

Host Ontogeny Structures Gut Microbiome Composition and Pathogen-Associated Bacterial Communities in the Endangered Ethiopian Wolf (*Canis simensis*)

Girma Ayalew Mengistu

girmasas@gmail.com

Ethiopian Wildlife Conservation Authority

Adey Feleke

Addis Ababa University

Helen Nigussie

Addis Ababa University

Anagaw Atickem

Addis Ababa University

Nigatu Kebede

Addis Ababa University

Alemayehu Lemma

Addis Ababa University

Chala Bashea

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Melak Getu

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Atsbeha Gebreegziabxier

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Gebremedhin Gebremicael

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Tolewak Sura

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Eleni Kidane

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Desta Ayana

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Abraham Ali

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Gemechu Tadesse

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Getachew Tollera

Ethiopian Public Health Institute, Integrated Pathogen Genomics and Bioinformatics facility for Surveillance and Research. Addis Ababa

Muktar Abute

Ethiopian Wolf Conservation Program

Yohannes Ayalew Mengistu

Sea Mar Community Health Centers Administrative Office

Girma Eshete

Bahir Dar University

Gurja Belay

Addis Ababa University

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Abstract

The Ethiopian wolf (*Canis simensis*), the world's rarest canid and a highly specialized Afroalpine carnivore, faces increasing threats from habitat fragmentation, human encroachment, and infectious disease. Understanding gut microbiome variation in this endangered species is important for elucidating host–microbe interactions, pathogen ecology, and conservation risks at the wildlife–livestock–human interface. We used shotgun metagenomic sequencing to characterize the gut bacterial microbiome of 31 Ethiopian wolves from Bale Mountains National Park, Ethiopia, and evaluated age- and pack-associated variation in community composition and pathogen burden. Shannon alpha diversity did not differ significantly among juvenile, subadult, and adult wolves. In contrast, beta diversity analyses revealed significant age-associated differences in gut microbial community composition, whereas pack membership had little influence. Species- and genus-level analyses identified several nominal age-associated abundance trends, but no taxa remained significant after false discovery rate correction, indicating that age-related variation reflected diffuse community-wide restructuring rather than large shifts in individual bacterial taxa. Fourteen bacterial taxa with documented pathogenic potential, including several zoonotic species, were detected. Pathogen burden differed significantly among age classes, with adult wolves exhibiting a higher cumulative abundance of potentially pathogenic bacteria than subadults, while no significant differences were observed among packs. These findings demonstrate that host development is an important determinant of gut microbiome composition and pathogen burden in Ethiopian wolves and provide the first shotgun metagenomic baseline for this endangered species, supporting future conservation and One Health surveillance initiatives.

Introduction

The gastrointestinal microbiome plays a fundamental role in host nutrition, immune function, metabolism, and disease susceptibility, thereby influencing health and fitness across vertebrate taxa [1], [2]. In wildlife, gut microbial communities are increasingly recognized as important components of host ecology and conservation biology because they mediate host–microbe interactions, influence pathogen dynamics, and reflect ecological adaptations [2], [3]. At the same time, wildlife species can serve as reservoirs or sentinels of zoonotic microorganisms, particularly in ecosystems where wildlife, domestic animals, and humans interact closely [4], [5]. Understanding gut microbiome structure is therefore increasingly important within a One Health framework for assessing disease risks at the wildlife–livestock–human interface and informing conservation strategies.

Despite rapid advances in microbiome research, most wildlife studies have focused on describing microbial taxonomic composition and diversity rather than examining the ecological processes that shape microbial communities or their relationship with pathogen carriage [6–8]. Although these studies have substantially expanded knowledge of microbial diversity across vertebrate hosts, comparative analyses of microbiome variation among age classes, social groups, and ecological contexts remain limited for many free-ranging mammals. Consequently, the ecological drivers of gut microbiome assembly and their implications for wildlife health remain poorly understood.

Large carnivores are particularly relevant for microbiome research because they occupy high trophic levels, interact with diverse prey species, and often occur in landscapes shared with domestic animals and humans

[3], [9]. Such ecological overlap creates opportunities for pathogen exchange and highlights the importance of understanding microbial communities in these species. Domestic dogs, for example, are recognized reservoirs of pathogens including rabies virus, canine distemper virus, *Campylobacter*, and *Salmonella* [10]. Although viral spillover between domestic dogs and wild carnivores has been extensively documented [10], [11], bacterial community composition and the occurrence of potentially zoonotic bacteria remain comparatively underexplored [12].

The Ethiopian wolf (*Canis simensis*), the world's rarest canid, is a highly specialized Afroalpine carnivore endemic to the Ethiopian Highlands [13], [14]. The largest remaining population occurs in Bale Mountains National Park, a biodiversity hotspot where Ethiopian wolves coexist with pastoralist communities, livestock, and large populations of free-ranging domestic dogs [14], [15]. This close ecological association increases opportunities for pathogen transmission across wildlife, livestock, and humans. Previous studies of Ethiopian wolves have primarily focused on viral diseases, parasites, population genetics, and conservation threats [14, 16–18], whereas gut bacterial communities have remained largely unexplored. Baseline information on the gut microbiome is therefore needed to better understand host–microbe interactions, pathogen ecology, and disease risk in this endangered species.

Shotgun metagenomic sequencing provides high-resolution characterization of microbial communities by simultaneously profiling taxonomic composition, functional potential, and genomic content, overcoming many of the limitations associated with marker gene-based approaches [19]. However, no study has previously applied this approach to characterize the gut bacterial microbiome of the Ethiopian wolf. We therefore used shotgun metagenomic sequencing to characterize gut bacterial community composition in Ethiopian wolves from Bale Mountains National Park, Ethiopia. Specifically, we evaluated alpha and beta diversity across age classes and wolf packs, investigated age-associated patterns in microbial community composition, and quantified variation in pathogen burden among host age classes and social groups. By providing the first shotgun metagenomic characterization of the Ethiopian wolf gut microbiome, this study establishes an important baseline for understanding microbiome ecology and supports future conservation and One Health surveillance efforts in this endangered carnivore.

Methods

Study Area

This study was conducted in Bale Mountains National Park, located approximately 400 km southeast of Addis Ababa in the Oromia National Regional State of southeastern Ethiopia [20]. The park lies within the Bale–Arsi massif of the southeastern Ethiopian Highlands and extends between 6°29'–7°10'N and 39°28'–39°57'E [20]. Covering approximately 2,200 km², BMNP contains the largest continuous Afroalpine ecosystem in Africa and comprises a mosaic of habitats, including extensive Afroalpine plateaus above 3,500 m above sea level (asl) [21].

