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# Dietary ecology structures gut microbiome assembly in sympatric wild mammals from Kruger National Park, South Africa

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## Abstract

**Background** The gut microbiome plays a critical role in host health, yet its composition in African wild mammals remains underrepresented. We investigated how dietary groups (carnivores, non-ruminant, and ruminant herbivores) influence gut microbiota composition and diversity in coexisting wild mammals from the Kruger National Park, South Africa.

**Results** After quality filtering, 89 samples (carnivores: $n=31$; non-ruminants: $n=37$; ruminants: $n=21$) were included. Gut microbial communities clustered strongly by dietary group (weighted: pseudo-F = 27.76, $p=0.0001$) with smaller but significant species-level differences within groups. Random Forest classification accurately distinguished dietary groups (93%), with six samples showing atypical clustering in beta-diversity analyses. Non-ruminants showed the highest alpha diversity (Shannon: median 6.89 [IQR 6.53–7.08]; Faith's PD: 18.02 [15.25–20.43]), followed by ruminants and carnivores (both $p<0.001$). Taxonomic profiling revealed group-specific signatures. Carnivores were dominated by Fusobacteriaceae, Clostridiaceae and Peptostreptococcaceae. Both ruminants and non-ruminants showed high levels of fibre-degrading bacteria, such as Rikenellaceae and Oscillospiraceae, while non-ruminants were additionally enriched in Spirochaetaceae and *p*-251-o5, reflecting hindgut fermentation strategies. Predicted functional profiling using PICRUSt2 revealed metabolic pathway enrichments, with carnivores enriched in amino acid biosynthesis and degradation pathways. In contrast, ruminants showed enrichment in branched amino acid and tryptophan biosynthesis, while non-ruminants were enriched in aerobic respiration, TCA cycle, and L-leucine degradation pathways. Focal species comparisons distinguished black from white rhinoceros and spotted hyaena from other carnivores, consistent with diet-driven metabolic specialisation. Species-level differences were evident at family and genus levels, with variable importance analyses identifying key predictive taxa.

**Conclusions** Diet strongly influenced gut microbiota composition and diversity, with herbivores exhibiting richer, more complex communities compared to carnivores. Among herbivores, non-ruminants harboured higher diversity than ruminants, likely due to their diverse, less specialised hindgut fermentation. These findings advance our





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understanding of wildlife microbiomes and provide a foundation for future conservation and health monitoring in African ecosystems.

**Keywords** Gut microbiome · Wildlife · African mammals · Diet · 16S rRNA · Microbial diversity

**Abbreviations**

| | |
|---|---|
| ASV | Amplicon Sequence Variants |
| PCoA | Principal Coordinate Analyses |
| PERMANOVA | Permutational Multivariate Analysis of Variance |
| RF | Random Forest |
| PD | Faith's Phylogenetic Diversity |
| SCFAs | Short-chain Fatty Acids |
| CLR | Centred Log-Ratio |
| LEfSe | Linear discriminant analysis Effect Size |
| LDA | Linear Discriminant Analysis |
| FC | Fold change |
| BH | Benjamini-Hochberg |

## Introduction

The gut microbiota is a complex microbial community that influences many host processes, including digestion, immune response, nutrient synthesis, and defence against pathogens [1, 2]. Indigestible polysaccharides are fermented by gut bacteria into short-chain fatty acids (SCFAs), which provide energy for colonocytes, act as signalling molecules that regulate immune responses, and contribute to a barrier against infections [2, 3]. While human and captive animal microbiomes have been well studied, those of wild mammals, especially large wildlife species in Africa, are less characterised [4, 5]. This gap in knowledge limits our understanding of host-microbiome coevolution in natural ecosystems and hampers efforts to integrate microbiome perspectives into conservation [6].

Among the factors driving gut microbiome variation, diet is one of the most dominant [7, 8]. Mammalian species consuming similar types of food tend to share similar gut microbial communities [9, 10]. Carnivores (meat-eaters) and herbivores (plant-eaters) have significantly different microbiotas that are adapted to their different diets [11]. Most gut communities of carnivorous animals typically have many protein- and bile-tolerant bacteria (e.g. Clostridiaceae, Fusobacteriaceae) that metabolise amino acids and fats, whereas herbivore guts are enriched in fibre-fermenting bacteria (e.g. Rikenellaceae, Oscillospiraceae, Prevotellaceae) that break down complex plant polysaccharides. Furthermore, as plant-rich diets provide a wider array of carbohydrate substrates, herbivores are generally more gut microbially diverse than carnivores [12, 13]. This trend is seen widely in mammals and even within human studies whereby controlled diet experiments show that plant-based diets increase polysaccharide-degrading microbes, whereas animal-based diets (meat) rapidly boost bile-tolerant taxa and reduce diversity of fibre specialists [13–15].

Within herbivores, digestive anatomy further modulates microbiome structure. Ruminants (e.g. African buffalo, impala) have a multi-chambered foregut (rumen) where microbial fermentation of cellulose occurs, while hindgut fermenters (non-ruminants such as African elephant, black and white rhinoceros, and warthog) have a simple stomach but an enlarged cecum and colon for fermentation [12, 13, 16]. Hindgut fermenters often consume a broader range of vegetation and rely on large-intestine fermentation, which can support even more diverse microbial niches than the specialised rumen environment [17, 18]. Theoretical arguments predict that diets richer in high fibre foods will create more ecological niches in the gut microbiome [19]. Empirical studies of zoo or

captive animals have shown, for instance, that hindgut-fermenting equids have different gut bacteria than ruminant bovids, and that microbial diversity patterns often align with these digestive strategies [4, 20].

The diverse megafauna of the Kruger National Park provides a natural setting to test diet and microbiome relationships. The Kruger National Park hosts a diverse assemblage of wild mammals including carnivores (African lion, spotted hyaena, and African wild dog), non-ruminants (warthog, African elephant, black rhinoceros, and white rhinoceros), and ruminant herbivores (African buffalo and impala) - all coexisting in the same ecosystem. Several of these species are of conservation concern: the African lion (vulnerable), African wild dog (endangered), black rhinoceros (critically endangered), and white rhinoceros (near threatened) are all listed on the IUCN Red list [21]. Because these species experience similar environmental conditions in Kruger, this setting minimises habitat confounders and emphasises the roles of diet and physiology. In this study, we used freshly frozen faecal samples from nine Kruger mammal species and sequenced the V3–V4 region of the 16S rRNA gene. We then compared gut microbiota composition and diversity across three dietary groups (carnivores, non-ruminant, ruminant) and among species within each group. This research will help fill gaps in our knowledge of African wildlife gut microbiomes by providing a baseline for multiple species in different dietary groups that coexist within the Kruger National Park, which includes underrepresented species such as African wild dog, warthog, and both rhinoceros species.

## Results

### Sequencing output and sample characteristics

Of the 94 faecal samples a total of 8,693,093 raw reads were obtained (min 41,434 - max 168,627 reads per sample) with a mean of 92,480 reads and median of 90,498 per sample. After quality filtering and read merging, the dataset consisted of 459,411 sequences (844–11,420 sequences per sample) with a mean of 4,887 sequences per sample. Detailed metadata for all samples, including SRA accession numbers and sequencing statistics, are provided in Table S1. Following the DADA2 pipeline in QIIME 2, we identified 7,536 different amplicon sequence variants (ASVs) across all samples. The most abundant classified ASV, assigned to the genus *Peptoclostridium*, was detected 1,899 times across 26 samples. Rarefaction analysis (Fig. S1) indicated sufficient coverage at 2,000 sequences per sample, resulting in the exclusion of five samples that fell below this threshold. The remaining 89 faecal samples (carnivores: $n=31$; non-ruminants: $n=37$; ruminants: $n=21$) were retained for downstream analyses.

### Gut microbial community structure differs strongly by diet

Principal coordinate analyses (PCoA) of UniFrac distances revealed clear separation by dietary group (Fig. 1). Weighted UniFrac (abundance-based) PCoA (Fig. 1A) showed distinct clusters for carnivores (orange), non-ruminants (purple), and ruminants (green), with the first three axes explaining 59.1% of variance. Unweighted UniFrac (presence/absence) PCoA (Fig. 1B) likewise separated the groups (38.3% variance). Six samples exhibited atypical clustering inconsistent with their dietary group in both weighted and unweighted UniFrac PCoA (Fig. S2, S3).

