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THE MICROBIOTA OF LONG-LIVING AND CANCER-FREE BLIND MOLERAT (*Nannospalax xanthodon*) FROM THE EDGE OF ITS DISTRIBUTION IN NORTHERN ANATOLIA

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ABSTRACT. The mammalian gut is colonized by microorganisms that affect development, immune system, energy metabolism, and reproduction. The majority of studies focused on laboratory or domestic animals in artificial setups, leaving the research focused on wild species underrepresented. The Anatolian Blind Molerat (hereafter ABMR), *Nannospalax xanthodon*, is a subterranean rodent that receives much attention due to its unique traits, such as tolerance to extreme hypoxic stress, resistance to cancer, and longer lifespan compared to similarly sized rodents. In this study, we characterize the gut microbiota of ABMR from its northernmost geographic distribution using 16S rRNA metabarcoding and compare our results with the microbiome characteristics of a few other ABMR populations studied previously, as well as other rodent species. The 16S rRNA barcode dataset revealed that approximately 90% of the ABMR gut microbiota comprises Firmicutes and Bacteriodota bacterial phyla, typical of most mammals. In addition, the ABMR gut microbiota has a high abundance of performance- and longevity-linked bacterial families. Overall, our results generally align well with the previous studies on blind molerats and emphasize the importance of studying the microbiome of natural populations.

1. INTRODUCTION

Microbial communities are integral to organism functioning in all mammals [1]. In animal-associated microbiota, the number of bacterial cells is more or less the same as the cell count of the host body [2]. Therefore, the effect of the gut microbiome, i.e. the microbial community occupying the gastrointestinal system, has a crucial role in the digestion, development, immunity, energetics, and fitness of animals [3–6].

The impact of gut microbiota on humans is a widely studied topic due to its association with various metabolic, autoimmune, and even psychological

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disorders [1,7,8]. The laboratory and domestic mammalian species serve as a good proxy model for this research, and there are many studies on their microbiota [9,10]. While studying captive animals is convenient, the effect of captivity conditions on microbiota composition cannot be ignored [11–14]. Besides the effect of captivity, the laboratory animal models often lack heterogeneity compared to their wild equivalents. Moving wild mice into a captive facility for a year dramatically changed the composition of the microbiota and made it more homogenous compared to the more diverse microbiota profiles observed in the wild [15]. Another study compared wild-caught with wild-derived, inbred strains of the house mouse (*M. musculus*) and found that approximately 16% of the bacteria differ between the wild and the inbred mice [16]. Therefore, using wild animals is crucial for understanding the ecological importance of the gut microbiome and revealing the details of the interactions among the host, the microbiome, and the environment that have been shaped by evolution during the course of their mutual coexistence [17–19].

The cause of differences in gut microbiota among individuals, groups, populations, and species is a topic of much interest. While the overall difference in microbiota is the largest at the species level [19], there is still a big room for questioning the within-species variation of microbiota [20] [15,20,21]. The Anatolian Blind Molerat (ABMR) *Nannospalax xanthodon* is a species of murid rodent that possesses many unique adaptations to obligate subterranean lifestyle. The AMBR is successful ecologically, as it is found from the sea level in the Aegean (warm Mediterranean climate) to the highlands of Taurus and Eastern Anatolian mountains (harsh cold alpine climate). The ABMR is in fact a taxonomic complex of multiple, cytogenetically distinct, and potentially genetically isolated geographic populations [22]. With its wide distribution range and potentially genetically isolated geographic populations living in different ecological conditions, the AMBR is a suitable model to study the within-species variation of microbiome composition. Besides its wide distribution range, the Blind Molerats have unique physiological traits such as resistance to extreme hypoxia and hypercapnia underground [23], resistance to cancer [24,25], and longer life span (~20 years) compared to similar sized rat [26]. These traits highlight the importance of intensive biomedical and ecophysiological research on Blind Molerats, including the microbiome.

In this pilot study, we aim to characterise the gut microbiome of wild ABMR in its two northernmost populations in Türkiye using 16S rRNA amplicon sequencing. Since the *Nannospalax* superspecies has a complex evolutionary history and is represented by multiple, cytogenetically distinct, and potentially genetically isolated geographic populations, studying the microbiota can help to understand the ecological difference between these species. Therefore, we aim to compare our results with a few previous studies on the Blind Molerat microbiome.