The study was conducted exclusively in the Web Valley, one of the principal strongholds for the Ethiopian wolf (*Canis simensis*) within Bale Mountains National Park [14, 22]. The valley is characterized by close ecological overlap among Ethiopian wolves, domestic dogs, livestock, wildlife, and human settlements,

making it an important setting for investigating host-associated microbiomes at the wildlife–livestock–human interface [15, 22, 23].

Fecal samples were collected between 24 March and 27 March 2025 from individually identified Ethiopian wolves belonging to five recognized social packs (Alo, Bowman, Megati, Mekena, and Tarura) monitored by the Ethiopian Wolf Conservation Programme and the Ethiopian Wildlife Conservation Authority.

Fecal sample collection

Ethiopian wolves were tracked on foot and horseback with assistance from trained wolf monitors of the Ethiopian Wolf Conservation Programme. Because wolves do not tolerate close human approach, defecation events were observed from a distance using binoculars (Olympus 8 × 40 PowerView). Fresh fecal samples were collected within 10–15 min of defecation, and only the upper and inner portions of each fecal sample were collected to minimize environmental contamination.

Approximately 100–200 mg of fecal material was transferred into sterile 20 × 76 mm screw-cap tubes containing 9 mL of DNA/RNA Shield (Zymo Research, Irvine, CA, USA) using the integrated collection spoon supplied by the manufacturer. Samples were thoroughly homogenized in the preservation buffer, transported in a cool box to the molecular laboratory of the Ethiopian Public Health Institute, and stored at – 80°C until DNA extraction.

A total of 31 fecal samples were collected from individually identified Ethiopian wolves. For each sample, the collection date, age class, sex (when known), pack affiliation, and individual identity were recorded. Individual wolves were identified using ear markings or plastic ear tags applied during previous vaccination campaigns, allowing duplicate sampling to be avoided. The study included 14 adults (> 2 years), 6 subadults (1–2 years), and 11 juveniles (< 1 year), as classified by the Ethiopian Wolf Conservation Programme. Sex was known for all adults and subadults (10 males and 10 females), whereas juveniles were of unknown sex because they lacked permanent individual markings.

DNA extraction, Library Preparation, and Sequencing

Genomic DNA was extracted from fecal samples using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. A42352) according to the manufacturer's instructions. DNA extraction was performed on a KingFisher™ magnetic particle processor (Thermo Fisher Scientific) using the standard automated protocol. Purified nucleic acids were eluted in 50–100 µL of elution buffer and stored at – 20°C until library preparation. DNA concentration was quantified using a Qubit™ Flex Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) with the Qubit dsDNA High Sensitivity Assay.

Shotgun metagenomic libraries were prepared from purified DNA using the Illumina DNA Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Genomic DNA was fragmented and adapter sequences were incorporated using bead-linked transposome technology, followed by PCR amplification with unique dual-index barcodes for sample identification. Libraries were purified and size-selected using Illumina purification beads, quantified using the Qubit™ Flex Fluorometer with the Qubit dsDNA High Sensitivity Assay,

normalized, pooled, and sequenced according to the manufacturer's protocol. Sequencing was performed at the Integrated Pathogen Genomics and Bioinformatics Facility for Surveillance and Research, Ethiopian Public Health Institute, Addis Ababa, Ethiopia, using an Illumina NovaSeq X platform with paired-end 150-bp sequencing (2 × 150 bp).

Bioinformatics analysis

Raw shotgun metagenomic sequencing data were generated as paired-end FASTQ files. Read quality was assessed using FastQC (v0.12.1) [24], followed by adapter trimming and quality filtering using fastp (v0.23.4) [25]. Sequencing quality metrics and insert size distributions generated by fastp were evaluated to assess sequencing performance and library consistency across samples.

Quality-filtered reads were aligned against the gray wolf (*Canis lupus*) reference genome (assembly mCanLor1.2; GenBank accession GCA_905319855.2) [26] using Bowtie2 (v2.5.5) [27] for host-read removal. Reads mapping to the host reference genome or External RNA Controls Consortium (ERCC) control sequences were removed, and unmapped reads were retained for downstream microbial analyses.

Host-depleted reads were subsequently uploaded to the Chan Zuckerberg ID (CZ ID) platform [28], which performs additional quality control, deduplication, and removal of residual host- and human-derived sequences. Because *Canis simensis* was not available as a selectable host reference within the CZ ID pipeline, *Canis lupus familiaris* was selected as the host reference for secondary host-read filtering.

Sequencing depth, quality-filtering statistics, and read-retention metrics for all samples are summarized in Online Resource 1, Sheet S1a.

Taxonomic classification and abundance estimation were performed using the CZ ID metagenomic analysis pipeline [28]. Microbial taxa were identified using nucleotide alignment against the NCBI nucleotide (NT) database with GSNAPL and protein alignment against the NCBI non-redundant (NR) database with DIAMOND [29, 30]. Analyses were performed using CZ ID pipeline version 8.3 with NT and NR database indexes dated 06 February 2024 (accessed January 2026).

Taxonomic abundance tables exported from CZ ID were analyzed in R (v4.5.2) [31]. Data preprocessing and manipulation were performed using the dplyr package (v1.2.1). Taxon-specific read counts were converted to relative abundances by dividing the reads assigned to each taxon by the total classified microbial reads within each sample. The resulting normalized bacterial relative abundance matrix is provided in Online Resource 2.

Statistical analysis

To further investigate age-associated microbial compositional patterns, bacterial taxa detected in at least 20% of Ethiopian wolf samples (≥ 7 of 31 samples) were retained to reduce sparsity and minimize the influence of rare taxa. The prevalence-filtered relative abundance matrices underwent count zero multiplicative (CZM) replacement using the zCompositions package to replace zero values while preserving

the compositional structure of the data [32]. The zero-replaced abundance data were subsequently transformed using the centered log-ratio (CLR) transformation implemented in the compositions package to account for the compositional nature of microbiome data [33, 34]. Differences in CLR-transformed taxon abundances among age classes were assessed using Kruskal–Wallis rank-sum tests, and resulting p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure [35, 36]. Species-level analyses were performed on prevalence-filtered taxa, after which species abundances were aggregated to the genus level and the same prevalence filtering, CZM replacement, CLR transformation, Kruskal–Wallis testing, and FDR correction workflow was applied to genus-level abundance data. These analyses were conducted to assess whether age-associated microbiome variation was driven by specific bacterial taxa or reflected broader community-level restructuring. Figures were generated in R (v4.5.2) using the ggplot2 package.

Results

Sequencing depth and read retention varied across samples. Following quality filtering and host-read removal, all samples retained sufficient sequencing depth for robust downstream analyses (Table 1).

