



## Analysis of Gut Microbiota and Assessment of Environmental Health in Western Anatolian Vole (*Microtus lydius* Blackler, 1916)

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**Abstract:** The significant risk posed by zoonotic pathogens to humans increases the need to better understand how these pathogens are maintained and transmitted within ecosystems. *Microtus* (voles), one of the important members of wildlife, are widely distributed in agricultural fields and can directly interact with humans through farmers or agricultural products. Microbiological studies on this genus have identified certain pathogenic bacteria. However, the microbiota of free-living voles in nature also includes members with significant probiotic effects. In this study, the gut microbiota of *Microtus lydius*, a species widely distributed in Western Anatolia, was characterized for the first time through DNA isolation from fecal samples and the use of 16S rRNA next-generation sequencing technology. The detected bacterial groups were examined across all taxonomic categories. According to the microbial analysis results of the species, the dominant bacteria in the gut microbiota are probiotic in nature, reflecting the species' dietary characteristics. Pathogenic bacteria, on the other hand, are present at low abundance and contribute to species diversity. The microbial records identified for *M. lydius* provide valuable insights for assessing the infectious risks of this species, which interacts with humans in the wild, as well as for understanding the probiotic health effects in social behavior models and contributing to phylogenetic research.

**Key words:** *Microtus*, wildlife, microbiota.

## Batı Anadolu Tarla Faresi (*Microtus lydius* Blackler, 1916)'nde Bağırsak Mikrobiyotası Analizi ve Çevresel Sağlık Değerlendirmesi

**Öz:** Zoonotik patojenlerin insanlar için önemli bir risk oluşturmaları, bu patojenlerin ekosistemlerde nasıl korunduğu ve bulaştığı hakkında daha fazla bilgi edinme gerekliliğini artırmaktadır. Yaban hayatının önemli üyelerinden biri olan *Microtus* (tarla fareleri), tarlalarda geniş bir yayılışa sahiptir ve doğrudan çiftçilerle veya tarım ürünleri aracılığıyla insanlarla etkileşime girebilmektedir. Bu cinsin mikrobiyolojik çalışmalarında bazı patojenik bakteriler tespit edilmiştir. Bununla birlikte, doğada serbest yaşayan tarla farelerinin mikrobiyotası, önemli probiyotik etkilere sahip üyeler de içermektedir. Bu çalışmada Batı Anadolu'da geniş bir yayılışa sahip *Microtus lydius* türünün bağırsak mikrobiyotası, dışkı örneklerinden DNA izolasyonu yapılarak ve 16S rRNA yeni nesil dizileme tekniği kullanılarak ilk kez karakterize edilmiştir. Tespit edilen bakteri grupları, tüm taksonomik kategorilerde incelenmiştir. Türün mikrobiyal analiz sonuçlarına göre, bağırsak mikrobiyotasındaki baskın bakteriler, türün beslenme özelliklerini yansıtacak şekilde ve probiyotik niteliktedir. Patojenik özellikteki bakteriler ise düşük yoğunlukta ve tür çeşitliliğine katkıda bulunmaktadır. Araştırma verileri, yaban hayatında insanlarla etkileşime giren bu türün enfeksiyöz risk değerlendirmesi, sosyal davranış modellerindeki probiyotik sağlık etkileri ve filogenetik araştırmalar için önemli bir katkı sağlayacak niteliktedir.

**Anahtar Kelimeler:** *Microtus*, yaban hayatı, mikrobiyota.

### INTRODUCTION

The mammalian gastrointestinal system, which harbors the most significant microbial biodiversity on Earth, hosts over 100 trillion microorganisms (Partrick et al., 2018). Any alteration in the composition of this microbial community termed the gut microbiota, can lead

to diseases such as inflammatory bowel disease, allergies, autoimmune disorders, diabetes, mental disorders, and cancer (Bhat & Kapila, 2017). The development of the gut microbiota is regulated by a complex interplay between host and environmental factors, including diet and lifestyle (Rothschild et al., 2018). Depending on dietary habits (frugivore, herbivore, folivore, carnivore, omnivore),