2. MATERIALS AND METHODS

2.1. Sampling

We captured three ABMR individuals: two in Ağlı and one in Taşköprü, Northern Türkiye, in July 2020 (Figure 1 and Table 1). The distance between the two populations is ~50 km. The genus *Nannospalax* is known for its complex taxonomy with numerous cytogenetic (=chromosomal) races distributed parapatrically in Anatolia. The Ağlı population studied here belongs to the "Kastamonu" cytotype (2N=60) and the Taşköprü population was designated as the "Taşköprü" cytotype (2N=58) in [22, 27]. Animals were captured alive by the hoe technique as described in [28]. We recorded the body mass and sex of the animals. We then dissected the animals, collected the caecum tissue, stored it in EtOh, and placed the sample to -80°C freezer next day. The procedure was approved by the Animal Ethics committee of Bülent Ecevit University (#91330202).

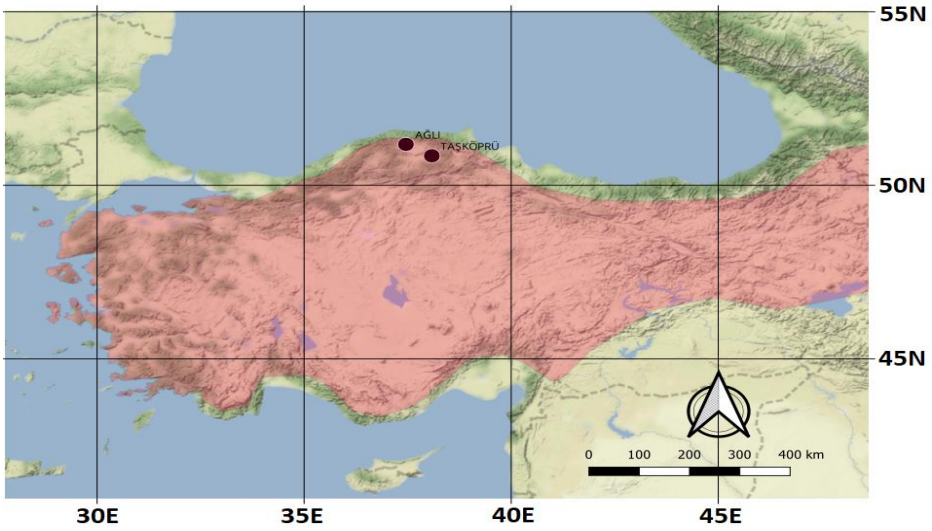

FIGURE 1. The distribution of ABMR and sampling locations. The red-shaded area represents the distribution range of *Nannospalax xanthodon* superspecies.

TABLE 1. List of frozen gut samples used in the study.

Sample	Population	Sex	Mass (gr)	Date of Collection	Latitude	Longitude
AGL1	Ağlı	female	161	03.07.2020	41.7139	33.6529
AGL2	Ağlı	female	212	03.07.2020	41.7139	33.6529
TAS1	Taşköprü	female	229	04.07.2020	41.4922	34.2147

2.2. DNA preparation and amplification

A small piece at the terminal end of the caecum (~5 g) was cut from the frozen sample with a flame-sterilised scalpel and used for DNA extraction. Whole metagenomic DNA was extracted from the caecum samples using DNEasy PowerSoil Kit (Qiagen, Cat No:47014).

The V3–V4 variable region in the bacterial 16S rRNA gene was amplified using the universal primers S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') [29]. We used Q5 High Fidelity DNA polymerase (NEB, Cat No: M0491S) to perform the PCR in 20 ul reaction volumes, with the following cycling conditions: initial denaturation at 98 °C for 5 min, 30 cycles of 98 °C (15 s), 55 °C for 20 s and 72 °C for 40 s and a final extension at 72 °C for 5 min. The PCR products were run in 1.5% agarose gel to evaluate the product quality and successfully amplified bands were purified using MiniElute Gel Extraction Kit (Qiagen, Cat No:28604). Then, PCR products were pooled according to the equimolar concentrations of each sample. The final pool was loaded on Pippin Prep automatic size selection system (Sage Science) targeting the amplicon size window of 350 - 550 bp.