Table 1
Summary statistics of sequencing depth and read retention across Ethiopian wolf fecal metagenomic samples

Metric	Mean ± SD	Median	Maximum
Total reads	52,856,213 ± 50,364,503	39,256,510	150,000,000
Quality-filtered reads	47,282,674 ± 45,514,224	35,811,022	138,904,626
Host-filtered reads	46,581,940 ± 45,075,302	34,767,878	137,613,392
Final reads	28,178,819 ± 27,104,231	18,941,532	113,142,214

Gut microbiome alpha diversity

Shannon alpha diversity showed substantial inter-individual variation among Ethiopian wolves (Fig. 1a). The relative abundance table used for downstream analyses is provided in Online Resource 2. When stratified by age class, Shannon diversity values showed overlapping distributions among juvenile, subadult, and adult wolves (Fig. 1b). Shannon alpha diversity did not differ significantly among age classes (Kruskal–Wallis: $\chi^2 = 2.53$, $df = 2$, $p = 0.28$), and no pairwise comparisons were significant after Benjamini–Hochberg correction (adjusted $p > 0.05$). Likewise, observed richness ($\chi^2 = 1.51$, $p = 0.47$) and Pielou's evenness ($\chi^2 = 2.27$, $p = 0.32$) did not differ significantly among age classes.

(a) Overall Shannon alpha diversity across all sampled Ethiopian wolves. (b) Shannon alpha diversity stratified by age class (juvenile, subadult, and adult). Sample sizes were juvenile ($n = 11$), subadult ($n = 6$), and adult ($n = 14$). Boxes indicate medians and interquartile ranges, and points represent individual wolves. 'ns' denotes non-significant pairwise comparisons

Gut microbiome beta diversity and age-associated compositional differences

Beta diversity analysis based on Bray–Curtis dissimilarity revealed age-associated differences in gut microbiome composition among Ethiopian wolves (Fig. 2). Principal coordinates analysis (PCoA) showed partial clustering of juvenile, subadult, and adult wolves with overlapping group centroids. PERMANOVA confirmed a significant effect of age class on gut microbiome composition (pseudo- $F = 2.02$, $R^2 = 0.126$, $p = 0.005$), indicating that age explained 12.6% of the observed variation. Homogeneity of multivariate dispersion did not differ among age classes (betadisper ANOVA: $F = 0.10$, $p = 0.90$; permutation test: $p = 0.91$), indicating comparable within-group dispersion.

Multivariable PERMANOVA analyses incorporating pack membership and sex produced consistent results, with age remaining significantly associated with gut microbiome composition after adjustment ($R^2 = 0.053$, $p = 0.042$). Pack membership was not significantly associated with microbiome variation ($R^2 = 0.125$, $p = 0.384$), whereas sex showed a modest association ($R^2 = 0.054$, $p = 0.032$). However, significant differences in multivariate dispersion between sex categories (betadisper ANOVA: $F = 3.85$, $p = 0.030$; permutation test: $p = 0.021$) suggest that this association should be interpreted cautiously.

Species- and genus-level differential abundance analyses were performed on prevalence-filtered, CLR-transformed abundance data (Online Resource 3, sheets S3f and S3g). Several taxa showed nominal age-associated abundance trends before multiple-testing correction, including *Helicobacter cholecystus*, *Phocaeicola vulgatus*, and *Clostridium perfringens* at the species level, and *Clostridium*, *Faecalicatena*, and *Phocaeicola* at the genus level. However, no species or genus remained significant after Benjamini–Hochberg FDR correction. Full PERMANOVA and differential abundance results are provided in Online Resource 3.

Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity illustrating differences in gut microbiome composition among Ethiopian wolf (*Canis simensis*) samples. Points represent individual wolves, with colors and point shapes indicating age class (juvenile, subadult, and adult). Ellipses represent 95% confidence intervals around group centroids. Axes indicate the percentage of variance explained by the first two principal coordinates

Dominant potentially pathogenic bacterial taxa

Fourteen bacterial taxa with documented zoonotic or non-zoonotic pathogenic potential were detected in Ethiopian wolf gut microbiomes (Table 2). *Escherichia coli* and *Clostridium perfringens* were detected in all individuals and contributed substantially to total relative abundance, whereas other pathogenic taxa occurred at lower prevalence or abundance. Overall, pathogen occurrence varied among individuals, indicating heterogeneity in pathogen burden across the population.

Table 2

Dominant bacterial taxa with documented zoonotic and non-zoonotic pathogenic potential detected in Ethiopian wolf gut microbiomes.

Taxon	Pathogenic Category	Prevalence %	Positive samples ^a	Mean relative abundance ^a	Total relative abundance
<i>Clostridium perfringens</i>	Zoonotic	100	31/31	0.0997	3.09
<i>Escherichia coli</i>	Zoonotic	100	31/31	0.0244	0.756
<i>Salmonella enterica</i>	Zoonotic	100	31/31	0.00015	0.00464
<i>Campylobacter upsaliensis</i>	Zoonotic	100	31/31	0.00343	0.106
<i>Paeniclostridium sordellii</i>	Zoonotic	100	31/31	0.0396	1.23
<i>Bacteroides fragilis</i>	Zoonotic	100	31/31	0.0127	0.393
<i>Anaerobiospirillum succiniciproducens</i>	Zoonotic	80.6	25/31	0.00653	0.163
<i>Clostridium botulinum</i>	Non-zoonotic pathogenic	100	31/31	0.000812	0.0252
<i>Peptacetobacter hiranonis</i>	Non-zoonotic pathogenic	100	31/31	0.0829	2.57
<i>Fusobacterium varium</i>	Non-zoonotic pathogenic/opportunistic	100	31/31	0.051	1.58
<i>Fusobacterium mortiferum</i>	Non-zoonotic pathogenic/opportunistic	100	31/31	0.0178	0.553
<i>Fusobacterium hominis</i>	Non-zoonotic pathogenic/opportunistic	100	31/31	0.029	0.899
<i>Helicobacter bilis</i>	Non-zoonotic pathogenic	93.5	29/31	0.00407	0.118
<i>Helicobacter cholecystus</i>	Non-zoonotic pathogenic	80.6	25/31	0.00352	0.0881

^a Positive samples indicate the number of Ethiopian wolf samples in which a taxon was detected. Mean relative abundance was calculated across positive samples only, while total relative abundance represents the summed relative abundance across all samples

Age-associated patterns in pathogen burden

Pathogen burden, calculated as the summed relative abundance of 14 bacterial taxa with documented pathogenic potential, differed significantly among juvenile, subadult, and adult Ethiopian wolves (Fig. 3; Kruskal–Wallis: $\chi^2 = 8.07$, $df = 2$, $p = 0.0177$). Pairwise comparisons showed significantly higher pathogen

burden in adult wolves than in subadults (adjusted Wilcoxon test, $p = 0.046$), whereas comparisons involving juveniles were not significant after Benjamini–Hochberg correction (adjusted $p > 0.05$).