PERMANOVA analyses confirmed significant group-level differences across dietary categories (weighted UniFrac: pseudo-$F=27.76$, $p=0.0001$; unweighted UniFrac: pseudo-$F=14.31$, $p=0.0001$). Pairwise PERMANOVA with Benjamini-Hochberg (BH) correction confirmed significant separation among all dietary group pairs across all distance metrics (Table S2). Using weighted UniFrac, the greatest differences were observed between carnivores and non-ruminants ($F=36.70$, $R^2=0.357$; $q<0.0001$), followed by carnivores vs. ruminants ($F=27.54$; $R^2=0.355$; $q<0.0001$), and non-ruminants vs. ruminants ($F=16.13$; $R^2=0.224$; $q<0.0001$). Identical significance was observed using unweighted UniFrac and Bray-Curtis distances. Within dietary groups, significant

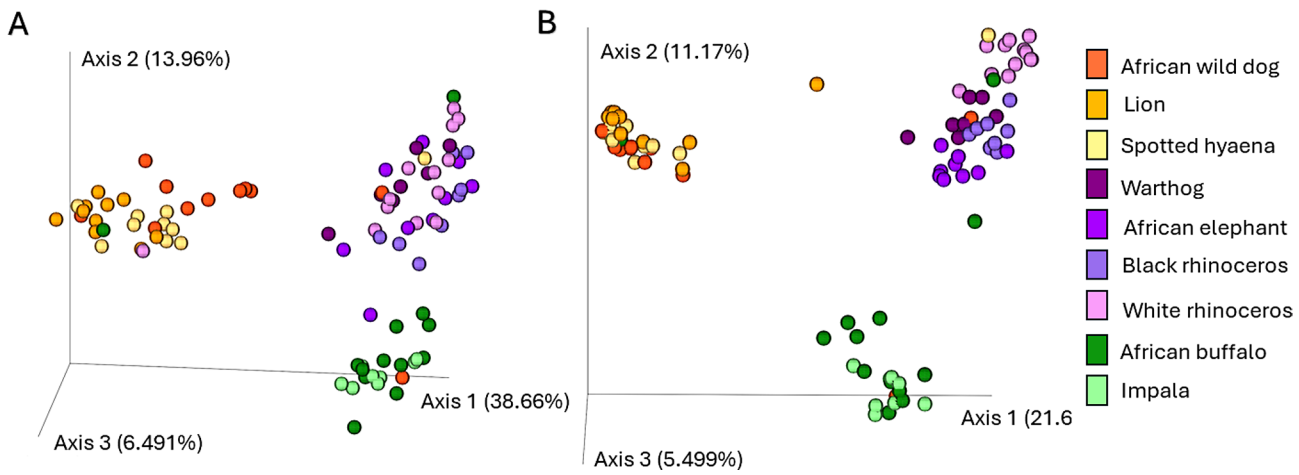


**Fig. 1** Principal coordinate analysis (PCoA) of weighted and unweighted UniFrac distances. Principal coordinates analysis (PCoA) of gut microbiota composition among wild mammals from Kruger National Park based on weighted (**A**) and unweighted (**B**) UniFrac distances. Points represent individual samples coloured by dietary group: carnivores (orange), non-ruminants (purple), and ruminants (green). Percentages indicate variance explained by each axis

species-level differences were also detected (weighted UniFrac): carnivores ($F=3.99$; $R^2=0.222$; $p<0.0002$), non-ruminants ($F=4.66$; $R^2=0.298$; $p<0.0001$), and ruminants ($F=1.81$; $R^2=0.087$; $p=0.029$), though effect sizes were substantially smaller than between-group differences.

Random Forest (RF) classification demonstrated high predictive performance, with an overall accuracy of 93.26% (Kappa = 0.8959; 95% CI: 0.859–0.975; $p<2\times10^{-16}$). The confusion matrix (Fig. S4A) showed that most samples were correctly assigned to their dietary group, while six samples were misclassified, corresponding to those with atypical beta-diversity clustering. Sensitivity and specificity were above 90% for all dietary groups, supporting the robustness of the model (Fig S4B). The specific family- and genus-level taxa driving these classifications are detailed in Figures S5 and S6.

## Alpha diversity patterns reflect digestive strategies

Alpha diversity, measured by both Shannon diversity index and Faith's Phylogenetic Diversity (PD), differed significantly across dietary groups (Kruskal-Wallis, Table S3; $p < 0.001$) (Fig. 2). Pairwise comparisons further highlighted these differences, emphasising diet as a major driver of gut microbial diversity among the studied wild mammals. Within each dietary group, alpha diversity did not significantly differ among host species, as confirmed by both Shannon and Faith's PD indices (Figs. 2B, 2D). We therefore only report significant pairwise contrasts across dietary groups, assessed using Dunn's tests with Benjamini-Hochberg correction (Table S4).

For the Shannon index, non-ruminants exhibited the highest median diversity (6.89 [IQR 6.53–7.08]), followed by ruminants (6.54 [5.63–6.81]), and carnivores (5.83 [5.6–6.11]). Post hoc Dunn's tests revealed significantly higher diversity in non-ruminants compared to carnivores ($Z=-5.70$, $p=1.18\times10^{-8}$, $q=3.55\times10^{-8}$) and to ruminants ($Z=2.61$, $p=0.009$, $q=0.014$). Ruminants also showed significantly higher diversity than carnivores ($Z=-2.39$, $p=0.017$, $q=0.017$).

At the host species level, carnivores had Shannon diversity of 6.02 [5.72–6.1] in African wild dogs, 5.91 [5.60–6.24] in spotted hyaena, and 5.65 [5.60–5.74] in lion. Among non-ruminants, higher values were observed across all species, including African elephant (6.89 [6.75–7.02]), black rhinoceros (6.87 [6.52–7.52]), warthog (6.81 [6.4–7.06]), and white rhinoceros (6.74 [6.64–7.01]). Ruminant species showed slightly lower but still elevated diversity, including impala (6.65 [6.14–6.82]) and African buffalo (6.19 [5.61–6.78]).

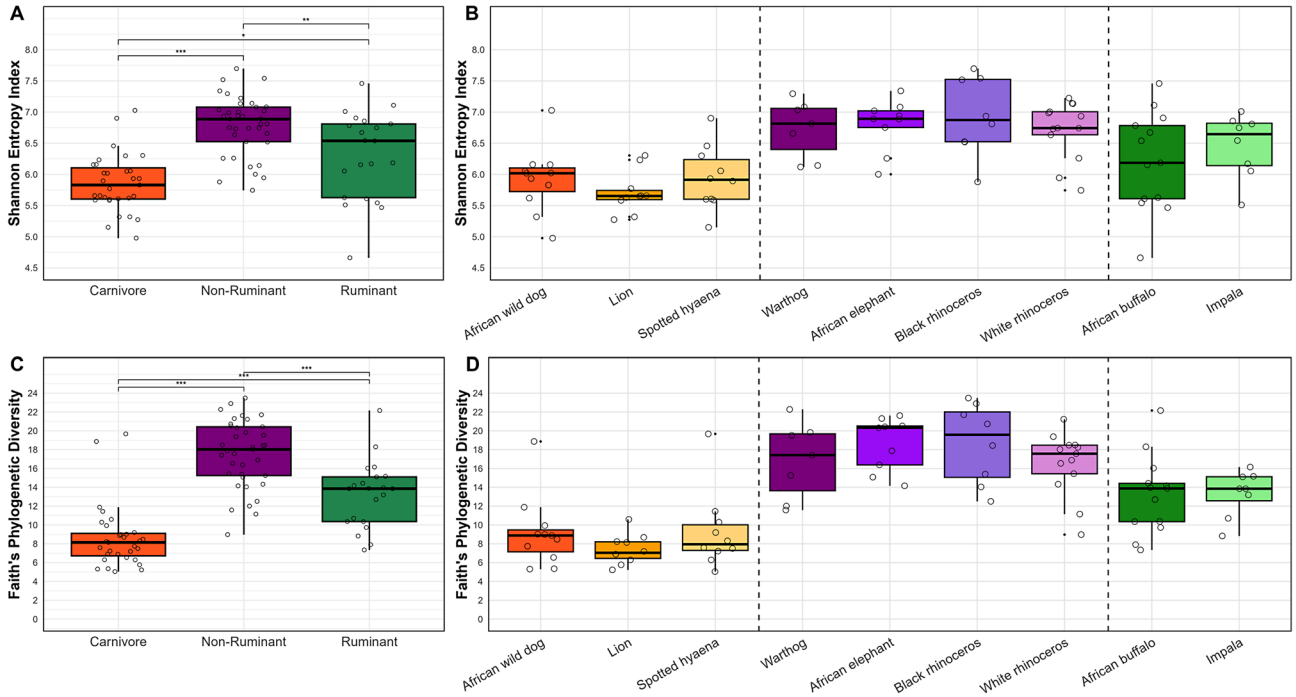


**Fig. 2** Alpha diversity of gut microbiota across dietary groups and host species. Boxplots display two measures of alpha diversity—Shannon entropy (**A, B**) and Faith's phylogenetic diversity (**C, D**)—among wild mammalian hosts from Kruger National Park. Panels **A** and **C** compare diversity across the three main dietary groups: carnivores, non-ruminants, and ruminants. Panels **B** and **D** show corresponding diversity metrics at the host species level within each dietary group. Individual data points represent samples per host, with horizontal lines indicating significance. These results highlight both dietary and species-level variation in gut microbial diversity

Faith's PD mirrored these patterns, revealing the highest phylogenetic diversity in non-ruminants (18.02 [15.25–20.43]), followed by ruminants (13.85 [10.36–15.11]), and lowest in carnivores (8.14 [6.72–9.11]). Non-ruminants had significantly higher Faith's PD compared to carnivores ($Z = -7.09$, $p = 1.37 \times 10^{-12}$, $q = 4.11 \times 10^{-12}$) and to ruminants ($Z = 2.98$, $p = 0.003$, $q = 0.003$). Ruminants also showed significantly higher diversity than carnivores ($Z = -3.23$, $p = 0.001$, $q = 0.002$). Detailed pairwise comparisons at the host species level are provided in Table S5 for Shannon diversity and Table S6 for Faith's PD.