mammalian microbiota hosts a broad microbial community across different animal species (Flemer et al., 2017). While viruses, fungi, and protozoa are present in the gut microbiota, bacteria dominate over other organisms (Valdes et al., 2018). According to microbial sequence data from humans and rats, Firmicutes and Bacteroidetes (93%) are similarly dominant in both (Lleala et al., 2019). However, the abundance and diversity of gut microorganisms vary depending on host species and their environment (Anwar et al., 2019). Most mammalian microbiota remains strongly associated with host phylogeny despite significant dietary changes (Amato et al., 2019). Comparative microbiota studies across animal species suggest that while gut flora initially and continuously forms through bacteria acquired from external sources, it is phylogenetically conserved over evolutionary timescales (Ley et al., 2008). Recent microbiota research supports the hypothesis that species-specific gut microbiota composition results in greater similarity among members of the same species than between different species, reflecting host phylogeny (Lombardo, 2008; Ochman et al., 2010; Nelson et al., 2013). However, the proposed link between gut microbiota changes and host phylogeny, based on studies in select animal species, lacks specificity at the bacterial species/subspecies level.

Unlike culture-based methods, culture-independent molecular techniques such as next-generation 16S rRNA metagenomic sequencing (16S NGS) offer high specificity and sensitivity, enabling detailed and precise identification of unculturable organisms, including pathogens (Liu et al., 2022). Although each mammalian species harbors a distinct gut microbiota, their contribution to mammalian evolution and diversity remains largely unexplored. Gut microbiota studies on humans and domestic animals have recently expanded to include wildlife species, aiming to understand a critical component of their bioecology (Pereira & Cunha, 2020). However, research characterizing the bacterial diversity of gut microbiota in *Microtus* species remains limited (He et al., 2020; Yang et al., 2020). In Turkey, 15 *Microtus* species are distributed: *M. socialis*, *M. obscurus*, *M. lydius*, *M. guentheri*, *M. majori*, *M. subterraneus*, *M. gud*, *M. nivalis*, *M. irani*, *M. roberti*, *M. dogramacii*, *M. anatolicus*, *M. daghestanicus*, *M. rossiaemeridionalis* (*levis*), and *M. schidlovski* (Aria, 2011). The species *Microtus lydius* has been recorded in Bilecik province, bordering Kütahya and Eskişehir in Western Anatolia (Yağcı, 2019). Species of the genus *Microtus* typically nest in uncultivated edge strips of sloping agricultural fields, fallow lands, areas with dense gramineous vegetation, and zones adjacent to agricultural fields along roadsides (Yavuz et al., 2011).

Zoonoses emerging from wildlife populations pose a significant and growing threat to human populations. Rodents, which harbor the highest number of zoonotic pathogens, frequently exhibit co-infections (Herrero-Cófreces, 2021). To prevent zoonotic diseases, it is critical to first identify the source of the pathogen or infection. Among the zoonotic pathogens associated with the genus *Microtus*, the most prominent include *Leptospira*, *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Francisella*, and *Rickettsia* (Rodríguez-Pastor et al., 2019). Members of the genus *Microtus* (voles), a key component of wildlife, are widely distributed in agricultural fields, leading to direct interactions with farmers or indirect contact via crops. Recent studies highlight that future research on probiotics in *Microtus* species could advance our understanding of gut microbiota integration into gut-brain communication and contribute to novel therapeutic approaches for human disorders (Nuccio et al., 2023).

In this study, the gut microbiota of *M. lydius* (Blackler, 1916) distributed in Bilecik province was characterized using next-generation sequencing and metagenomic approaches. Based on metagenomic analyses, the microbiota of *Microtus* was comprehensively profiled to reveal variability in specific probiotic/commensal and pathogenic bacteria, as well as the effects of species-specific and dietary traits on gut microbial composition. Additionally, a species-specific microbial record was established to enable global comparisons of gut microbiota across *Microtus* species worldwide.