Each sample was amplified and genotyped twice (in duplicates) to account for the effect of possible amplification stochasticity. In the following analyses, the data from duplicates are treated as individual samples. After that, the dual-indexed sequencing adaptors were ligated using a TruSeq nano DNA library preparation kit (Illumina), and the resulting amplicon libraries were outsourced for sequencing using the Illumina MiSeq instrument, Reagent Kit v2 (2 x 300 bp) at CEITEC Genomics Core Facility (Brno, Czech Republic).

2.3. Bioinformatics analysis

The raw sequencing data were trimmed and demultiplexed using Skewer and reads with low quality were eliminated by setting the expected error rate per paired-end read >1 [30]. The bacterial 16s rRNA haplotypes (Amplicon Sequence Variants, hereafter ASVs) were quality checked, identified, and analyzed using the software DADA2 [31]. Software UCHIME [32] was used for the identification and removal of sequence chimeras. The gold.fna (available at: https://drive5.com/uchime/gold.fa) database is used as a reference for chimera filtering. Silva database version 138.1 (updated in March 2021, [33] was used as a reference in DADA2 software [31]. Finally, *phyloseq* [34] package in R (version 4.2) was used to create a database that contains the OTU table, OTU sequences, taxonomic annotations, and phylogeny of bacterial OTUs.

2.4. Statistical analysis

The microbiome database comprised 61274 high-quality sequences grouped in 4841 non-chimeric OTUs. PROTEST (Procrustes Rotation of Two

Configurations in R package *vegan*) was used to compare duplicates. We used the observed number of OTUs, the Shannon index, and the Simpson index to estimate alpha diversity via the estimate_richness command in the phyloseq package in R. Because Taşköprü population was represented by only one sample, we used duplicates of each sample as pseudo-samples to calculate beta diversity. The Bray-Curtis dissimilarity index was used to calculate the divergence in microbiota composition between samples. Then, we applied PERMANOVA (adonis2 function from the *vegan* R package) to test the difference between the gut microbiota composition of samples.

3. RESULTS

We successfully genotyped the 16S rRNA amplicons from three ABMR caecum samples. After quality filtering, the numbers of reads per sample were 20421, 20426, and 20427 in samples TAS1, AGL2, and AGL1, respectively. PROTEST showed no significant difference between duplicates (number of permutations= 999; p-value=0.001).

The bacteriome database was dominated by Firmicutes (50% of all reads), Bacteriodota (39%), and Desulfobacterota (2%) (Figures 2 and 3A). At the family level, the data was dominated by Muribaculaceae (35%), Lacnospiraceae (28%), Oscillospiraceae (12%), Ruminococcaceae (7%), Desulfovibrionaceae (3%), Christensenellaceae (1%), and Rikenellaceae (1%) (Figure 3B). The bacterial phyla or families with less than 1% abundance were grouped into a "remainder" category.

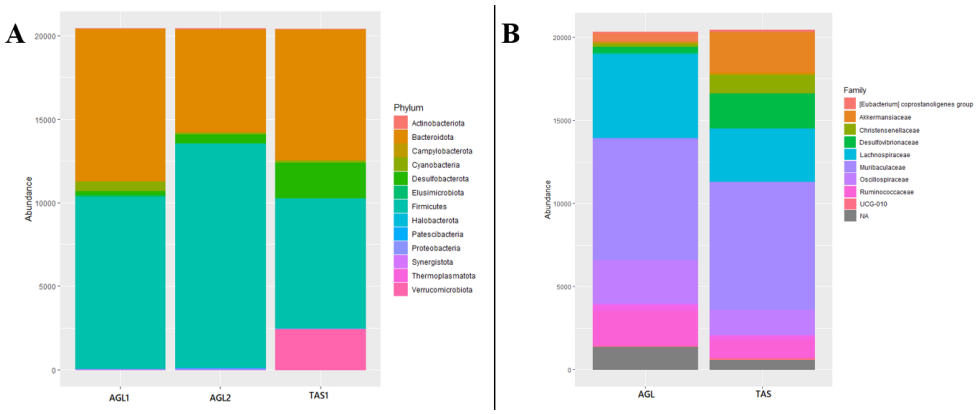

FIGURE 2. Relative abundance of bacterial phyla by sample.