## Taxonomic composition is distinct across dietary groups

Taxonomic profiling revealed distinct diet-associated microbial signatures among wild mammalian hosts (Fig. 3). The average relative abundances of dominant bacterial families across all dietary groups summarised in Table S7 and Figure S7, and the breakdown for each host group is provided in Table S8. Lachnospiraceae emerged as a core taxon across all dietary groups. Carnivores were enriched in Fusobacteriaceae (average relative abundance of 11.98%), Clostridiaceae (8.51%), and Peptostreptococcaceae (8.46%), driven by high relative abundances of *Fusobacterium*, *Clostridium sensu stricto 1*, and *Peptoclostridium*, the latter representing the most frequently classified ASV. Elevated Bacteroidaceae in carnivores and ruminants was primarily attributable to *Bacteroides*, whereas Prevotellaceae, specialised in fibre degradation, occurred across all groups but at significantly lower relative abundance in carnivores. Ruminants and non-ruminants harboured higher proportions of the fibre-degrading Rikenellaceae (ruminants: 11.32%, non-ruminants: 10.62%) and Oscillospiraceae (15.00%, 7.11%, respectively), reflected in the prominence of *Rikenellaceae RC9 gut group*. Non-ruminants displayed greater genus-level diversity, including enrichment of *Treponema* (Spirochaetaceae), *NK4A214 group* (Oscillospiraceae), *Prevotellaceae*

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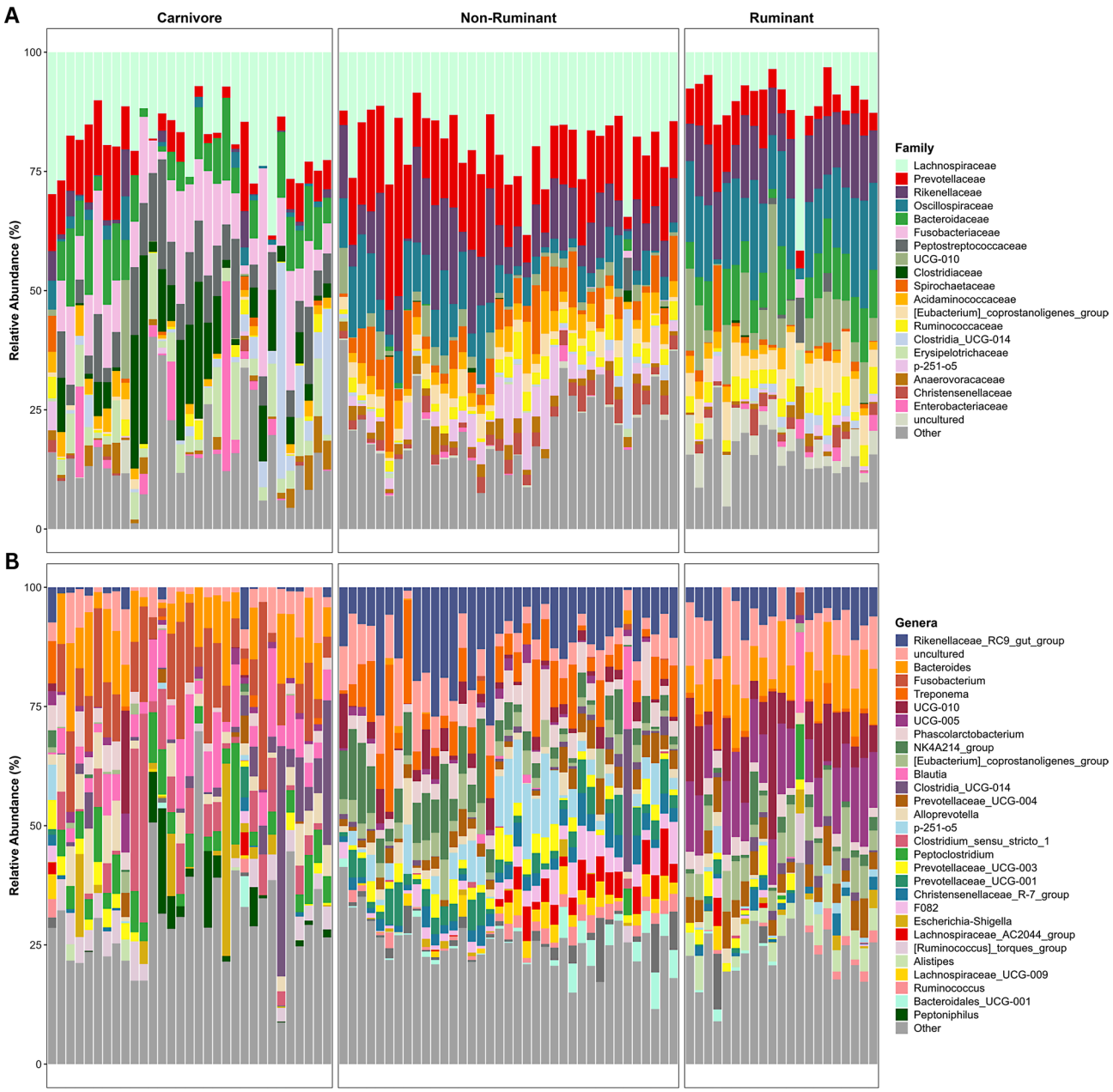


**Fig. 3** Taxonomic composition of gut microbiota across mammalian dietary groups on a family-level. Stacked bar plots show the relative abundance of the top 20 most prevalent bacterial families (**A**) and top 30 genera (**B**) In individual gut microbiome samples from carnivores, non-ruminants, and ruminants. Samples are grouped by dietary category, with each bar representing an individual sample. Taxa below the top 20/30 threshold are grouped under 'other'

*UCG-001* and *UCG-003*, and members of the *p*-251-o5 family. Several Lachnospiraceae genera, such as *AC2044 group*, *UCG-009*, and *Bacteroides UCG-001*, occurred almost exclusively in subsets of non-ruminants, indicating finer-scale specialisation. Ruminants exhibited broadly consistent community composition, with *Rikenellaceae RC9 gut group*, *Bacteroides*, *UCG-005*, and *UCG-010* as the most prominent genera. Genus-level distributions across dietary groups were further visualised using a ternary plot (Fig. 4). Most genera were positioned along the non-ruminant and ruminant axes, with only few genera clustering near the carnivore corner, reflecting distinct taxonomic composition of carnivore microbiomes relative to herbivores.

