Metabarcoding of bacteria and parasites in the gut of *Apodemus agrarius*

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Metabarcoding of bacteria and parasites in the gut of *Apodemus agrarius*

Directed by Professor Tai-Soon Yong

The Master's Thesis
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This certifies that the Master's Thesis of
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This thesis is dedicated to my mom. ☺

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ABSTRACT

Metabarcoding of bacteria and parasites in the gut of *Apodemus agrarius*

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Apodemus agrarius is a wild rodent found in fields in Korea, and it is known to carry various pathogens. To analyze the bacterial microbiome, amplicon next-generation sequencing (NGS) targeting the 16S rRNA gene is most widely used. Although many bacterial microbiome analyses have been attempted using feces of wild animals, there are still few studies have used NGS to screen for parasites. This study aimed to rapidly detect bacterial, fungal, and parasitic pathogens in the guts of wild mice using NGS-based metabarcoding analysis. We conducted 18S/16S rDNA-targeted high-throughput sequencing was conducted on cecal samples from *A. agrarius* (n = 48) collected in May and October, 2017. Taxa of protozoa, fungi, helminths, and bacteria in the cecal content were then identified. Of the protozoa, *Tritrichomonas* sp. was found in all the cecal samples, followed by *Monocercomonas* sp. (95.8%; 46/48 of samples) and *Giardia* sp. (75%; 36/48).

For helminths, *Heligmosomoides* sp. was found in 85.4% (41/48) of samples, followed by *Hymenolepis* sp. (10.4%; 5/48) and *Syphacia* sp. (25%; 12/48). In 16S rRNA gene analysis, the microbial composition changed by season (p = 0.005). Linear discriminant analysis effect size analysis showed that *Escherichia coli* and *Lactobacillus murinus* were more abundant and that *Helicobacter rodentium* was less abundant in the mice collected in spring. *Helicobacter japonicus* was more abundant and *Prevotella*_uc was less abundant in males. Finally, microbial composition changed based on the *Heligmosomoides* sp. infection status (p = 0.019). Specifically, *Lactobacillus gasseri* and *Lactobacillus intestinalis* were more abundant in the *Heligmosomoides* sp.-positive group than in the negative group.

This study demonstrated that bacterial abundance changed based on the season, specific parasitic infection status of collected mice. These results highlight the advantages of NGS technology in monitoring zoonotic disease reservoirs.

Key words : Metabarcoding, *Apodemus agrarius*, Parasite, Microbiome

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I. INTRODUCTION

Zoonotic diseases are commonly transmitted by wild animals around the world and can spread rapidly [1, 2]. Zoonotic diseases are caused by pathogenic organisms such as bacteria, viruses, protozoa, and parasites. The emergence of novel zoonoses is generally unpredictable [3], therefore, it is necessary to develop a preemptive pathogen screening method for the surveillance of infected animals.

The striped field mouse, *Apodemus agrarius*, is a common type of wild rodent in the Republic of Korea [4]. This wild rodent can spread a great number of infectious bacteria and parasites through its feces [1, 5]. Furthermore, rodents live in various types of habitats, including agricultural regions and man-made environments [3], and have high reproductive rates, which is a well-known characteristic of r-selected species. Owing to these specific characteristics, wild rodents are considered one of the most dangerous reservoirs of infectious organisms among wild animals.

Previously, molecular, serological, and microscopic methods such as polymerase chain reaction (PCR) and ELISA have been used to detect pathogens in wild rodent samples. For example, *Orientia tsutsugamushi*, *Anaplasma phagocytophilum*, and *Leptospira interrogans* were identified in the spleens and the blood of striped field mice [6]. In addition,

zoonotic helminths such as *Hymenolepis diminuta* were identified using light microscopy [7-10]. However, those conventional methods have limitations when screening a large number of samples of a variety of pathogens.

Recently, studies have analyzed the microbiomes of feces of wild animals using next-generation sequencing (NGS) for a more integrated and rapid screening approach [11-13]. To analyze the bacterial microbiome, amplicon NGS targeting the 16S rRNA gene is most widely used [14]. The NGS technique can be applied when detecting veiled pathogens because of its untargeted nature and ability to investigate non-culturable organisms [15]. Although many bacterial microbiome analyses have been attempted using the feces of wild animals [16], only a few studies have used NGS to screen for parasites. [17-19]. Therefore, we decided to use NGS to detect various kinds of pathogens.