Pathogen burden was calculated as the summed relative abundance of 14 bacterial taxa with documented pathogenic potential (including zoonotic and non-zoonotic taxa) for each sample. Juvenile ($n = 11$), subadult ($n = 6$), and adult ($n = 14$) wolves are shown. Violin plots depict the distribution of pathogen burden within each age class, boxes represent the interquartile range (IQR) with median values indicated, and whiskers extend to $1.5 \times$ IQR. Individual points represent single wolf samples. Asterisks (*) and 'ns' denote statistically significant and non-significant pairwise comparisons, respectively

Gut microbiome variation across wolf packs

Beta diversity analysis based on Bray–Curtis dissimilarity showed limited pack-level differentiation in gut microbiome composition among Ethiopian wolves (Fig. 4). Principal coordinates analysis (PCoA) revealed substantial overlap among packs, and PERMANOVA indicated that pack membership was not significantly associated with gut microbiome composition (pseudo- $F = 1.16$, $R^2 = 0.151$, $p = 0.186$). Homogeneity of multivariate dispersion did not differ among packs (betadisper ANOVA: $F = 0.67$, $p = 0.619$; permutation test: $p = 0.633$).

To evaluate sex-specific effects independently of age-related confounding, PERMANOVA was repeated using only individuals of known sex ($n = 20$). In this subset, sex showed a modest association with gut microbiome composition (pseudo- $F = 1.75$, $R^2 = 0.089$, $p = 0.042$). However, homogeneity of multivariate dispersion differed significantly between sex categories (betadisper ANOVA: $F = 3.85$, $p = 0.030$; permutation test: $p = 0.021$).

Principal coordinates analysis (PCoA) of Bray–Curtis dissimilarities showing gut microbial community composition across Ethiopian wolf packs. Points represent individual wolves, with colors and point shapes indicating wolf pack (Alo, Bowman, Megati, Mekena, and Tarura). Crosses indicate pack centroids. Axes represent the first two principal coordinates

Pathogen burden across wolf packs

Pathogen burden did not differ significantly among wolf packs (Fig. 5; Kruskal–Wallis: $\chi^2 = 7.96$, $df = 4$, $p = 0.093$).

Boxplots represent the median and interquartile range, with individual samples overlaid as points. Point shapes indicate wolf pack (Alo, Bowman, Megati, Mekena, and Tarura)

Discussion

This study presents the first shotgun metagenomic characterization of the gut microbiome of the endangered Ethiopian wolf (*Canis simensis*) from Bale Mountains National Park, Ethiopia. By integrating community-level diversity analyses with pathogen-focused profiling, our findings provide new insights into how host age structure shapes gut microbiome composition and pathogen burden in an endangered carnivore inhabiting a human–livestock–wildlife interface.

Stability in alpha diversity masks age-related microbiome restructuring

Despite substantial inter-individual variation, Shannon diversity, richness, and evenness did not differ significantly among juvenile, subadult, and adult Ethiopian wolves. Similar age-independent patterns of within-sample diversity have been reported in other wild mammals, including carnivores, suggesting that stabilization of gut microbial diversity following early-life development may be a common feature of wild mammal microbiomes rather than a species-specific pattern [37]. In Ethiopian wolves, whose diet consists predominantly of Afroalpine rodents, dietary specialization and functional redundancy within the gut microbiome may contribute to maintaining stable within-sample diversity across developmental stages. Although alpha diversity remained stable, significant differences in beta diversity indicate that host age influenced microbial community composition through taxonomic turnover rather than changes in overall within-sample diversity.

Age and pack effects on gut microbiome composition

Beta diversity analyses demonstrated significant age-associated differences in gut microbiome composition, with age explaining approximately 12.6% of the observed variation. The high taxonomic resolution of shotgun metagenomic sequencing enabled detailed characterization of these community-level differences, consistent with previous studies reporting modest but biologically meaningful age-associated variation in wildlife gut microbiomes [38]. Because multivariate dispersion did not differ among age classes, the observed PERMANOVA effect likely reflects genuine age-associated compositional differences rather than unequal within-group variability.

Although several bacterial species and genera exhibited nominal age-associated trends before multiple-testing correction, none remained significant following Benjamini–Hochberg FDR correction. Together with the significant beta-diversity results, this suggests that age-associated microbiome differences primarily reflect diffuse community-wide restructuring rather than large abundance shifts in individual taxa. Such restructuring is consistent with developmental changes in host physiology, diet, environmental exposure, and microbial succession during maturation [38]. Juvenile wolves likely experience distinct microbial exposures associated with denning behavior and maternal contact, whereas adults encounter broader environmental and anthropogenic microbial sources through territorial movements and increased interactions with domestic dogs [23]. The persistence of the age effect after adjustment for pack membership and sex further supports host age as an independent factor associated with gut microbiome composition.

In contrast, gut microbiome composition did not differ significantly among wolf packs. The substantial overlap in beta-diversity patterns and comparable multivariate dispersion among packs suggest limited pack-level microbiome structuring, likely reflecting the Ethiopian wolf's specialized rodent-based diet, shared Afroalpine habitat, and frequent ecological interactions among neighboring packs [23]. However, the relatively small and uneven sample sizes among packs warrant cautious interpretation, and broader sampling will be needed to evaluate the contribution of social structure to gut microbiome variation.

Sex-specific analyses restricted to individuals of known sex suggested a modest association between sex and gut microbiome composition. However, significant differences in multivariate dispersion indicate that this result should be interpreted cautiously and may partly reflect unequal within-group variability rather than a consistent sex-associated compositional shift.

Pathogen exposure and burden across age classes and packs

The detection of bacterial taxa with documented pathogenic potential, including several of zoonotic importance, highlights the complex microbial landscape inhabited by Ethiopian wolves. Several taxa were highly prevalent and contributed substantially to the overall pathogen burden, suggesting widespread exposure within the population. Similar patterns have been reported in other free-ranging carnivores inhabiting human-modified environments, where interactions with livestock, domestic animals, and contaminated habitats facilitate circulation of enteric pathogens [39].

Adult wolves exhibited a significantly higher pathogen burden than subadults, whereas comparisons involving juveniles were not significant. This pattern may reflect cumulative ecological and behavioral exposure associated with age. Compared with subadults, adult Ethiopian wolves participate more extensively in territorial patrols, scent marking, and movements across pack territories [40], increasing opportunities for contact with environmental reservoirs and fecal contamination shared with free-ranging domestic dogs and livestock [18, 41]. Adults may also encounter carrion more frequently, providing additional opportunities for exposure to bacterial taxa associated with carcasses, including *Clostridium perfringens* and *Escherichia coli*, which were also detected in the present study [42, 43]. Because alpha diversity did not differ among age classes, the increased pathogen burden in adults likely reflects changes in microbial composition rather than overall within-sample diversity.