## MATERIAL AND METHOD

**Collection of *Microtus* Fecal Samples:** *Microtus lydius* specimens (7♂) widely distributed in agricultural fields and gardens of the Western Anatolia Region (Bilecik province) were collected using live traps (Figure 1). Animals collected from their habitats were transported to the laboratory on the day of capture. Following a 4-hour fasting period, fecal samples were collected from each individual under aseptic conditions using sterile swabs. The samples were immediately flash-frozen in liquid nitrogen and stored in sterile tubes at -80°C until further processing (Flemer et al., 2017). No experimental procedures were performed on the animals, and they were released back into their natural habitat following sample collection (General Directorate of Nature Conservation and National Parks, Permit No.: E-21264211-288.04-5841683).

**Microbial DNA Isolation from Feces:** Fecal samples from each animal were pooled for DNA isolation. For each pool, DNA was extracted using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Catalog No.: D6010; Zymo Research, USA) according to the

manufacturer's protocol. The quantity and purity of the isolated DNA were fluorometrically assessed using a Qubit Fluorometer (Invitrogen, USA). To optimize performance, 0.5% (v/v)  $\beta$ -mercaptoethanol was added to the final dilution of the genomic lysis buffer. Using this technique, a general microbiota profile characteristic of the species was established; however, due to sample pooling, individual variations could not be assessed. Due to the small sample size and the pooling of samples, statistical analyses could not be performed, as individual-level data necessary for meaningful comparisons were not available.



**Figure 1.** Sampling site of *Microtus lydius* in Bilecik (40°12.23'N, 29°57.80'E), with habitat and representative image of the species.

**Library Preparation:** The V3-V4 hypervariable region of the 16S rRNA gene was amplified using region-specific primers, followed by purification. During the index PCR step, Illumina dual indexes and adapters were added using the Nextera XT Index Kit (Illumina, USA), and the product was purified. The concentration of the generated libraries was measured via real-time PCR, normalized by dilution to 4 nM, and pooled for sequencing.

**Amplicon Sequencing:** After library preparation, sequencing-by-synthesis (SBS) was performed. Fluorescent signals emitted during the incorporation of each dNTP were optically detected and recorded.

**Raw Data Processing:** Post-sequencing data were converted into raw sequence files (FASTA format) for downstream analysis. The metagenomic data have been deposited in NCBI under the bioproject accession number PRJNA1281174.

Raw sequencing data in FastQ format were processed through the following steps:

- **Quality Control:** Overall sequence quality was assessed using *FastQC*. Additionally, read quality profiles were examined via *QIIME2* to guide downstream filtering steps.
- **Chimera Detection:** Chimeric (artificial) sequences were identified using the *DADA2* algorithm.
- **Filtering:** Reads with Phred scores below 20, as well as barcode and primer regions, were removed. Chimeric reads detected by *DADA2* were also excluded from further analysis.

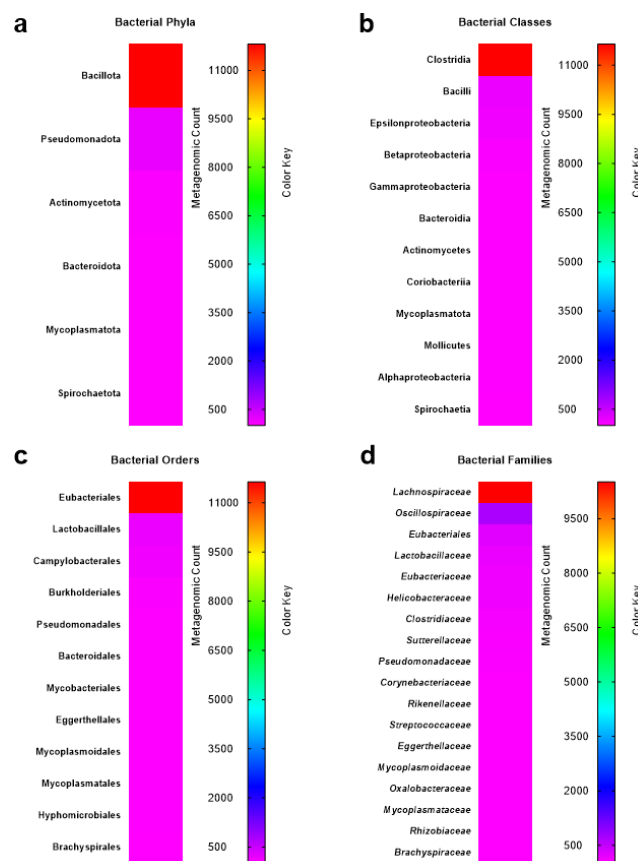
- **Taxonomic Assignment:** The taxonomic composition of each sample was determined using *QIIME2* based on reference databases.