In this study, we used 18S rRNA gene amplicon NGS to screen fungi and parasites and 16S rRNA gene amplicon NGS to screen bacteria in the gut of *A. agrarius* for the first time in Korea, to the best of our knowledge. Some studies have revealed the interaction between parasites and host microbiota [20-23]. Interestingly, some parasites need host microbiota alterations to promote the successful survival and control of parasite numbers [24, 25]. In another research, the host microbiota is a resistance factor for parasitic infection [26]. Hence, we compared differences in the microbial composition of hosts based on their parasite infection status. In addition, seasonal variation could affect the supplementation of food to host; this results in microbial differences and affects the chance of infection of a parasite [27, 28]. Thus, we further conducted comparisons of the microbial composition based on seasonality. Cecal contents were used because these contain assorted organisms, including pathogens, and are appropriate for analyzing the interaction between parasites and the bacterial microbiome.

II. MATERIALS AND METHODS

1. Sample collection

In total, 48 *A. agrarius* animals were captured using Sherman Live Traps (H. B. Sherman Traps, Inc., Tallahassee, FL, USA) from Gangneung and Wonju, Gangwon-do, Korea in May and October, 2017. Information of wild rodents used in this study is included in Supplementary Table 1. Traps were opened after 24 h and all mice were alive and euthanized on that day. All 48 rodents were euthanized using a CO_2 chamber and dissected to collect their ceca, which were immediately stored at −70 ℃. Six months after collection, the cecal contents were extracted from the cecal lumen using disposable sterile bacterial spreaders. Cecal DNA was extracted using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Carlsbad, USA) according to the manufacturer's protocol. The DNA samples were stored at −80 °C until needed.

Table 1. Date (season), location, sex, weight, and *Heligmosomoides* sp. infection status of 48 *Apodemus agrarius*

	Date	Location	Sex	Weight(g)	*Heligmosomoides* sp. infection
1	2017-05-19 (Spring)	N 37°72'94" E128°89'81"	M	31.65	Positive
2	2017-05-19 (Spring)	N 37°72'94" E128°89'81"	M	35.6	Positive
3	2017-05-19 (Spring)	N 37°72'94" E128°89'81"	M	23.7	Positive
4	2017-05-19 (Spring)	N 37°72'94" E128°89'81"	F	16.93	Positive
5	2017-05-19 (Spring)	N 37°72'94" E128°89'81"	F	19	Positive
6	2017-05-19 (Spring)	N 37°72'97" E 128°89'69"	M	32.24	Positive
7	2017-05-19 (Spring)	N 37°72'97" E 128°89'69"	F	42.23	Positive
8	2017-05-19 (Spring)	N 37°72'97" E 128°89'69"	F	24.89	Positive
9	2017-05-19 (Spring)	N 37°72'97" E 128°89'69"	F	24.36	Positive
10	2017-05-19 (Spring)	N 37°68'79" E128°91'11"	M	38.95	Positive
11	2017-05-19 (Spring)	N 37°68'79" E128°91'11"	M	27.68	Positive
12	2017-05-19 (Spring)	N 37°72'97" E 128°89'69"	M	28.34	Positive
13	2017-05-19 (Spring)	N 37°68'99" E 128°91'41"	M	35.93	Positive
14	2017-05-19 (Spring)	N 37°68'99" E 128°91'41"	M	31.41	Negative
15	2017-05-19 (Spring)	N 37°68'99" E 128°91'41"	F	37	Positive
16	2017-05-19 (Spring)	N 37°68'99" E 128°91'41"	F	18.33	Positive
17	2017-05-19 (Spring)	N 37°68'99" E 128°91'41"	M	22.82	Negative
18	2017-05-19 (Spring)	N 37°72'97" E 128°89'69"	M	42	Positive
19	2017-05-15 (Spring)	N 37°26'94" E 127°90'41"	M	23.13	Positive
20	2017-05-15 (Spring)	N 37°26'94" E 127°90'41"	F	23.32	Negative
21	2017-05-15 (Spring)	N 37°26'94" E 127°90'41"	M	51.64	Negative
22	2017-05-15 (Spring)	N 37°26'94" E 127°90'41"	F	25.49	Negative
23	2017-05-15 (Spring)	N 37°26'94" E 127°90'41"	M	40.44	Negative
24	2017-05-15 (Spring)	N 37°26'94" E 127°90'41"	F	25.5	Positive
25	2017-05-15 (Spring)	N 37°26'94" E 127°90'41"	M	37.41	Positive
26	2017-10-17 (Fall)	N 37°68'79" E128°91'11"	M	22.87	Positive
27	2017-10-17 (Fall)	N 37°68'79" E128°91'11"	F	37.38	Positive