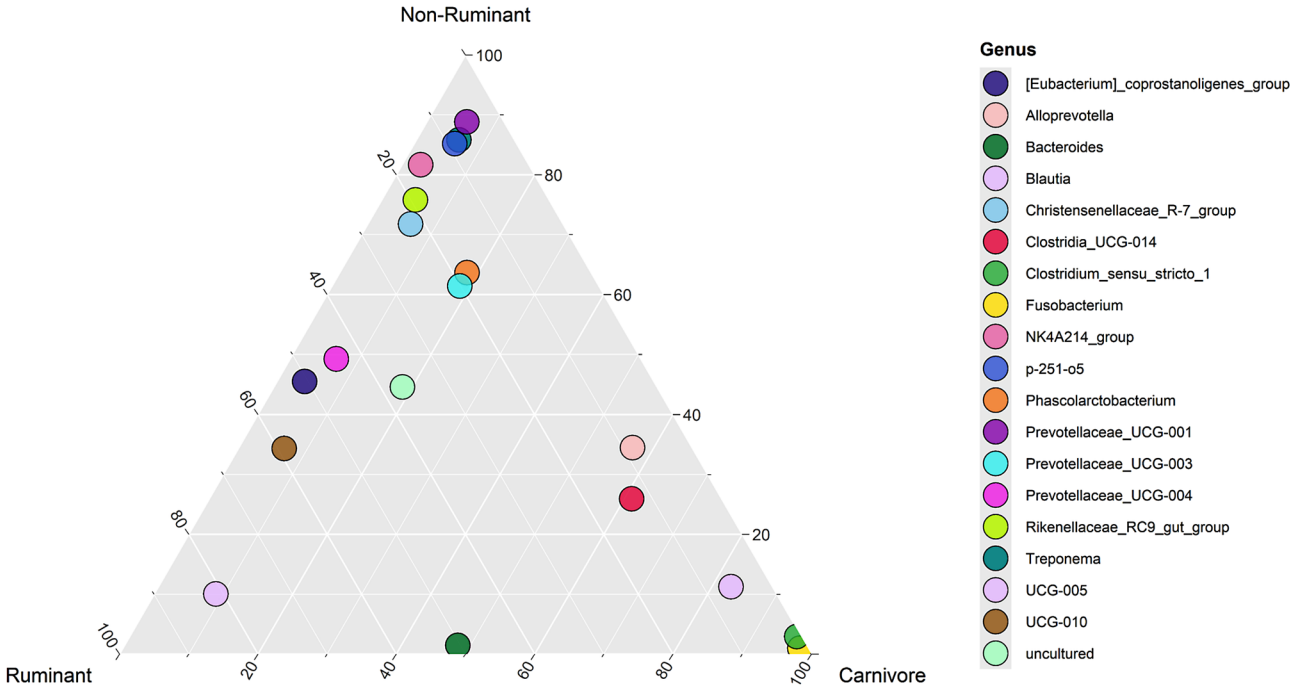


**Fig. 4** Ternary plot of genus-level taxonomic distribution across dietary groups. Each point represents a bacterial genus positioned according to its relative abundance across carnivore, non-ruminants, and ruminant hosts. Axes correspond to percentage contributions to each dietary group, with corners representing 100% contribution

To examine the distribution of genera with known or potential pathogenic roles, we generated a heatmap of absolute read counts across host species (Fig. 5). All host species showed some level of *Escherichia-Shigella*, with lions exhibiting the highest abundance (1,338 reads). *Streptococcus* was detected in six of nine species (Median: 188 reads). *Pseudocitrobacter* were detected exclusively in African wild dogs (86 reads), while *Chlamydia* was found only in black rhinoceros (29 reads) and *Mycobacterium* only in lions (20 reads). Notably, spotted hyaena contained four genera exclusively in this species. These included *Helicobacter* (199 reads), *Enterococcus* (76 reads), *Staphylococcus* (63 reads) and *Lactococcus* (54 reads). A comprehensive list of animal-associated and zoonotic bacterial families detected in this study, including their biological roles and clinical relevance, is provided in Table S9. The annotation panel above the heatmap highlights the dietary category associated with each species, however no distinct patterns emerged.

## Identification of host ost speciesspecies-specific microbiota profiles

At the host species level, distinct differences in gut microbiota composition emerged within each dietary group (Fig. 6). Among carnivores, substantial inter-individual and inter-species variability was present. African wild dogs were dominated by *Bacteroides* and *Fusobacterium*, with two individuals exhibiting notably high *Clostridium sensu stricto 1*, a taxon of potential clinical interest. Lion exhibited the highest relative abundance of Peptostreptococcaceae (13.08%), consistent with protein-rich diets, and also showed prominent *Bacteroides*, *Fusobacterium*, and *Blautia*. One lion exhibited high *Escherichia-Shigella*, while *Peptoriphilus* remained consistently low across individuals. Spotted hyaena were uniquely characterised by an exclusive presence of *Clostridia UCG-014* (12.79%) across individuals, alongside *Bacteroides* and low levels of *Clostridium sensu stricto 1* and *Fusobacterium*. Carnivores overall exhibited greater individual-level variability than herbivores.

Among non-ruminants, warthogs showed a pronounced enrichment of the hindgut-associated family *p*-251-o5 (10.82%) and shared the *Lachnospiraceae AC2044 group* with white rhinoceroses. African elephants showed a

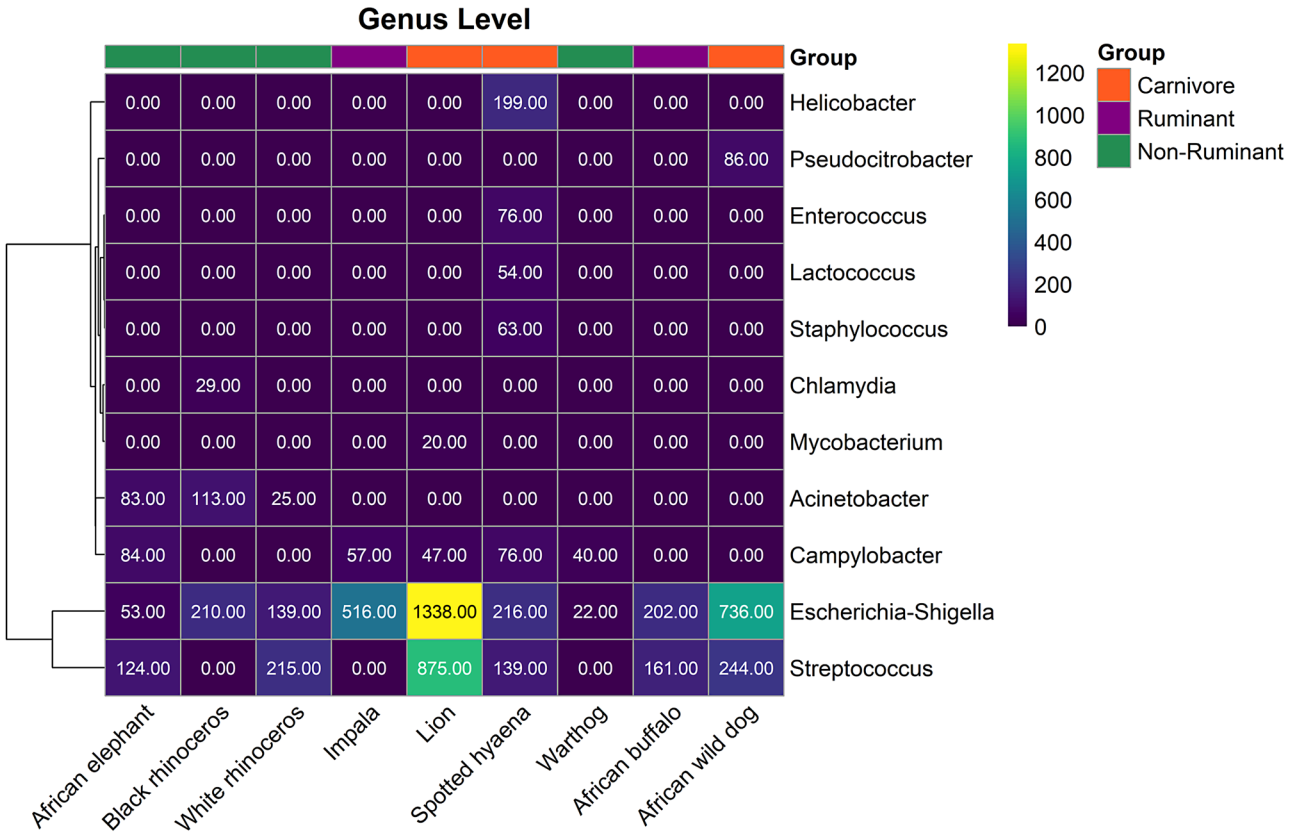


**Fig. 5** Heatmap of potential pathogens across host species. Heatmap displays absolute read counts for genera with known or potential pathogenic roles (rows) across individual host species (columns). The top annotation panel indicates each species' dietary group

community structure broadly similar to black rhinoceroses but with higher levels of *Treponema*. Notably, black and white rhinoceroses, despite sharing the same non-ruminant digestive strategy, showed divergent genus-level profiles. Black rhinoceroses were characterised by higher proportions of *Rikenellaceae RC9 gut group* and Prevotellaceae-affiliated genera, while white rhinoceroses exhibited greater representation of Lachnospiraceae members including *UCG-009* and *AC2044 group*, *Prevotellaceae UCG-004*, and a larger fraction of unclassified taxa.