We observed higher relative abundances of Bacteroidota, Firmicutes, and Cyanobacteria in Ağlı compared to Taşköprü. Conversely, Taşköprü exhibited higher relative abundances of Desulfobacterota and Verrucomicrobiota (Figure 2). At the bacterial family level, the relative abundances of Lachnospiraceae, UCG-010, and Ruminococcaceae were higher in Ağlı, while the relative

abundances of Akkermansiaceae, Christensenellaceae, Desulfovibrionaceae, and Muribaculaceae were higher in Taşköprü (Figure 2B).

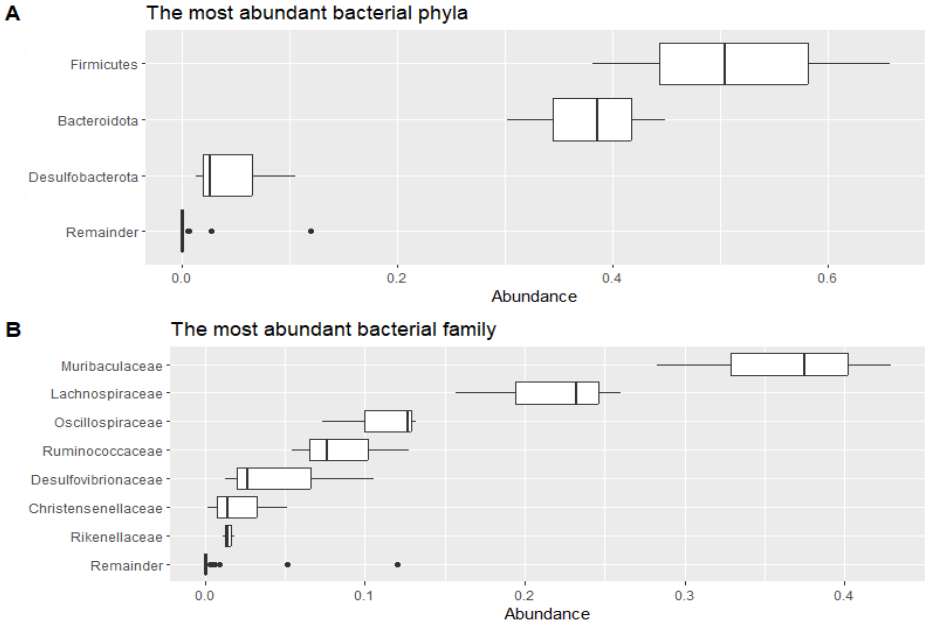

FIGURE 3. **A:** The most abundant bacterial phyla and **B:** The most abundant bacterial families for all samples together (X-axis represents % of the abundance of all read and "remainder" represents the taxa with <1% abundance).

We estimated the alpha diversity by using (i) the exact number of observed OTUs, (ii) the Shannon index, and (iii) the Simpson index. With a higher number of observed OTUs and higher values of Shannon and Simpson index values, all the alpha diversity indexes showed that samples from Ağlı have more diverse microbiota compared to the Taşköprü (Table 2). The relative abundance-based Bray-Curtis index values are used to calculate the difference in gut microbiota difference. On the PCoA plot based on the Bray-Curtis index, the first axis showed a clear separation of populations (Figure 4), however, the PERMANOVA test p-value was only marginally significant (permutation: 999 and p-value: 0.06).

TABLE 2. Variation in gut bacteriome diversity between samples.

Sample ID	Observed number of OTUs	Shannon index	Simpson index
AGL1	232	4.772	0.986
AGL2	235	4.834	0.985
TAS1	191	4.271	0.967