28	2017-10-17 (Fall)	N 37°68'79" E128°91'11"	M	51.54	Positive
29	2017-10-17 (Fall)	N 37°68'79" E128°91'11"	M	40.68	Positive
30	2017-10-17 (Fall)	N 37°68'79" E128°91'11"	F	43.81	Positive
31	2017-10-17 (Fall)	N 37°72'97" E 128°89'69"	M	16.8	Positive
32	2017-10-17 (Fall)	N 37°72'97" E 128°89'69"	F	22.63	Negative
33	2017-10-17 (Fall)	N 37°72'97" E 128°89'69"	F	37	Positive
34	2017-10-17 (Fall)	N 37°73'10" E 128°90'96"	F	50.82	Positive
35	2017-10-17 (Fall)	N 37°73'10" E 128°90'96"	F	19.87	Positive
36	2017-10-17 (Fall)	N 37°73'10" E 128°90'96"	M	50.85	Positive
37	2017-10-17 (Fall)	N 37°73'10" E 128°90'96"	F	16.82	Positive
38	2017-10-17 (Fall)	N 37°73'10" E 128°90'96"	F	48.37	Positive
39	2017-10-17 (Fall)	N 37°73'10" E 128°90'96"	F	40.97	Positive
40	2017-10-17 (Fall)	N 37°72'94" E128°89'81"	M	42.18	Positive
41	2017-10-17 (Fall)	N 37°72'94" E128°89'81"	F	17.6	Positive
42	2017-10-17 (Fall)	N 37°68'99" E 128°91'41"	F	40.74	Positive
43	2017-10-17 (Fall)	N 37°68'99" E 128°91'41"	F	20	Positive
44	2017-10-17 (Fall)	N 37°68'99" E 128°91'41"	M	37.5	Positive
45	2017-10-17 (Fall)	N 37°68'92" E 128°91'46"	F	28	Positive
46	2017-10-17 (Fall)	N 37°68'92" E 128°91'46"	F	16.7	Positive
47	2017-10-17 (Fall)	N 37°68'92" E 128°91'46"	M	19.13	Positive
48	2017-10-17 (Fall)	N 37°68'92" E 128°91'46"	M	19.81	Positive

2. Illumina iSeq sequencing

For the eukaryotic microbiome study, the 18S rRNA gene V9 region was amplified by PCR using the primers 1391f (5′-TCGTCGGCAGCGTCAGATG TGTATAAGAGACAG GTACACACCGCCCGTC-3′) and EukBr (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGATC CTTCTGCAGGTTCACCTAC-3′) [29]. For the bacterial microbiome study, the 16S rRNA gene V4 region was amplified by PCR using the primers 515F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTA AT-3′) [30]. KAPA HiFi HotStart ReadyMix (Roche Sequencing Solutions, Pleasanton, CA, USA) was used for PCR amplification. PCR amplification was performed as follows: 95 °C for 5 min; 25 cycles of 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min. AMPure XP (Beckman Coulter, Brea, CA, USA) was used for DNA purification. A limited-cycle (eight cycles) amplification step was performed to add multiplexing indices and Illumina sequencing adapters. Mixed amplicons were pooled and sequenced on an Illumina iSeq 100 sequencing system using the Illumina iSeq™ 100 i1 Reagent v2 kit (San Diego, CA, USA) according to the manufacturer's protocol.

3. iSeq100 data processing and bioinformatics

Geneious Prime® 2022.0.2 (Biomatters Ltd., Auckland, New Zealand) was used to process and assemble raw 18S V9 reads as previously described [31, 32]. Briefly, low-quality sequences (<Q25) were filtered using BBDuk (v38.84). The forward and reverse reads were merged to produce a single consensus sequence using BBMerge (v38.84) using

the ‘high rate’ setting. Sequences of 120 bp to 260 bp in length were sorted. The UCHIME algorithm was used to detect and remove chimeric sequences [33]. Closely related sequences were clustered into separate operational taxonomic units (OTUs) using de novo assembly and the default setting, which is a “Minimum Overlap Identity” of 98%. To create a sequence classifier database, the OTUs were aligned via sequence clustering using the Basic Local Alignment Search Tool (BLAST) and the NCBI “nt” GenBank database (November 2021 version). Then, the full sequences of BLAST hits from the NCBI were downloaded, and only the regions of the BLAST hits were extracted in order to create the sequence classifier database. The Geneious Sequence Classifier plugin was used to classify the merged amplicon dataset, using the created sequence classifier database. The ‘very high sensitivity/slow’ mode was used, with a minimum overlap of 90 bp. The sequences in the database that showed the highest homology were selected in the final taxonomic identification result [34].