Within ruminants, microbial profiles were strikingly similar, dominated by *Rikenellaceae RC9 gut group*, *Bacteroides*, *UCG-005*, *UCG-010*, and *Prevotellaceae UCG-004*. African buffalo showed marginally higher levels of *UCG-010* compared to impala, but the overall homogeneity of ruminant profiles reflects the homogenising influence of foregut fermentation.

## Predicted functional profiles and differential abundance reveal species-specific signatures

At the dietary group level, predicted functional profiles were largely conserved across carnivores, non-ruminants, and ruminants, dominated by biosynthesis (~75%), generation of precursor metabolites and energy (~10%), and degradation/utilisation/assimilation (~8%), with no meaningful categorical differentiation (Fig. S8). Analysis of the 30 most variable predicted MetaCyc pathways by ANOVA revealed pronounced functional divergence driven primarily by carnivore samples (Fig. 7A; Table S10). The most strongly differentiated pathways were enriched in opposing directions between dietary groups. The superpathways of L-tyrosine biosynthesis (enrichment score = 1.95) and L-phenylalanine biosynthesis (1.95), polyamine biosynthesis I (1.91), cob(II)yrinate a, c-diamide biosynthesis I (1.83), L-lysine biosynthesis II (1.82), and N-acetylglucosamine/N-acetylneuraminate

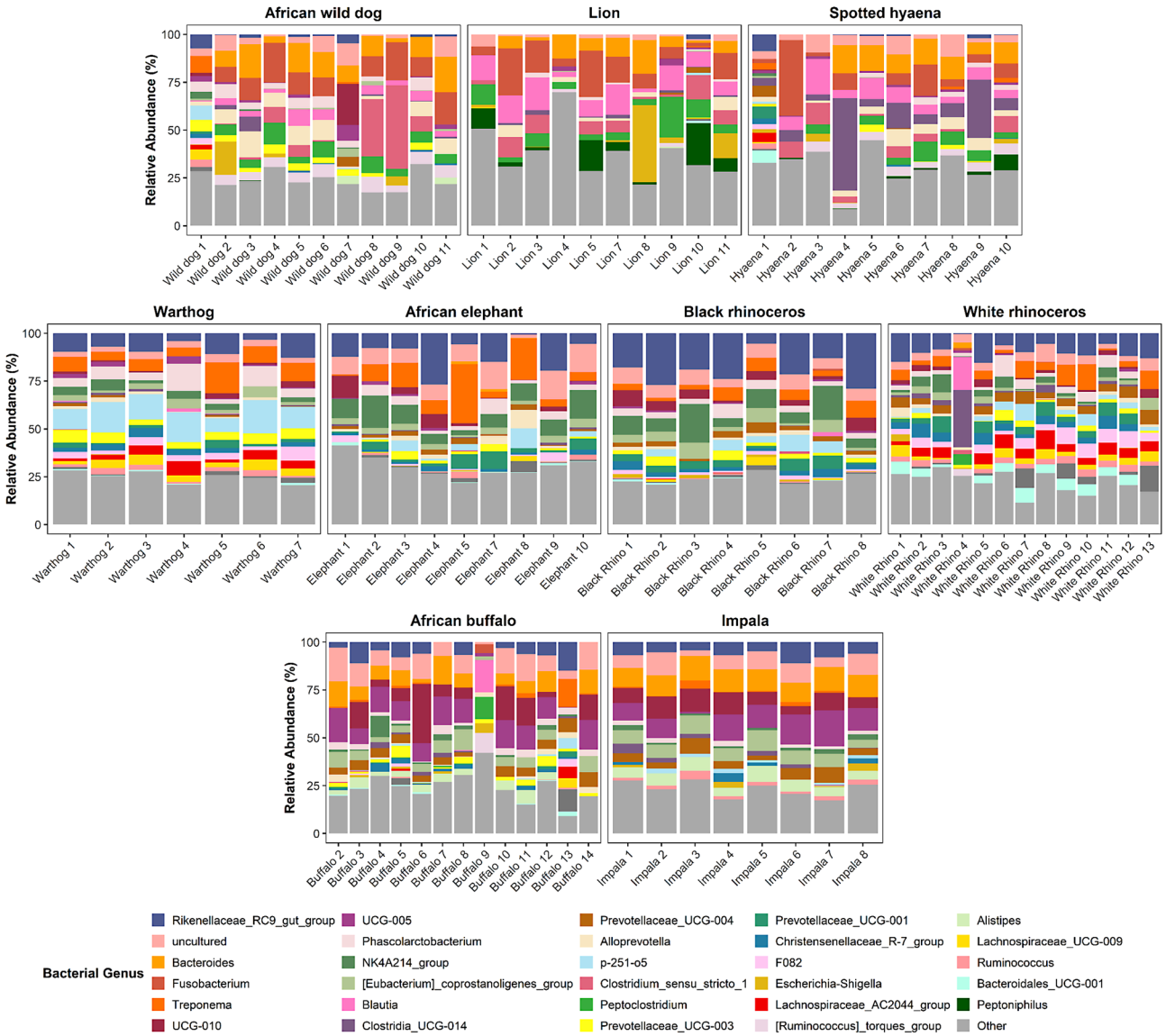


**Fig. 6** Genus-level taxonomic composition of gut microbiota across host species. Genus-level taxonomic composition of gut microbiota across host species within each dietary group. Stacked bar plots display relative abundances of the 30 most prevalent bacterial genera in individual samples; genera outside the top 30 are grouped as "Other." Samples are organised by host species within carnivores (row 1), non-ruminant (row 2), and ruminants (row 3)

degradation (1.81) were enriched in carnivores relative to ruminants. Conversely, L-tryptophan biosynthesis (1.98), L-histidine biosynthesis (1.87), the superpathway of branched amino acid biosynthesis (1.82), and L-serine and glycine biosynthesis I (1.80) were depleted in carnivores and enriched in ruminants and non-ruminants.

Within the rhinoceros comparison (Fig. 7B; Table S11), 15 pathways were significantly differentiated between species (all $p<0.02$). Black rhinoceros showed enrichment in acetyl-CoA fermentation to butanoate II (FC = 4.87; $p<0.001$), tetrapyrrole biosynthesis I and II (FC = 1.17 and 1.18 respectively; $p<0.001$ and $p=0.001$), the superpathway of branched amino acid biosynthesis (FC = 1.04; $p=0.003$), L-isoleucine biosynthesis III (FC = 1.06; $p=0.003$), 2-methylcitrate cycle I (FC = 21.00; $p=0.008$), and photorespiration (FC = 8.64; $p=0.012$). White rhinoceros were enriched in homolactic fermentation (FC = 0.85; $p<0.001$), glycolysis I from glucose 6-phosphate (FC = 0.87; $p<0.001$), glycolysis II from fructose 6-phosphate (FC = 0.80; $p<0.001$), the superpathway of (R,R)-butanediol biosynthesis (FC = 0.29; $p<0.001$), cis-vaccenate biosynthesis (FC = 0.96; $p=0.002$), superpathway of

