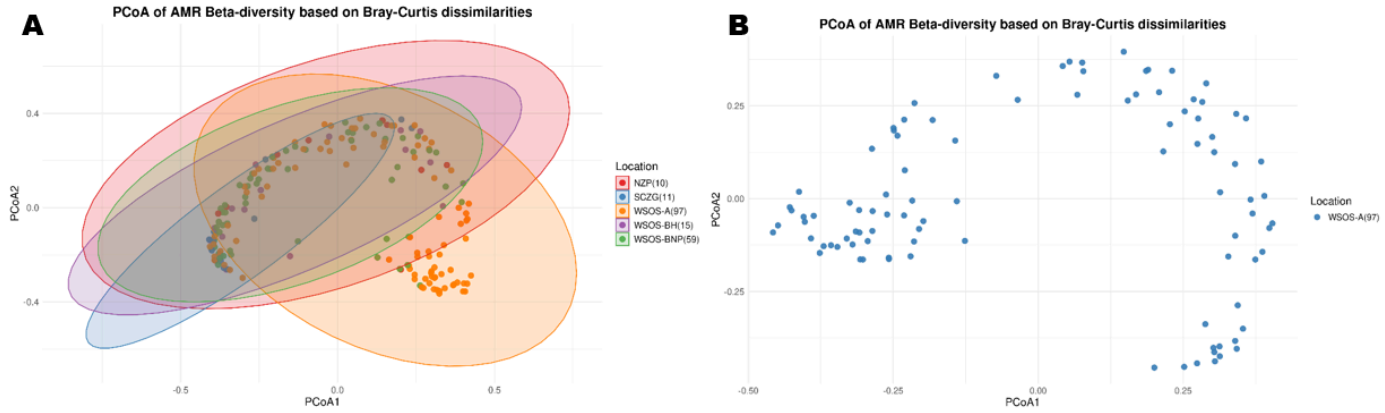


Figure 4

(A) AMR β-diversity analysis based on Bray-Curtis dissimilarity matrix in sloth bear samples from Nehru Zoological Park, Hyderabad (n=10), Sri Chamarajendra Zoological Gardens, Mysuru (n=11), Wildlife SOS, Bhopal (n=15), Wildlife SOS, Bannerghatta (n=59), and Wildlife SOS, Agra (n=97); and **(B)** only from Wildlife SOS, Agra (n=97), showing a horseshoe pattern.

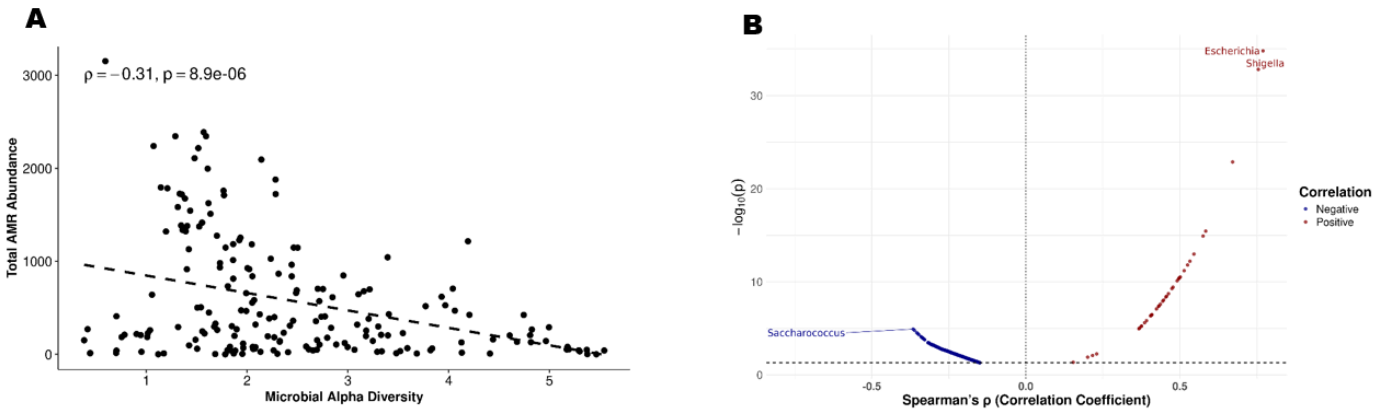


Figure 5

(A) Spearman's correlation indicating a negative association (ρ = -0.31, p-value = 8.9e-0.6) between Shannon indices of microbial diversity and AMR abundance, and (B) Volcano plot showing bacterial genera whose abundances significantly correlate to AMR abundance (padj-value<0.05 corrected for

multiple testing using Benjamini–Hochberg false discovery rate) in captive sloth bear samples (n=215) from 14 locations.