The absence of significant differences between adults and juveniles may reflect the greater variability observed among juvenile wolves. Differences in developmental stage, dietary transition, environmental exposure, and immune maturation may have contributed to this heterogeneity, although these mechanisms were not directly evaluated in the present study [44].

In contrast, pathogen burden did not differ significantly among wolf packs, suggesting that exposure to bacterial taxa with documented pathogenic potential is influenced primarily by shared environmental and dietary conditions rather than pack-specific dynamics. Importantly, detection of bacterial taxa with documented pathogenic potential does not imply disease, as many such bacteria can occur as asymptomatic members of the gut microbiome and their health significance depends on strain-level characteristics, functional gene content, and host immune status [45, 46]. These findings emphasize the need for future studies integrating taxonomic, phylogenetic, and functional analyses to better understand pathogen ecology in Ethiopian wolves.

Methodological advances enabled by shotgun metagenomics

Unlike most previous wildlife microbiome studies, which have relied primarily on 16S rRNA gene sequencing for taxonomic profiling [47, 48], this study applied shotgun metagenomic sequencing to characterize the gut

microbiome of free-ranging Ethiopian wolves. The higher taxonomic resolution of shotgun metagenomic sequencing enabled more precise identification of microbial taxa, including bacteria with documented pathogenic potential, thereby facilitating ecological interpretation beyond presence–absence patterns. Previous studies of wild mammals have reported age-associated shifts in gut microbial communities [49, 50], but these have often relied on amplicon sequencing with limited species-level resolution. In contrast, shotgun metagenomic sequencing enabled detailed characterization of community composition across age classes while emphasizing that taxonomic identification alone does not demonstrate infection or functional activity.

Although shotgun metagenomics also enables investigation of functional traits such as antimicrobial resistance and virulence genes, these analyses were beyond the scope of the present study. Future integration of taxonomic, phylogenetic, and functional analyses will provide a more comprehensive understanding of the ecological, epidemiological, and conservation significance of the microbial taxa identified in Ethiopian wolves.

Conservation and One Health implications

The Ethiopian wolf inhabits a dynamic human–livestock–wildlife interface, where increasing human settlement and livestock grazing within Bale Mountains National Park have intensified ecological overlap among wolves, domestic dogs, livestock, and people [22, 23]. This setting creates opportunities for the environmental dissemination and cross-species transmission of microbial taxa and pathogens, highlighting the importance of a One Health approach to wildlife disease surveillance and conservation [51, 52].

These findings are particularly relevant given the conservation status of the Ethiopian wolf. Bale Mountains National Park supports approximately half of the global population, and repeated rabies and canine distemper virus outbreaks transmitted by domestic dogs have caused substantial population declines in recent decades [23]. Although the present study focused on bacterial communities rather than active disease, the observed age-associated differences in gut microbiome composition together with the higher pathogen burden detected in adult wolves suggest that host age may influence microbial exposure and pathogen carriage within this endangered species.

The absence of strong pack-level differences in either gut microbiome composition or pathogen burden suggests that microbial dynamics operate at broader ecological scales rather than being confined within individual social groups. Consequently, disease surveillance and conservation strategies should adopt landscape-scale approaches that integrate wildlife, livestock, domestic dogs, and human populations. Future studies incorporating strain-level phylogenetic analyses together with antimicrobial resistance, virulence gene profiling, and other functional genomic approaches will be important for clarifying pathogen transmission pathways, zoonotic potential, and the conservation significance of the microbial taxa identified in Ethiopian wolves.

Conclusion

This study provides the first shotgun metagenomic characterization of the gut bacterial microbiome of the endangered Ethiopian wolf, establishing an important baseline for understanding host–microbiome

interactions in this species. Host age emerged as the primary factor independently associated with gut microbiome variation, influencing community composition and pathogen burden despite stable alpha diversity. These findings indicate that microbiome maturation in Ethiopian wolves is characterized by community-level compositional restructuring rather than large changes in overall microbial diversity.

The detection of bacterial taxa with documented pathogenic potential, together with age-associated differences in pathogen burden, highlights the ecological and conservation relevance of the Ethiopian wolf gut microbiome. In contrast, the limited influence of pack membership suggests that broader ecological processes and host-related factors play a greater role in shaping gut microbial communities than social grouping.

Although limited to cross-sectional bacterial community profiling, this study provides a foundation for future longitudinal, strain-level, and functional metagenomic investigations. Integrating microbiome research into One Health surveillance will improve understanding of pathogen ecology and support evidence-based conservation of Ethiopian wolves at the wildlife–livestock–human interface in the Bale Mountains ecosystem.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Author Contributions

Girma Ayalew Mengistu contributed to study conceptualization, methodology, sample collection, data curation, data analysis, visualization, and writing and revision of the manuscript. Adey Feleke, Helen Nigussie, Anagaw Atickem, Nigatu Kebede, and Alemayehu Lemma contributed to supervision and revision of the manuscript. Chala Bashea, Melak Getu, and Atsbeha Gebreegziabxier contributed to laboratory analysis and revision of the manuscript. Muktar Abute contributed to sample collection and revision of the manuscript. Gebremedhin Gebremicael, Tolewak Sura, Eleni Kidane, Desta Ayana, Abraham Ali, Gemechu Tadesse, Getachew Tollera, Yohannes Ayalew Mengistu, and Girma Eshete contributed to revision of the manuscript. Gurja Belay contributed to study conceptualization, supervision, and revision of the manuscript. All authors read and approved the final manuscript.

Ethics Approval

The research project was evaluated and approved by the Addis Ababa University, College of Natural and Computational Sciences Institutional Review Board (CNC-IRB) Committee (Approval No. CNCSDO/711/16/2024) and authorized by the Ethiopian Wildlife Conservation Authority (Permit No. Sg8/02/2024). The study involved non-invasive collection of fecal samples from free-ranging Ethiopian wolves, and all sampling and field procedures were conducted in accordance with the requirements and guidelines of the relevant institutional and wildlife authorities.

Data availability

Raw shotgun metagenomic sequencing data and associated sample metadata have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1463541. The BioProject includes both Ethiopian wolf (*Canis simensis*) and domestic dog samples submitted under separate accession numbers; however, only Ethiopian wolf samples were included in the analyses presented in this study. Ethiopian wolf samples correspond to BioSample accessions SAMN59502226–SAMN59502256.