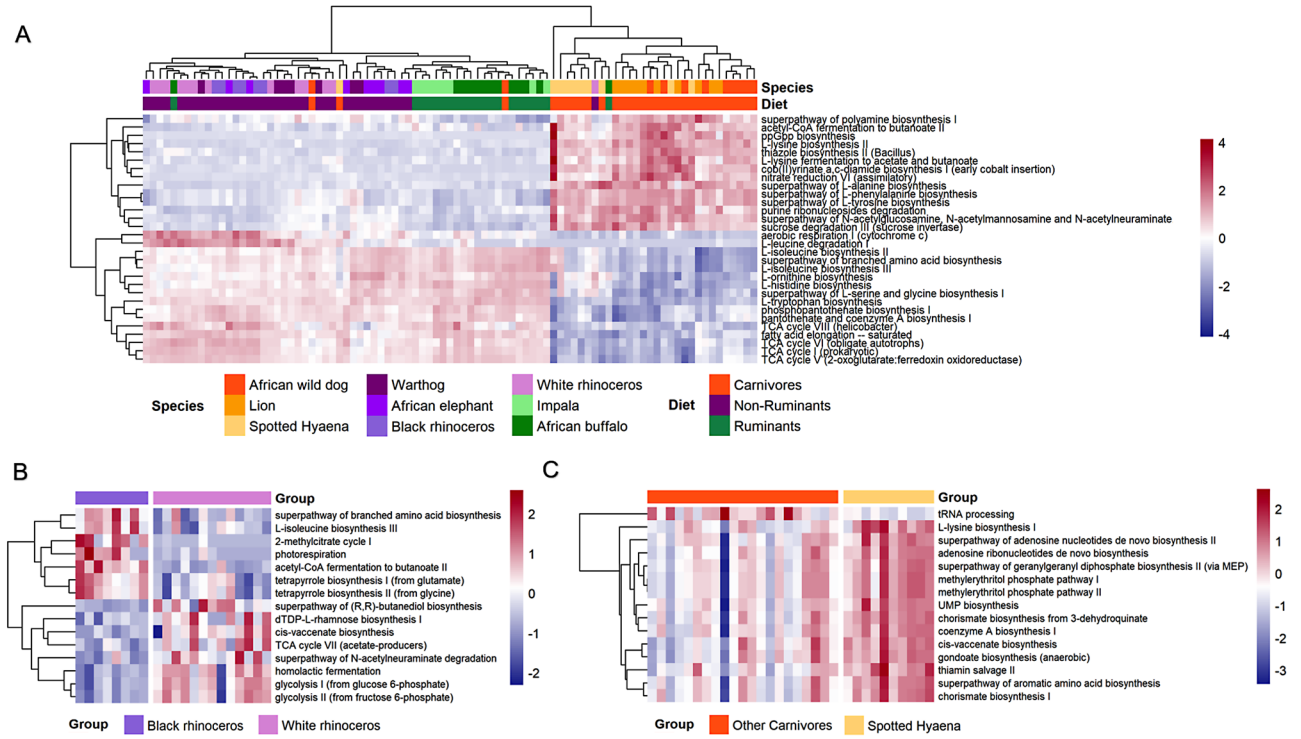


**Fig. 7** Predicted functional profiles of gut microbial communities across host species. Heatmaps display Z-scaled relative abundances of the top most variable predicted MetaCyc pathways across individual samples. **A.** Top 30 pathways selected by one-way ANOVA F-statistic across all 89 samples. Samples are annotated by host species (top bar) and dietary group (second bar): carnivores (orange), non-ruminants (purple), and ruminants (green). **B.** Top 15 most variable pathways within the rhinoceros subset, comparing black rhinoceros (dark purple) and white rhinoceros (light purple). **C.** Top 15 most variable pathways within the carnivore subset, comparing other carnivores (lion and African wild dog; orange) and spotted hyaena (yellow). In all panels, red indicates enrichment relative to the row mean and blue indicates depletion; rows are clustered hierarchically. Pathway abundances were predicted using PICRUSt2 and Z-scaled per row

N-acetylneuraminate degradation (FC = 0.77; $p = 0.004$), dTDP-L-rhamnose biosynthesis I (FC = 0.94; $p = 0.004$), and TCA cycle VII acetate-producers (FC = 0.58; $p = 0.004$).

Within the carnivore comparison (Fig. 7C; Table S12), spotted hyaena were enriched in 14 of the 15 most variable pathways. The strongest discriminating pathways included chorismate biosynthesis from 3-dehydroquinate (FC = 1.16; $p < 0.001$), gondoate biosynthesis anaerobic (FC = 1.15; $p < 0.001$), thiamin salvage II (FC = 1.23; $p < 0.001$), and L-lysine biosynthesis I (FC = 1.21; $p = 0.001$). Additional enrichment was observed across nucleotide biosynthesis, isoprenoid, and fatty acid pathways. Other carnivores were exclusively enriched in tRNA processing (FC = 0.24; $p < 0.001$). LEfSe analysis further identified *Rikenellaceae RC9 gut group* (LDA = 4.83; $p < 0.05$) and *Lachnospiraceae AC2044 group* (LDA = 4.64; $p < 0.001$) as the strongest taxonomic discriminators of black and white rhinoceros respectively, while *Clostridia* UCG-014 (LDA = 5.01; $p < 0.001$) was the dominant taxon enriched in spotted hyaena relative to other carnivores (Fig. S9).

## Discussion

We performed a comparative analysis of gut microbiota across nine wild mammal species in the Kruger National Park, and our findings highlight diet as the primary driver of microbial diversity and composition. Previous microbiome research has primarily focused on single species such as African buffalo, African elephant, lion, and impala [22–28]. To our knowledge, this is the first 16S rRNA-based study to characterise gut microbiota across

multiple coexisting species in Kruger, including underrepresented taxa such as African wild dog, warthog, and both rhinoceros species, thereby filling an important gap in African wildlife microbiome knowledge.

Consistent with previous research, our beta diversity analyses revealed strong clustering by dietary category, affirming that diet exerts a greater influence on gut microbial communities than host species identity [8, 23, 29]. The Random Forest classification model demonstrated high predictive accuracy (93.26%; Kappa = 0.8959). Six samples were misclassified by the RF model, consistent with their atypical clustering in beta-diversity ordinations; these samples also appeared separate in PICRUSt2 predicted functional profiling, lending additional but non-definitive support to the presence of technical or biological sources of variation whose origin remains unclear. This performance nonetheless highlights the potential of microbiome-based classification for discerning dietary signatures across diverse hosts, aligning with trends in microbiome-driven machine learning applications [30–33]. Notably, both sensitivity and specificity exceeded 90%, reinforcing the biological relevance of our models and their applicability in conservation contexts [34, 35]. Alpha diversity patterns aligned with theoretical expectations, indicating that herbivores support richer microbial communities compared to carnivores. Non-ruminant hindgut fermenters exhibited the highest diversity, followed by ruminants and carnivores, reflecting the ecological niches created by fibre-rich diets [12, 13, 17, 36]. These findings are consistent with studies on elephants in Nepal, which similarly demonstrated higher microbial diversity than sympatric ruminants [12].

Taxonomic profiles further reflect digestive strategies. Carnivores were enriched in Fusobacteriaceae, Clostridiaceae, and Peptostreptococcaceae, families associated with protein and bile metabolism, represented predominantly by *Fusobacterium*, *Clostridium sensu stricto 1*, and *Peptoclostridium* [12, 13]. Herbivores harboured Rikenellaceae, Oscillospiraceae, and Spirochaetaceae, specialised for fibre degradation. These were largely driven by *Rikenellaceae RC9 gut group*, *NK4A214 group*, and *Treponema*, all of which are well adapted for plant polysaccharide utilisation and have been linked to elevated dietary fibre intake across mammalian taxa [37]. Prevotellaceae, represented by *Prevotellaceae UCG-001* and *UCG-003*, were widespread but at significantly lower abundance in carnivores, consistent with reduced fibre intake. Lachnospiraceae emerged as a core family across all dietary groups, with genera such as *AC2044 group* and *UCG-009* suggesting finer-scale functional specialisation, particularly among non-ruminants. The consistent presence of Lachnospiraceae emphasises its fundamental role in fermenting complex polysaccharides into SCFAs, an essential energy source irrespective of host diet [38]. By contrast, Bacteroidaceae, dominated by *Bacteroides*, was abundant in carnivores and ruminants but nearly absent in non-ruminants, highlighting divergent fermentative pathways [13]. These differences appear to correspond to the dietary preferences of the herbivore species in this study; African buffalo, warthogs, and white rhinoceros are considered grazers, impala and elephants are mixed-feeders, and black rhinoceros are browsers [39].

Host-specific adaptations were also evident. Warthogs exhibited enrichment of the hindgut-associated *p*-251-o5 group, a bacterial family previously linked to fibre-rich, low-protein diets, suggesting that warthogs follow a fibre-oriented diet, consistent with their predominantly grazing ecology [40]. Distinct microbiota profiles in black and white rhinoceroses support their dietary and ecological divergence, with direct implications for conservation management. As a browser, black rhinoceros require different vegetation and habitat from white rhinoceros, which are grazers. These dietary requirements can impact the success of translocations, responses to climate-associated habitat change, and the conservation of these threatened and endangered species [41, 42]. The strongest taxonomic discriminators identified by LEfSe reflected these dietary differences clearly. The enrichment of *Rikenellaceae RC9 gut group*, *Pseudobutyrivibrio*, and *Faecalibacterium* in black rhinoceros is consistent with a browse-based diet rich in tannins and plant secondary compounds, as these taxa are associated with butyrate production and degradation of complex plant substrates [37, 43, 44]. The higher abundance of *Lachnospiraceae UCG-009* and *AC2044 group* in white rhinoceros aligns with a grass-dominated diet, as Lachnospiraceae genera are characteristic constituents of grass-adapted hindgut communities and have been reported as dominant taxa in white rhinoceros gut microbiomes [45, 46].