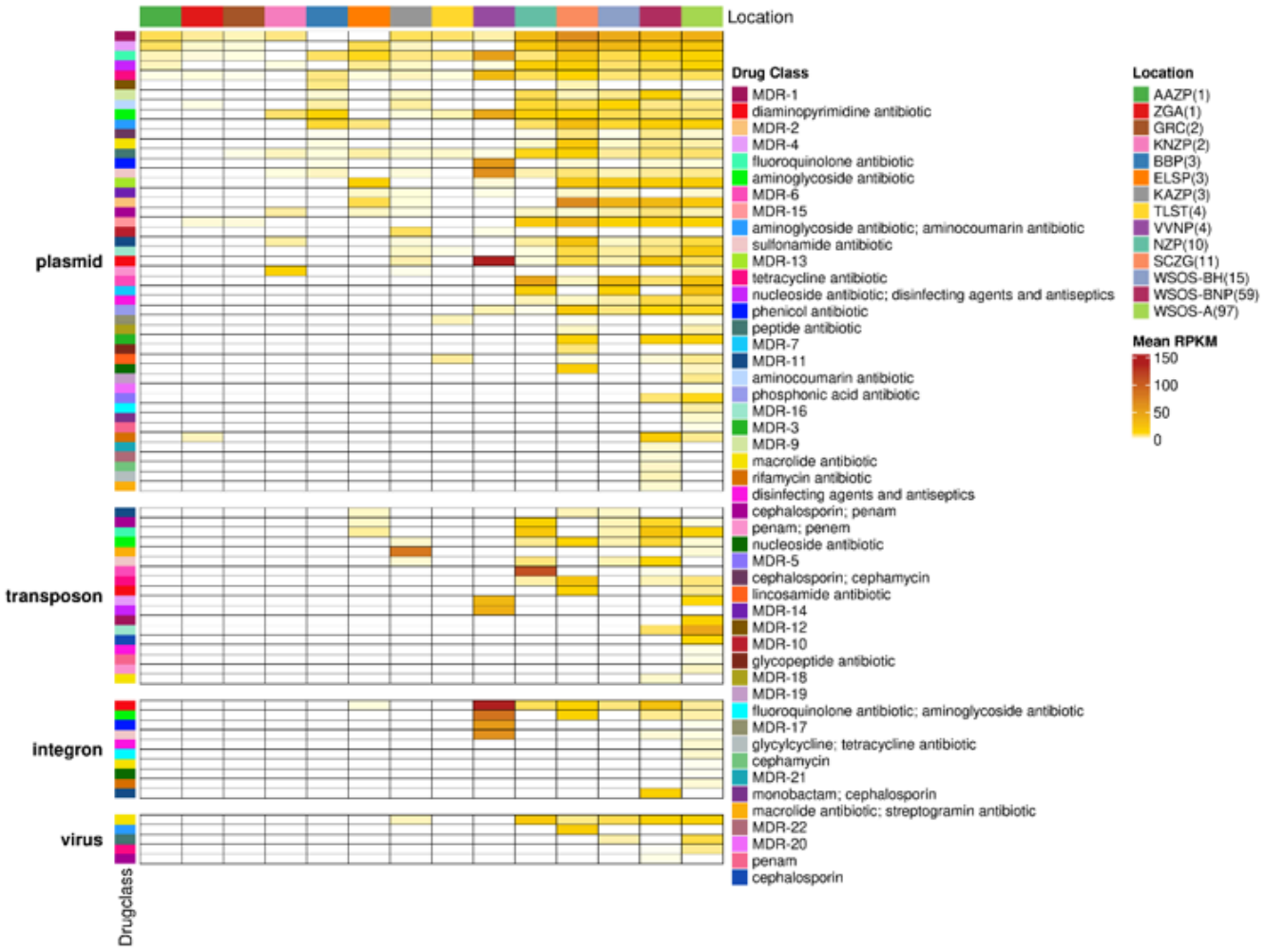


Figure 6

Heat map depicting the co-occurrence of mobile genetic elements (plasmids, pro-viral elements/ phages, integrons and transposons) with ARGs grouped by antibiotic drug classes in captive sloth bear samples (n=215) from 14 locations.