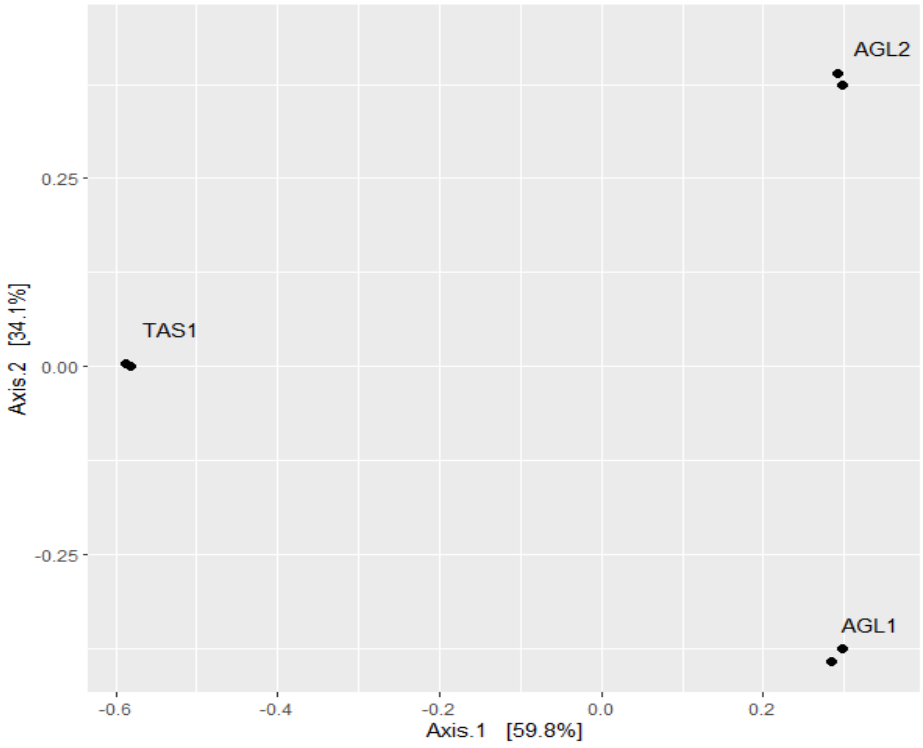

FIGURE 4. Principal Coordinate Analysis (PCoA) ordination of gut microbiota composition divergence between samples (based on the Bray-Curtis index).

4. DISCUSSION

In this study, we characterised the gut microbiota of wild-caught ABMRs from two populations in North Anatolia. We investigated the bacterial diversity of the ABMR microbiota at the class, phylum, and family levels. Taxonomic assignment of the 16S rRNA sequences revealed the most abundant bacterial classes: Clostridia (50%), Bacteroidia (38%), and Desulfovibrionia (2%). At the level of phyla, approximately 90% of the ABMR gut microbiota was dominated by Firmicutes and Bacteriodota (Figure 3A). These are typical components of mammalian microbiota and play a role in various processes, such as immune regulation, metabolism, and storage of fat [35–39].

The Desulfobacterota was the third most abundant phylum in our dataset, more abundant (~12%) in Taşköprü compared to Ağlı (~5%). While the Taşköprü population is represented by only one sample, we used duplicates as pseudo-samples and calculated dissimilarity using the relative abundance-based Bray-Curtis index. The difference in the gut microbiome composition between populations was only marginally above the significance level, which may be attributed to reduced test power caused by the small sample size. The differences between the locations (ie. relative abundances of bacterial phyla and alpha

diversity) could have been caused by differences in soil, climate, diet, vegetation, or host genetics. The effect of these factors on the animal microbiome has been discussed in many studies [40–43]. Further investigations are necessary to explore the intriguing variations in the relative abundances of bacterial taxa observed between populations. To gain a better understanding of these changes, it is important to conduct more extensive sampling across a broader range of geographical areas and consider the influence of multiple environmental factors. Additional efforts in these areas could shed light on the potential explanations for these observed differences.

TABLE 3. Comparison of the relative abundance of bacterial phyla between studies (Desulfobacterota is a synonym for Proteobacteria). N represents the sample size for each study. C (=caecal), F (=fecal), and GI (=gastrointestinal) represent the source of microbiota. *The study by Sibai et al. (2020) documented the change in fecal microbiome composition over a 1–2-month period, therefore the results are presented as a range of % values.

Study	Species	*N*	source	Relative abundance of most abundant bacterial phyla (%)			
				Firmicutes	Bacteriodota	Desulfobacterota	Actinobacteria
This study	*N. xanthodon*	3	C	50	39	2	>1
Kuang et al. 2022	*N. ehrenbergi*	12	C	59.6	10.7	17.4	7.6
Sibai et al. 2020 *	*N. xanthodon*	34	F	~31-32	~50-65	~12	>1
Weldon et al. 2015	*M. musculus*	39	C	68	22	1	NA
Kreisinger et al. 2015	*A. flavicollis*	15	GI	67	27	4	>1
Debebe et al. 2017	*H. glaber*	35	F	40.8	38.8	2.6	2.7