Predicted functional profiling further supported this dietary divergence. Black rhinoceros showed greater predicted capacity for SCFA production and propionate metabolism from branched-chain substrates, consistent with fermentation of browse-derived structural polysaccharides and plant secondary metabolites [47]. Tetrapyrrole

biosynthesis enrichment in black rhinoceros may additionally reflect greater microbial porphyrin synthesis capacity, previously associated with communities processing tannin-rich plant material [48, 49]. White rhinoceros predicted profiles were instead characterised by enrichment in glycolytic and homolactic fermentation pathways, consistent with grass-dominated diets supplying readily fermentable hexose sugars; homolactic fermentation is characteristic of lactic acid bacteria prevalent in grass-adapted hindgut communities [48, 49]. Together, these convergent taxonomic and functional lines of evidence support the interpretation that rhinoceros microbiota divergence reflects dietary specialisation rather than phylogenetic divergence alone and underscore the value of microbiome characterisation as a tool for informing species-specific conservation and habitat management decisions [42].

Within carnivores, the most strongly discriminatory taxon in spotted hyaena was *Clostridia UCG-014,* consistent with their scavenging ecology and capacity to consume decomposed carcasses and bone. This is a nutritional niche that may select for fermentative taxa capable of processing collagenous and recalcitrant substrates [27, 50, 51]. This aligns with prior work showing that Kruger lions rely primarily on hunted prey, whereas spotted hyaenas scavenge a substantial proportion of their diet, driving divergent gut communities within the carnivore group [52]. Predicted functional profiling corroborated this distinction, with spotted hyaena showing enrichment in anaerobic long-chain fatty acid biosynthesis pathways, consistent with the bone marrow and fat-rich dietary components that hyaena uniquely exploit among the carnivores studied [53]. Enrichment of thiamin salvage pathways in hyaena is also ecologically coherent, as microbial thiamin salvage is selectively advantageous in communities exposed to protein- and bone-rich substrates deficient in plant-derived thiamin precursors [27, 54].

At the full cohort level, carnivore samples drove the greatest predicted functional divergence among the topmost variable pathways, consistent with the distinct protein-adapted taxonomic profiles observed in this group. The ternary plot provided further support for dietary structuring, with most genera aligning along the non-ruminant and ruminant axes, reflecting the dominance of fibre-associated taxa across the studied communities, even among species with differing digestive strategies. The distribution of potentially pathogenic genera across host species likely reflects a combination of commensal carriage, host ecology, and environmental exposure. *Escherichia-Shigella* occurred in every species, with highest abundance in lions. While consistent with its role as ubiquitous gut flora, elevated abundance in apex predators may warrant monitoring for stress-related overgrowth or antimicrobial resistance [55, 56]. Host-restricted detections are ecologically noteworthy but require cautious interpretation. The detection of *Mycobacterium* exclusively in lions and *Chlamydia* only in black rhinoceros is of interest; however, 16S rRNA sequencing cannot resolve species-level taxonomic identity, and targeted culture or PCR-based confirmation would be required before any pathogenic or zoonotic significance can be attributed [57–63]. While these detections do not confirm active infection, they highlight the value of non-invasive microbiome surveillance as an early-warning system for zoonotic and wildlife pathogens. Similarly, *Pseudocitrobacter* only in African wild dogs may reflect species-specific exposure or susceptibility. The four unique genera observed in spotted hyaenas are striking and may relate to their scavenging niche and digestive physiology. The absence of dietary patterns suggests that social structure, habitat use, or environmental reservoirs may be more influential than diet for spotted hyaenas. Collectively, these findings demonstrate that wildlife harbour diverse potentially pathogenic bacteria in the absence of clinical disease, highlighting the value of non-invasive microbiome surveillance for early pathogen detection within a One Health framework [32, 64].

Variable importance analysis reinforced these dietary distinctions by identifying key bacterial predictors at both the family and genus levels. In carnivores, Erysipelotrichaceae, Fusobacteriaceae, and Peptostreptococcaceae, together with *Peptoclostridium* and *Blautia*, best defined their gut microbiota. Non-ruminants were primarily distinguished by Spirochaetaceae, F082, and Bacteroidaceae, with *Treponema* and *Prevotellaceae UCG-001* as signature genera, consistent with fibre-rich hindgut fermentation. Ruminants, in turn, were characterised by Akkermansiaceae, [Eubacterium] coprostanoligenes group, and uncultured families, with *Alistipes*, *UCG-005*, and *dgA gut group* representing dominant taxa. The presence of overlapping families and genera across groups further points to a set of generalist or functionally flexible taxa, coexisting with highly diet-specialised lineages.

Together, these results corroborate prior evidence that host diet is a primary determinant of gut microbial composition in mammals [8, 33].

From a conservation perspective, establishing baseline microbiomes for Kruger's mammals is crucial for detecting ecological stress, shifts in forage quality, or emerging diseases. Key fibre-degrading taxa may serve as candidate biomarkers for habitat condition, while increases in pathogenic taxa could signal compromised resilience [65]. In addition, these provide a foundation for comparison of diet and microbiome shifts in captive wildlife which may be associated with abnormal health. Integrating microbiome profiling with machine learning, as demonstrated through our RF analysis, enhances its potential as a rapid, non-invasive diagnostic tool for conservation efforts [66]. Changes in microbiome composition associated with chronic stress, e.g., capture, translocation and placing in holding facilities with an altered diet [4, 24], can result in pathogenic bacteria becoming more prominent and causing disease [2, 24].

Our study has several limitations. Sampling was restricted to a single time point, limiting inference on seasonal or temporal microbiome variation, which is known to be substantial in wild mammals. Metadata such as sex, age, and individual health status were unavailable; these are recognised covariates of gut microbiome composition and their absence limits interpretation of within-group variation [47]. Sampling occurred opportunistically during routine veterinary interventions (e.g., snare removal, dehorning), management procedures (e.g., collaring), or post-mortem examinations following severe poaching-related injuries. The acute physiological stress associated with these specific events, alongside the use of chemical immobilization agents, may transiently alter microbiota composition, representing an inherent limitation of opportunistic wildlife sampling. All animals were sampled within a single ecosystem; microbiome composition is likely to differ across habitats and geographic populations, and findings from Kruger should not be assumed to generalise broadly. Finally, 16S rRNA sequencing provides taxonomic rather than functional resolution; species-level pathogen identification and community metabolic capacity cannot be determined without culture, targeted PCR, or metagenomic approaches. While we utilised PICRUSt2 for functional prediction, we acknowledge that its utility relies on default databases heavily biased toward human and model organism genomes. As noted by Sun et al. (2020), the accuracy of PICRUSt2 predictions is likely limited outside of human samples, and the development of tools for gene prediction specific to non-human and environmental samples is warranted [67]. Therefore, our functional results should be interpreted as hypothetical predictions requiring validation via metagenomics or metabolomics. Future work should employ longitudinal designs and functional approaches such as metagenomics or metabolomics advance beyond the taxonomic baseline established here.

In summary, dietary groups strongly structure the gut microbiomes of Kruger mammals, with carnivores maintaining protein-adapted communities and herbivores hosting diverse fibre-degrading consortia. Hindgut fermenters displayed the highest richness, underscoring digestive physiology as a driver of microbial diversity. By establishing microbiome baselines across multiple coexisting species, this study provides both ecological insight and a foundation for microbiome-based conservation management in African wildlife.

## Conclusion

Our research revealed a comprehensive overview of the bacterial composition and diversity of the gut microbiome of various wild animals from the Kruger National Park, revealing distinct community structures shaped by host diet and physiology. Carnivores had less diverse, protein-adapted microbiomes, whereas ruminants and non-ruminants exhibited communities enriched with fibre-degrading taxa, reflecting digestive specialisation and trophic ecology. These patterns show the central role of the host dietary niche in structuring gut microbial ecosystems in natural settings. By characterising the microbiota of multiple species within a shared environment, this work establishes a valuable ecological baseline for future wildlife microbiome research. Expanding these comparative approaches to include environmental, seasonal, and host health factors will advance understanding

of how gut microbial diversity contributes to adaptation, resilience, and ecosystem function in free-ranging animal populations.

## Methods

### Sample collection and research permits

Fresh faecal samples from nine wild mammal species inhabiting Kruger National Park, South Africa, were collected between 27 and 31 August 2023. Sampling occurred from the rectum during standard immobilizations (for research, disease surveillance, veterinary treatments like dehorning or snare removal, or management like collaring) or necropsies following euthanasia for severe, typically poaching-related, injuries. Consequently, individual-level clinical metadata and precise health statuses at the time of sampling were not uniformly available. Animals were handled by registered wildlife veterinary staff according to the South African National Parks (SANParks) Standard Operating Procedures for the Capture, Transportation and Maintenance in Holding Facilities of Wildlife. Targeted species were lion (*Panthera leo*; $n=11$), spotted hyaena (*Crocuta crocuta*; $n=10$), African wild dog (*Lycaon pictus*; $n=11$), warthog (*Phacochoerus africanus*; $n=7$), African elephant (*Loxodonta africana*; $n=10$), black rhinoceros (*Diceros bicornis*; $n=9$), white rhinoceros (*Ceratotherium simum*; $n=14$), African buffalo (*Syncerus caffer*; $n=14$), and impala (*Aepyceros melampus*; $n=8$). Fresh droppings were collected in sterile containers, immediately placed on ice packs in the field for transport to Veterinary Wildlife Services (VWS) laboratory, then stored at −80 °C until processing. Samples were acquired after approval of a biomaterials transfer agreement approval (BMTA 013/23) granted by the South African National Parks Veterinary Wildlife Services Biobank (Skukuza, South Africa). Section 20 approval was received from the Department of Agriculture, Land Reform & Rural Development 12/11/1/7/2 (4011KL).