- **Diversity Analysis:** Alpha and beta diversity metrics were calculated to assess microbial community structure. Rarefaction analysis was also performed to normalize sequencing depth across samples.

**Statistical Analysis:** Heatmap analysis of metagenomic bacterial counts was conducted using GraphPad Prism 8 (GraphPad Software, USA).

## RESULTS

The bacterial community constituting the microbiota of pooled fecal samples from *Microtus lydius* (7♂), widely distributed in Bilecik province, was analyzed across taxonomic units. At the phylum level, Bacillota was identified as the most abundant bacterial group, comprising 97.82% of the population (Figure 2a). At the class level, *Clostridia* dominated with 96.38%, while at the order level, *Eubacteriales* exhibited a high prevalence (Figure 2b, 2c). Among the 18 bacterial families detected at the family level, *Lachnospiraceae* formed the dominant population (87.02%), followed by *Oscillospiraceae* (5.67%) and *Eubacteriales* (2.02%). Other bacteria (5.28%), though present at lower proportions, significantly contributed to microbial diversity (Figure 2d).



**Figure 2.** Bacterial Distribution at Taxonomic Levels (a-Phylum, b-Class, c-Order, d-Family) in *Microtus lydius*.

Analysis of the genus-level microbiota composition in *M. lydius* samples revealed *Kineothrix* as the dominant genus (67.33%), followed by *Roseburia* (8.53%), *Variimorphobacter* (6.48%), and *Lawsonibacter* (4.79%). Although bacteria detected at 0–2% levels were present in lower proportions, they significantly enhanced population diversity (Figure 3).

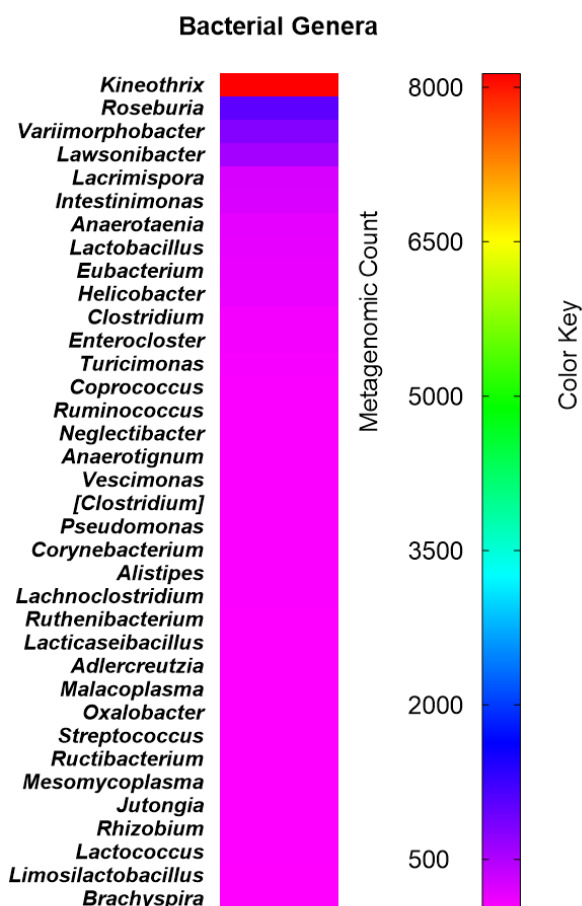


Figure 3. Bacterial Distribution at the Genus Level in *Microtus lydius*.