Bacterial microbiome analysis of the 16S rRNA gene sequence data was performed using EzBioCloud, a commercially available ChunLab bioinformatics cloud platform for microbiome research (https://www.ezbiocloud.net/). Bioinformatic analyses were performed as previously described [35, 36]. Briefly, raw reads were quality checked, and low-quality (<Q25) reads were filtered using Trimmomatic 0.32 [37]. Paired-end sequence data were then merged using PandaSeq [38]. Primers were then trimmed using the ChunLab in-house program (ChunLab, Inc., Seoul, Korea), which applied a similarity cut-off of 0.8. Sequences were denoised using the Mothur pre-clustering program, which merges sequences and extracts unique sequences, allowing up to two differences between sequences [39]. The EzBioCloud database (https://www.ezbiocloud.net/) [36] was used for taxonomic assignment using BLAST 2.2.22, and pairwise alignments were generated for

similarity calculations [40, 41]. The UCHIME algorithm and non-chimeric 16S rRNA database from EzTaxon were used to detect chimeric sequences for reads with a best-hit similarity rate of <97% [33]. Ninety-seven percent similarity is generally used as the cutoff for species-level identification. Sequence data were then clustered using CD-Hit and UCLUST [42, 43]. All the following analyses were performed using EzBioCloud.

Rarefaction for the obtained OTUs was calculated using the ChunLab pipeline, in accordance with a previous protocol [44]. The reads were normalized to 8,000 for diversity analyses. We computed Shannon index [45] and performed principal coordinates analysis (PCoA) [46], permutational multivariate analysis of variance (PERMANOVA) [47], and permutational multivariate analysis of dispersion (PERMDISP) [48] based on the generalized Bray-Curtis distance. The PERMANOVA and PERMDISP tests were used to assess the differences in the microbial community structure based on factors including season and parasitic infection status. We used the Wilcoxon rank-sum test to test for differences in the number of OTUs and Shannon index to compare microbiome diversity between the groups divided by the season and parasite infection status. We used linear discriminant analysis effect size (LEfSe) analysis to identify significantly different taxa between the groups [49]. In addition, we used the theoretical framework of a previous study to investigate the impacts (synergistic, neutral, or antagonistic) of parasitic co-infection on bacterial diversity change when mice were infected by multiple parasites [50].

III. RESULTS

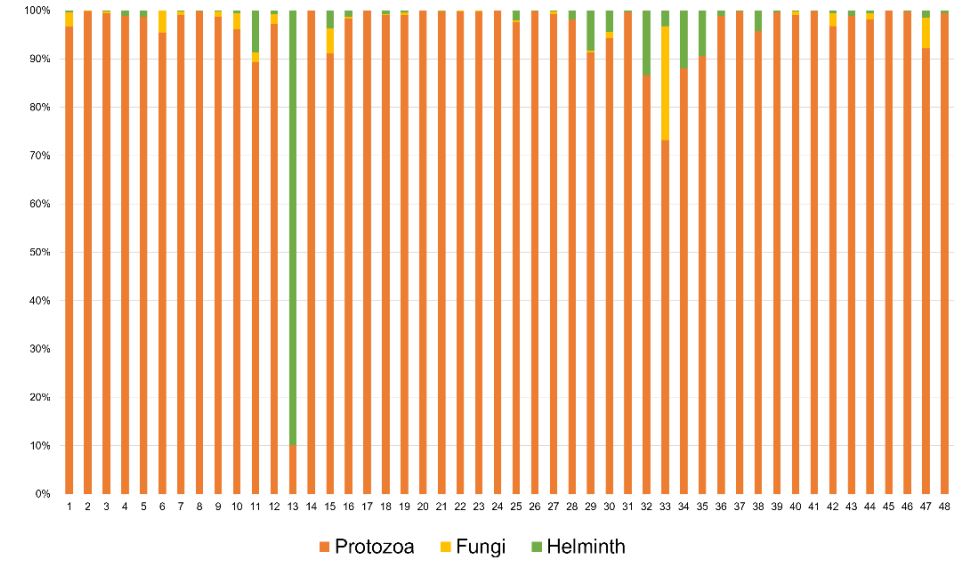
1. Eukaryotic organisms in the *A. agrarius* gut