### DNA extraction and 16S rRNA gene sequencing

We extracted DNA from 150 to 250 mg of each faecal sample using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, R2002, USA) following the manufacturer's instructions, with the exception that the bead-beating step was omitted. Samples were homogenised in 750 µL of DNA/RNA Shield (Zymo Research) and centrifuged at 16,000 × g for 30 s to remove debris, after which 400 µL of supernatant was transferred to a nuclease-free tube. DNA was eluted in 50 µL of DNase/RNase-free water after a 5 min incubation and stored at − 80 °C until downstream processing. A negative extraction control was included to monitor potential contamination.

The variable V3 and V4 regions of the prokaryotic 16S ribosomal RNA (16S rRNA) gene were amplified from extracted microbial DNA using gene-specific sequences selected from the Klindworth et al. publication [68]. Each 25 µL PCR reaction consisted of 12.5 µL of PrimeSTAR® GXL Premix (2×) (Takara Bio, Shiga, Japan), 5 µL of 16S forward primer (1 µM), 5 µL of 16S reverse primer (1 µM), and approximately 2.5 µL of template DNA. Amplification was carried out on a thermal cycler under the following conditions: initial denaturation at 98 °C for 10 s, annealing at 60 °C for 15 s, and extension at 70 °C for 30 s, for a total of 30 cycles.

Amplicon size (~550 bp) and quantity were verified using the LabChip® GX Touch 24 system (PerkinElmer, USA) and the Qubit™ 1X dsDNA HS Assay Kit on the Qubit™ Flex Fluorometer (Thermo Fisher Scientific, USA).

Indexed sequencing adapters were ligated to the amplicons during an indexing PCR step, followed by bead-based purification to remove unincorporated primers and adapter dimers. The resulting amplicon libraries (~630 bp average fragment size) were evaluated again on the LabChip® system, normalised to equimolar concentrations, and pooled. The final pooled library, supplemented with a 15% (v/v) PhiX Control v3 (Illumina, USA) spike-in, was diluted to 10 pM and loaded onto an Illumina® MiSeq v3 flow cell. Sequencing was performed as 2 × 301 bp paired-end reads on the Illumina® MiSeq platform.

Following sequencing, samples were demultiplexed using the Generate FASTQ module on the Illumina® MiSeq instrument. Run quality and performance metrics were evaluated using Illumina® Sequence Analysis Viewer software (v2.4.7).

## Data processing and taxonomic analysis

Raw paired-end 16S rRNA gene sequencing reads were processed using the QIIME 2 pipeline (version 2024.10) [69]. After demultiplexing, the DADA2 plugin [70] was used to denoise the paired-end reads by trimming, merging, and removing chimeric sequences. This approach resolves ASVs at single-nucleotide resolution, providing high-resolution identification without clustering similar sequences based on arbitrary similarity thresholds [71]. Taxonomic assignment of ASVs was performed using a pre-trained naive Bayes classifier trained on the SILVA 138 full-length reference sequences [72].

Ternary plots were generated using the ggtern package in R (v4.3.3) to visualise the proportional distribution of dominant bacterial families across carnivores, non-ruminants, and ruminants. Heatmaps were created with the ComplexHeatmap package to display abundance patterns of the most prevalent taxa across samples, facilitating comparative visualisation of taxonomic profiles between host groups. To minimise background noise, only taxa with a minimum threshold of $>20$ reads across samples were included in the analysis.

## Alpha and beta diversity analysis

We assessed microbial diversity within (alpha diversity) and between (beta diversity) different host categories using the QIIME 2 core diversity analysis plugins. To ensure fair comparisons across samples, we applied rarefaction at a depth of 2,000 sequences per sample; a threshold considered sufficient to capture most of the microbial diversity present. Samples were grouped based on dietary groups: carnivores, non-ruminants, and ruminants.

For alpha diversity, we used two complementary metrics: Faith's phylogenetic diversity (PD), which accounts for the evolutionary relationships among taxa, and the Shannon diversity index, which reflects both richness and evenness of taxa. Statistical differences in alpha diversity across groups were tested using pairwise Kruskal-Wallis tests ($\alpha = 0.05$), followed by post hoc pairwise Dunn's tests with Benjamini-Hochberg correction. The results were visualised using the ggplot2 package in R (v4.3.3).

Beta diversity was examined using both weighted and unweighted UniFrac distance metrics, which incorporate phylogenetic information to compare overall community composition between samples. We tested significant differences among host groups using PERMANOVA (Permutational Multivariate Analysis of Variance) with 9999 permutations implemented via the adonis2 function in the vegan package (v2.6) in R (v4.3.3). Pairwise PERMANOVA *p*-values were corrected for multiple comparisons using the Benjamini-Hochberg (BH) false discovery rate method, implemented via p.adjust() in R. To explore and illustrate clustering patterns among samples, we generated principal coordinate analysis (PCoA) plots using the Emperor visualisation tool in QIIME 2.

## Machine learning classification

We applied a Random Forest (RF) classifier to test the predictive power of gut microbial community composition for distinguishing host dietary groups. Family-level counts were transformed using a centred log-ratio (CLR) approach to account for the compositional nature of microbiome data. CLR-transformed abundance profiles were used as input features. RF models were trained with 500 trees and evaluated using 5-fold cross-validation implemented via caret package (R v4.3.3). The number of variables randomly sampled at each split (mtry) was tuned during model training; the optimal value selected was mtry $= 65$ out of 128 predictors, based on cross-validation accuracy. Classification accuracy, confusion matrices, and variable importance (measured as mean decrease in accuracy) were computed using the randomForest package.

### Identification of atypical samples and random forest validation

Six samples exhibited atypical beta-diversity clustering inconsistent with their assigned dietary group, comprising two African wild dogs, one spotted hyaena, two African buffalo, and one white rhinoceros. These were identified by two independent and concordant lines of evidence: (1) inconsistent clustering with the expected dietary group in both weighted and unweighted UniFrac PCoA ordinations, and (2) misclassification by the RF dietary group classifier. All 89 samples passing rarefaction were retained for downstream analyses; the six atypical samples are noted where relevant to the interpretation of within-group variation.

### Differential abundance and functional prediction

To explore the predicted functional potential of gut microbial communities, PICRUSt2 (v2.5.1) was applied. Pathway abundances were normalised by predicted 16S rRNA gene copy number and expressed as relative abundances per sample. Broad functional profiles were summarised by aggregating pathways into official MetaCyc top-level categories. To identify the most variable predicted pathways across all samples, one-way ANOVA was applied and pathways ranked by F-statistics, with the top 30 selected for visualisation. Species-level functional variation was further explored within the same two focal comparisons by selecting the top 15 most variable pathways within each subset, with significance assessed using Welch's t-tests.

Furthermore, to identify differentially abundant taxa between ecologically distinct species pairs, LEfSe analysis was performed at family and genus level using the microbiomeMarker package in R [73]. Two comparisons were conducted: black rhinoceros versus white rhinoceros, and spotted hyaena versus other carnivores (lion and African wild dog combined). Taxa were considered significant at Kruskal-Wallis $p < 0.05$, Wilcoxon $p < 0.05$, and a minimum LDA score of 2.0.

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1186/s42523-026-00630-0.

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**Data availability** Code and data are available at the GitHub repository: https://github.com/sheylle1/16S_Kruger_Comparative_Analysis_and_Microbial_Diversity. Raw sequence data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1248980. Individual SRA accession numbers for each sample are provided in the metadata file (Table S1).

## Declarations

**Ethics approval and consent to participate** Samples were acquired after approval of a biomaterials transfer agreement approval (BMTA 013/23) granted by the South African National Parks Veterinary Wildlife Services Biobank (Skukuza, South Africa). Section 20 approval was received from the Department of Agriculture, Land Reform & Rural Development 12/11/1/7/2 (4011KL). The study was approved by the Research Ethics Committee: Animal Care and Use at Stellenbosch University (reference number ACU-2025-35204).

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.



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