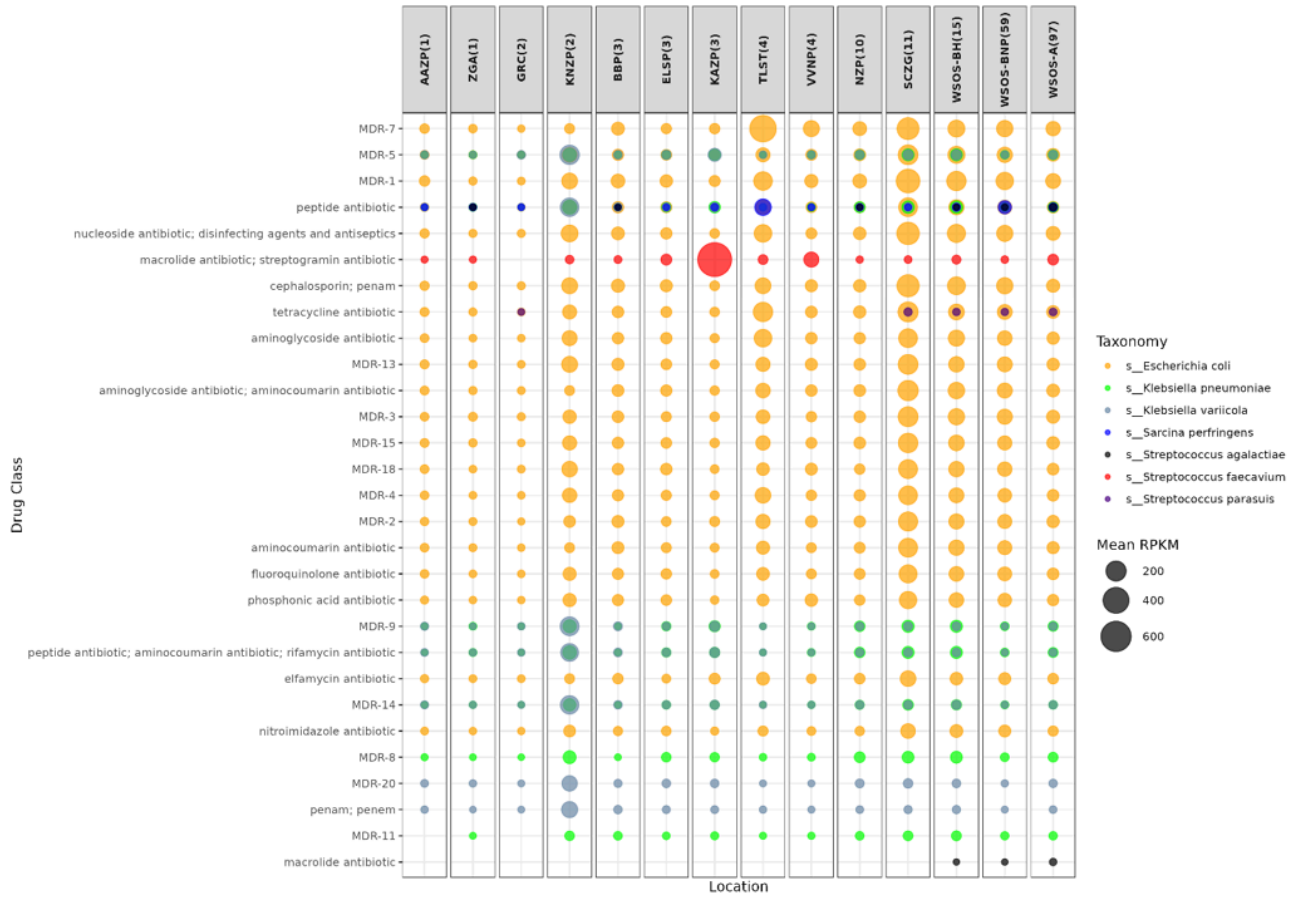


Figure 7

Bubble plot depicting the co-occurrence of Antibiotic Resistance Gene (ARGs) classes identified in metagenome-assembled genomes (MAGs), annotated by microbial species in sloth bear samples (n=215) from 14 locations. Bubble colour indicates species identity, while bubble size represents ARG abundance measured as RPKM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.docx
- TableS2.docx
- TableS5.csv
- TableS3.xlsx
- TableS1.csv
- TableS4.xlsx

- SupplementaryData.docx