## DISCUSSION AND CONCLUSION

Rodents are the most prevalent among mammalian wildlife populations and carry numerous potential pathogens and zoonotic agents that can threaten humans (Herrero-Cófreces, 2021). For example, due to their close interactions with humans and domestic animals, wild rats have been reported to harbor opportunistic and pathogenic bacteria that can severely threaten public health (Shah et al., 2023). Meanwhile, voles (*Microtus* sp.), which are rodents closely associated with humans in agricultural areas, are considered agricultural pests due to their consumption of crop leaves, roots, and stems, despite providing benefits such as soil aeration and fertilization (Jacop et al., 2013). Studies on *Microtus* species (e.g., *Microtus agrestis*, *Microtus oeconomus*) have identified

clinically dangerous bacterial families, such as *Mycoplasmataceae*, *Bartonellaceae*, *Anaplasmataceae*, and *Francisellaceae*, in liver samples, also reflecting their gut flora. Dominant bacterial families contributing to this diversity include *Anaplasmataceae* (9%), *Lachnospiraceae* (7%), *Ruminococcaceae* (6%), *Porphyromonadaceae* (6%), and *Mycoplasmataceae* (4%) (Koskela et al., 2017).

In this study, *Lachnospiraceae* constituted the majority (87.02%) of bacterial diversity, while *Mycoplasmataceae* accounted for less than 2%, with the remaining 5.28% contributing to total microbial diversity. This suggests that species differences may act as a distinguishing factor in microbial diversity. Phylogenetic studies have highlighted the role of host genetics in shaping microbiota composition, showing that microbiota profiles are species-specific and that individuals of the same species share greater similarities than those of different species (Knowles et al., 2019). Species-specific research on the gastrointestinal microbiota of voles remains limited. Furthermore, based on our literature review, no data exist on the fecal microbiota of *Microtus lydius*. Curtis et al. (2018) characterized the fecal microbiota of female prairie voles (*Microtus ochrogaster*) using 16S rRNA gene sequencing. *Ruminococcaceae* and *Lachnospiraceae* were reported as the most abundant families in the gut microbiota of wild voles. Other dominant bacterial groups, such as *Allobaculum* species and members of *Clostridiales*, were identified as major phylotypes in the vole microbiota. The dominant fecal OTUs (operational taxonomic units) detected in all voles aligned with their dietary preferences. Researchers noted that the absence of clinically significant pathogens, previously identified in liver samples from wild animals, might relate to the sample type. Despite studying wild *Microtus* species, this study did not find any significant pathogenic bacteria in their gut.

At the phylum level, *Bacillota*; class level, *Clostridia*; order level, *Eubacteriales*; family level, *Lachnospiraceae*; and genus level, *Kineothrix* were the most dominant phylotypes. At the species level, *Kineothrix alysoides*, *Roseburia intestinalis* L1-82, *Variimorphobacter saccharofermentans*, and *Lawsonibacter asaccharolyticus* represented significant contributors to diversity. *K. alysoides* has shown potential in treating metabolic dysfunction-associated steatotic liver disease (MASLD) in mice fed high-fat diets (Choi et al., 2023). Increased abundance of *R. intestinalis*, linked to whole-grain diets, has been shown to improve interleukin-6 (IL-6) concentrations associated with metabolic dysfunction (Nie et al., 2021). *V. saccharofermentans*, a sugar-fermenting, anaerobic, spore-forming member of *Lachnospiraceae*, and *L. asaccharolyticus*, a recently identified butyrate-producing species, were also prominent



in the microbiota of *M. lydius*. It was reported that probiotic supplementation during *Toxoplasma gondii* infection significantly altered gut microbiota, increasing the relative abundance of *L. asaccharolyticus* (Lee et al., 2024).