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Figures

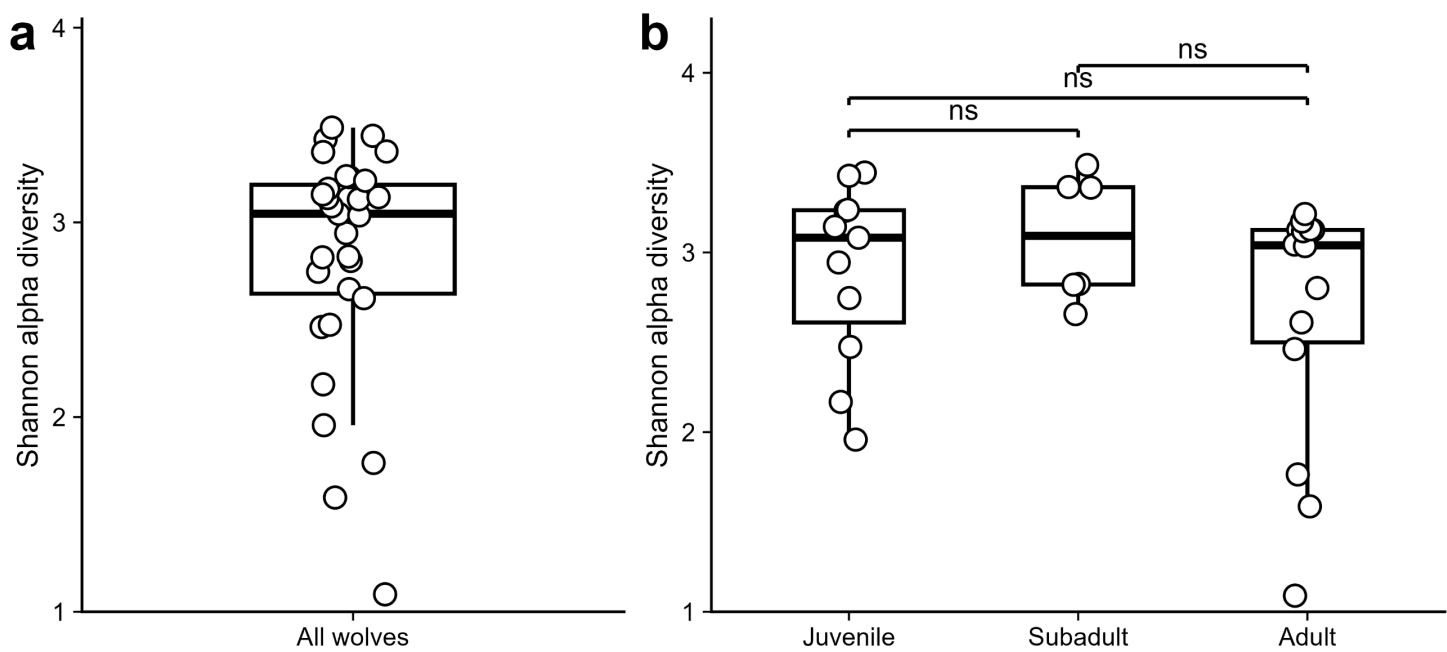


Figure 1

Beta diversity of Ethiopian wolf gut microbiota across age classes

(a) Overall Shannon alpha diversity across all sampled Ethiopian wolves. (b) Shannon alpha diversity stratified by age class (juvenile, subadult, and adult). Sample sizes were juvenile (n = 11), subadult (n = 6), and adult (n = 14). Boxes indicate medians and interquartile ranges, and points represent individual wolves. 'ns' denotes non-significant pairwise comparisons

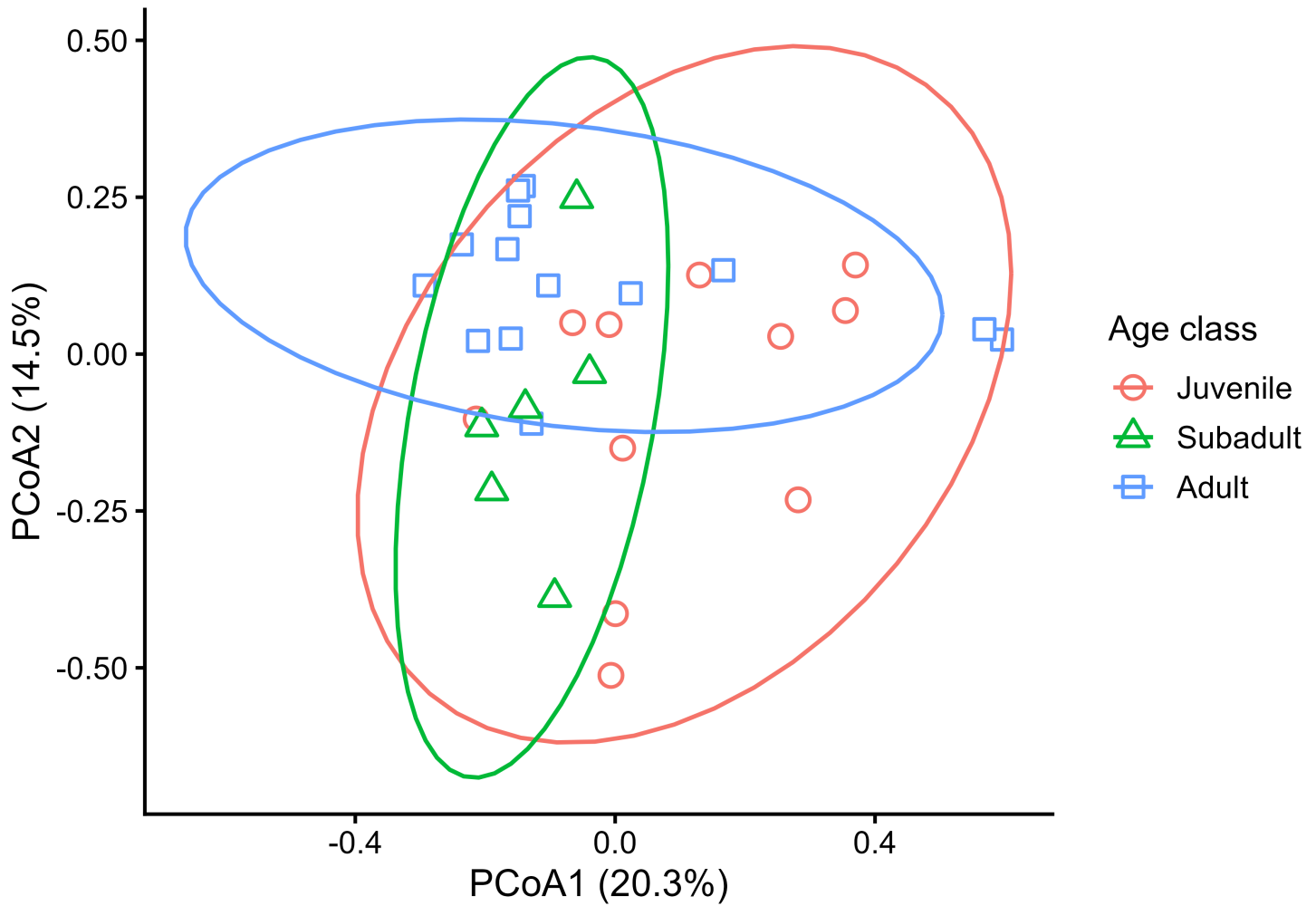


Figure 2

Beta diversity of Ethiopian wolf gut microbiota across age classes

Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity illustrating differences in gut microbiome composition among Ethiopian wolf (*Canis simensis*) samples. Points represent individual wolves, with colors and point shapes indicating age class (juvenile, subadult, and adult). Ellipses represent 95% confidence intervals around group centroids. Axes indicate the percentage of variance explained by the first two principal coordinates