The average number of assigned read counts was 34,957 ± 19,899 standard deviation (SD). The maximum number of reads was 88,107 and the minimum was 2,128. These reads included only protozoa, helminths, and fungi, as the host reads (average 221 ± 203 SD) were removed before analysis. The relative abundances of fungi, protozoa, and helminths in individual *A. agrarius* animals are shown in Fig. 1a. The relative abundances of protozoal taxa were greater than those of fungi and helminths in all of the *A. agrarius* samples except for one. All cecal samples were infected with *Tritrichomonas* sp. Furthermore, the prevalence of infection with *Monocercomonas* sp. (95.8%; 46/48) was second highest, followed by that of *Giardia* sp. (75%; 36/48, Fig. 1b). In addition, *Isospora* sp. were found in six samples, *Cryptosporidium* sp. were found in five samples, and *Blastocystis* sp. were found in one sample. Furthermore, *Entamoeba* sp., *Spironucleus* sp., and *Retortamonas* sp. were identified.

In this study, 27 of the 48 cecal samples were found to contain fungi, and *Kazachstania* sp. was the most dominant species (Fig. 1c). *Mucor* sp. were found in two samples and Candida sp., *Rhizopus* sp., *Cladosporium* sp., and *Periconia* sp. were found in one sample (Fig. 1c).

The relative abundance of helminth species in the cecal samples was as follows: *Heligmosomoides* sp., 85.4% (41/48); *Syphacia* sp., 25% (12/48); *Hymenolepis* sp., 10.4% (5/48); *Raillietina* sp., 8.3% (4/48); *Strongyloides* sp., 6.3% (3/48); *Plagiorchis* sp., 4.2% (2/48); *Oscheius* sp., 2.1% (1/48); *Panagrolaimus* sp., 2.1% (1/48) (Fig. 1d). Five of 23 mice were infected with *Hymenolepis* sp. in the fall, but there were no cases of *Hymenolepis* sp. infection in the spring.

a



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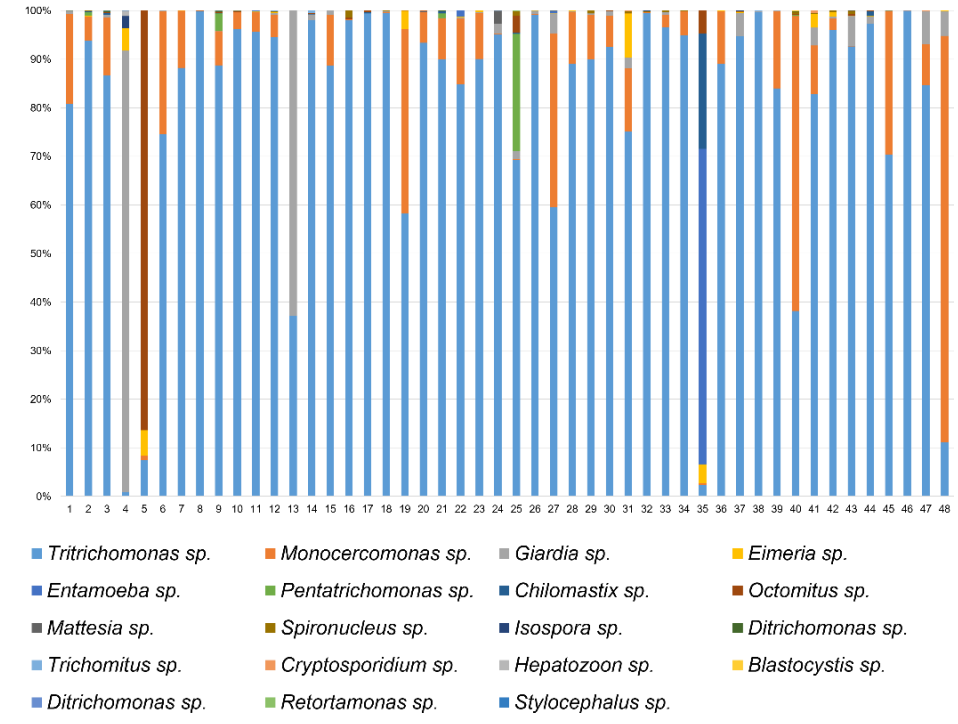