In this study, the bacterial diversity of *M. lydius*, analyzed across taxonomic levels, did not reveal bacteria directly linked to human infectious diseases. However, some low-abundance bacteria detected in the diversity profile are pathogenic. For instance, *Brachyspira* infection causes cell membrane disruption, glycocalyx defects, and mitochondrial swelling involving macrophages and phagolysosomes (Tidwell et al., 2024). *Streptococcus* species produce toxins that impair immune responses and disrupt host physiology (Barnett et al., 2015). *Ruthenibacterium*, observed in COVID-19 patients with reduced immune cells and hypoxemia (Kovtun et al., 2022), and *Helicobacter pylori*, a recognized carcinogen colonizing over half the global population (Kusters et al., 2006), were also noted. Alterations in gut microbiota composition, termed dysbiosis, can lead to inflammatory bowel disease, allergies, autoimmune disorders, diabetes, mental disorders, and cancer (Bhat & Kapila, 2017). Although the bacterial populations identified in *M. lydius* gut microbiota are not currently viewed as infectious agents, shifts in their composition could pose risks. Thus, this study underscores the importance of periodically monitoring species-specific microbiota profiles in wildlife to preempt potential disease outbreaks.

The dominant bacteria constituting the gut microbiota of *M. lydius* are those with protective and therapeutic properties for intestinal health. These animals typically inhabit open areas such as meadows and pastures, and their diet consists of grasses, seeds, leaves, stems, and roots (Jacob et al., 2014). The composition of their gut microbiota confirms that it originates from their pollution-free natural habitats and plant-based diet. Microbial studies on wild small mammals in urban and rural areas emphasize that habitat differences, driven by pollution or access to varied food sources, significantly alter gut microbiota (Gurbanov et al., 2022; Kauer et al., 2024).

Research on the microbiota of *M. lydius* highlights their value for advancing microbiota-gut-brain-behavior studies due to their highly social nature. Using 16S rRNA gene sequencing and molecular strain typing, thirty *Lactobacillus* strains isolated from the intestines of prairie voles (*Microtus ochrogaster*) demonstrated antibacterial and antifungal properties, strong adhesion capabilities, resistance to bile salts, and low pH. These strains, which also show potential for inorganic mercury detoxification, represent a promising probiotic model for alleviating disorders involving social withdrawal symptoms (Assefa et al., 2015). In this study, members of the order *Lactobacillales*, specifically *Lactobacillus rodentium*,

*Lacticaseibacillus paracasei*, and *Lactococcus cremoris*, were identified as the second most abundant taxa at the order level. These findings, which characterize the gut bacterial composition of *M. lydius*, lay a critical foundation for advancing future research into the interaction between gut microbiota and the social brain, a pivotal area in understanding microbiota-gut-brain axis dynamics. This study is the first to characterize the fecal microbiota of *M. lydius* using next-generation sequencing (NGS) technology. The current study was conducted exclusively on male individuals. However, due to the challenges of capturing wild specimens from natural habitats, only males could be included, and the limited sample size prevented the assessment of sex- and individual-level variations in microbiota composition. Despite these limitations, dominant bacterial phylotypes were identified in the fecal microbiota of *M. lydius*, and the findings are consistent with previous microbial studies demonstrating relationships between host identity, habitat, and diet. (Jacob et al., 2014). Although sex has clear effects on physiology and behavior, several studies have reported that sex-based differences in gut microbiota composition are difficult to detect. Some researchers have suggested that sex has little to no effect on microbiota composition (Org et al., 2016). Nevertheless, broader studies including both sexes are needed for a more comprehensive and accurate characterization of a species microbial structure. Despite these constraints, this research is anticipated to contribute to zoological and public health studies in Türkiye by advancing current knowledge on the biology and ecology of *Microtus* species and human-animal-environment interactions. Moreover, it provides complementary data for the screening and monitoring of wildlife-associated pathogens that may pose risks to global public health.

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