Previously, Sibai et al. [44] examined faecal and skin microbiomes of AMBR sourced from geographically close populations 140 km SW of Ağlı and Taşköprü (Gerede, Bolu province). Regardless of the distance between sampling locations, our samples belong to the "Kastamonu" (2N=60) and the "Taşköprü" (2N=58) cytotypes, while they used another chromosomal race of *N. xanthodon* named "Abant" cytotype (2N=52) by [22]. In addition, the animals used in Sibai et al. [44] were housed in captivity for several weeks prior to metabarcoding, and even then showed a progressive temporal change of microbiome composition in a series of samples taken over the course of 1-2 months. In our study, the sampling of gut content was performed on the same day the animals were captured, therefore our results represent a snapshot of the actual microbiome composition in the wild. A comparison of our results with the above-mentioned study showed that the three main bacterial phyla (Firmicutes, Bacteriodota, and Desulfobacterota) were always the most abundant. Even though Sibai et al. used the same species (*N. xanthodon*) with different chromosomal races, Bacteriodota was more abundant in their study compared to Firmicutes. Rather than a different evolutionary history of the hosts, different sampling seasons may also explain the differences in the relative abundance of the phyla. Another study of closely

related Blind Molerat species (*N. ehrenbergi*) showed the exact same order of the most abundant phyla, while the relative abundances of the phyla are slightly different [45] (Table 3). The same three bacterial phyla dominate the microbiota of other rodents, such as house mice [17,46], rats [47], and naked molerats [18,48].

At the family level, Muribaculaceae (phylum Bacteriodota) and Lachnospiraceae (phylum Firmicutes) were the most common bacterial families in all samples, 35% and 28% respectively (Figure 3B). While bacteria from the Lachnospiraceae family have numerous functions, they share a few common roles in the maintenance of gut health, act as active degraders of plant material in the gut [49], and take a role in butyrate production [50]. The Muribaculaceae family contributes to propionate, succinate, and acetate production [51,52]. Interestingly, the possible role of the Muribaculaceae family on extended life span was discussed by [44]. It should be noted, however, that Muribaculaceae also has a high abundance in short-living rodents [47,53,54] and other (possibly long-living) members of the family Spalacidae [55]. The association of this family with the host longevity thus deserves a more thorough and focused study.

The bacterial phyla Oscillospiraceae (phylum Firmicutes) and Ruminococcus (phyla Firmicutes) were the third and fourth most abundant phyla with 12% and 7% abundance, respectively. While [44] reported that these bacterial families comprised >5% of their data too (Oscillospiraceae used to categorise under Ruminococcus), [45] did not mention the abundance of bacterial families in their study. Together with the second most abundant bacterial family Lachnospiraceae, Oscillospiraceae, and Ruminococcus were found to be abundant in performance-associated hosts such as human athletes and racehorses [56,57]. Overall, the ABMR caecum microbiome is comparable to that of several other terrestrial rodents (Table 3).

A deeper investigation of the multiple functions of ABMR microbiome could provide better insight into its role in the extreme physiology of this unique animal species.

In conclusion, this study investigated the gut microbiome of ABMRs in two populations from the edge of its distribution in Northern Anatolia. The results revealed that the ABMR gut microbiota is dominated by Firmicutes, Bacteroidota, and Desulfobacterota, which aligns well with previous studies on closely related species and other rodent species, albeit with slight differences in the relative abundances of bacterial taxa. Alpha diversity analysis indicated that the microbiota of the Ağlı population is more diverse than that of Taşköprü. However, the differences in microbiota composition between populations were only marginally significant, possibly due to the small sample size. Therefore, further research with larger sample sizes and consideration of environmental factors is necessary to gain a better understanding of the factors influencing the variations in the ABMR gut microbiome. The findings of this study contribute to the knowledge of within-species variation of microbiota and underscore the

importance of using wild animal models to study the ecological significance of the gut microbiome.

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Author Contribution Statements HMS, AY, and ES designed the study, and HMS drafted the manuscript. FÇ and AY performed the sampling. HMS, JK, DC, and OÇ performed laboratory analyses and HMS, JK, and DC performed data analyses. AY, ES, JK, and DC secured the funding. All authors provided helpful comments and recommendations and approved the final version of the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest.

Data availability The bacteriome 16S rRNA dataset is available at the European Nucleotide Archive under the accession number of the study PRJEB61312 and the accession numbers for the samples are ERS14903192-ERS14903197.

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