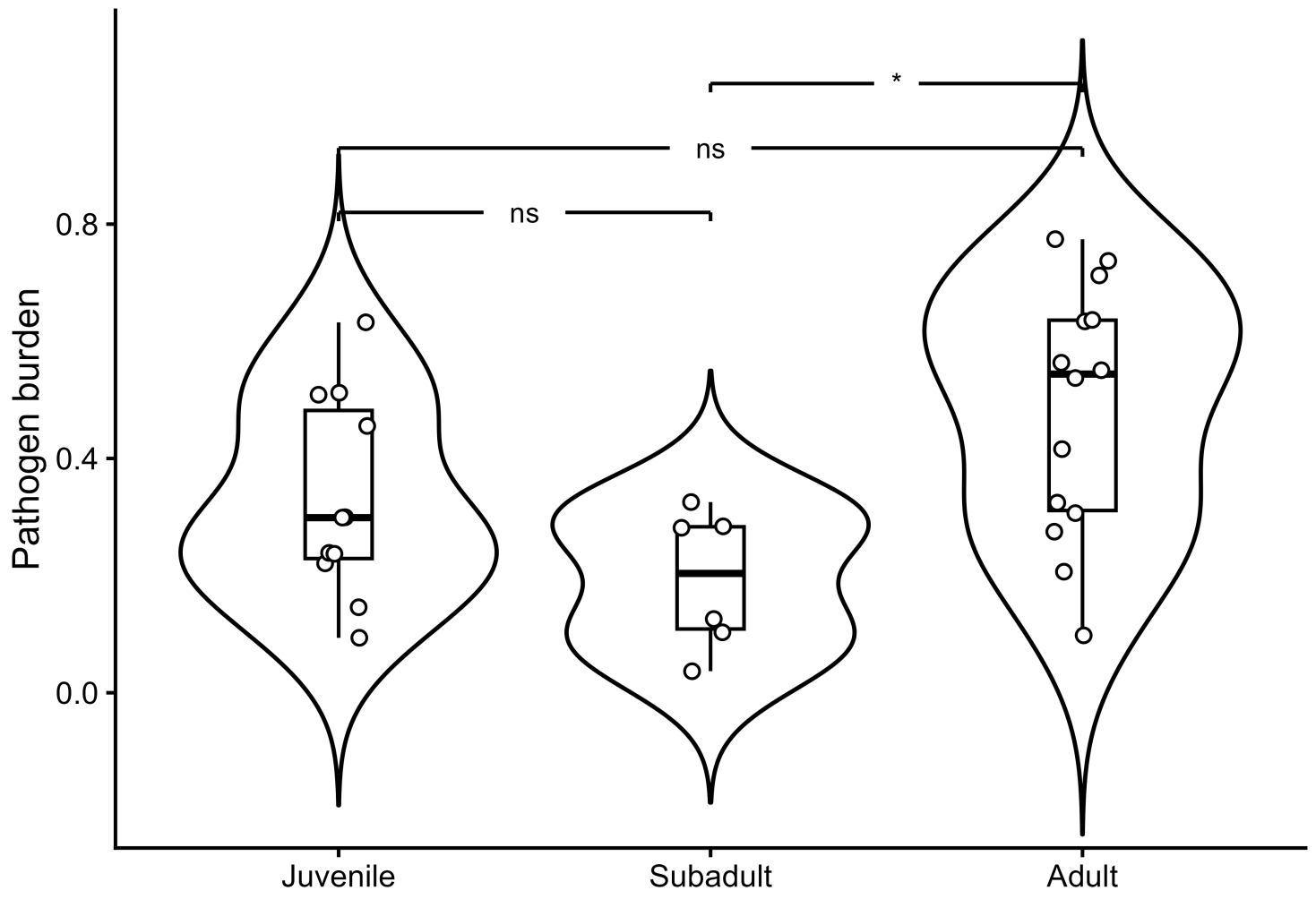


Figure 3

Pathogen burden across Ethiopian wolf age classes

Pathogen burden was calculated as the summed relative abundance of 14 bacterial taxa with documented pathogenic potential (including zoonotic and non-zoonotic taxa) for each sample. Juvenile (n = 11), subadult (n = 6), and adult (n = 14) wolves are shown. Violin plots depict the distribution of pathogen burden within each age class, boxes represent the interquartile range (IQR) with median values indicated, and whiskers extend to 1.5× IQR. Individual points represent single wolf samples. Asterisks (*) and 'ns' denote statistically significant and non-significant pairwise comparisons, respectively

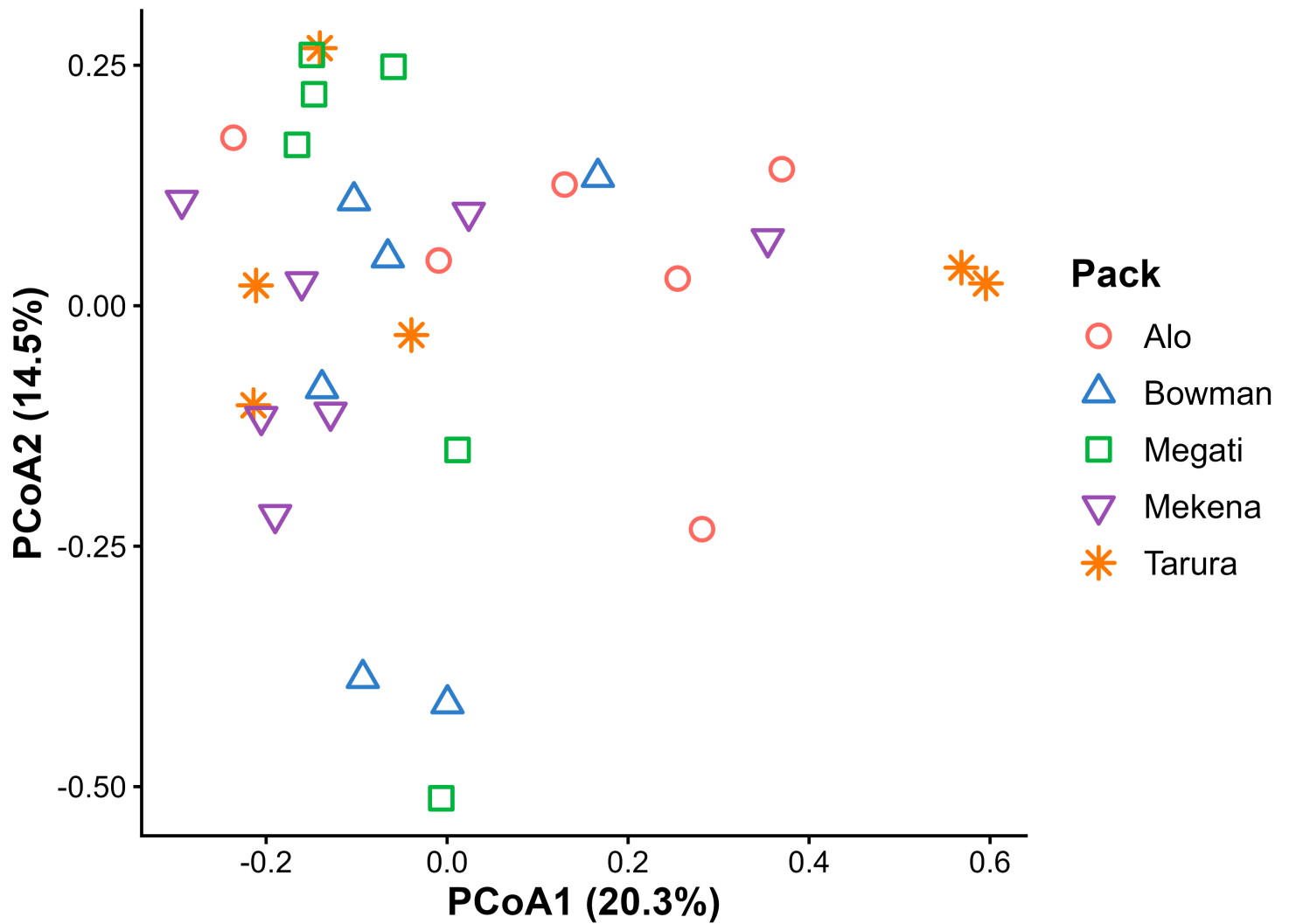


Figure 4

Beta diversity of Ethiopian wolf gut microbiota across wolf packs

Principal coordinates analysis (PCoA) of Bray–Curtis dissimilarities showing gut microbial community composition across Ethiopian wolf packs. Points represent individual wolves, with colors and point shapes indicating wolf pack (Alo, Bowman, Megati, Mekena, and Tarura). Crosses indicate pack centroids. Axes represent the first two principal coordinates

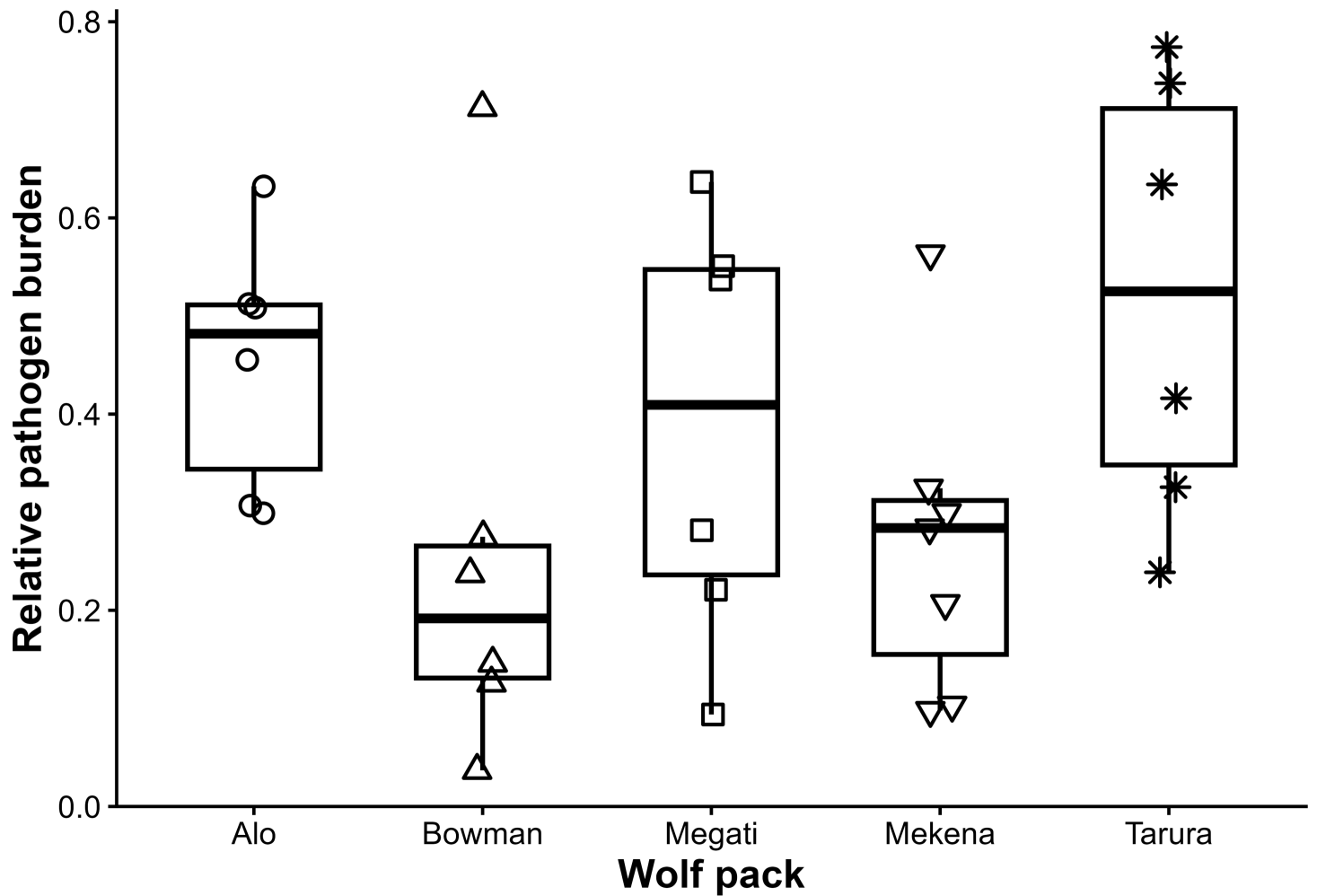


Figure 5

Pathogen burden across Ethiopian wolf packs

Boxplots represent the median and interquartile range, with individual samples overlaid as points. Point shapes indicate wolf pack (Alo, Bowman, Megati, Mekena, and Tarura)

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