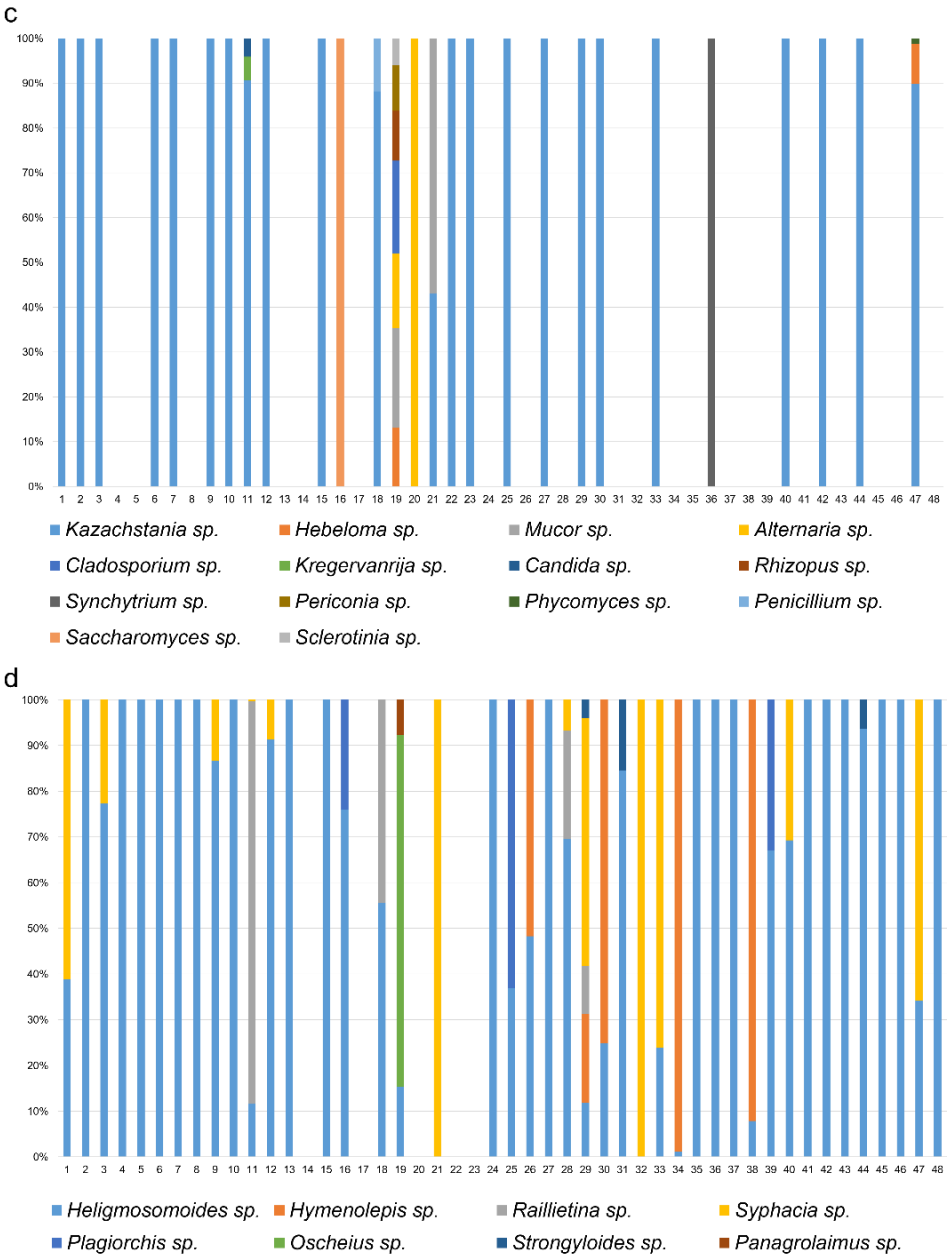


Figure 1. Composition of cecal eukaryotic organisms in *Apodemus agrarius* (n = 48). (a) Composition of taxa of protozoa, fungi, and helminths based on 18S rRNA. Taxa of (b) protozoa, (c) fungi, and (d) helminths at the species level for each sample (n = 48)

2. Bacteria in the *A. agrarius* gut

High-throughput sequencing of the 16S rRNA gene of 48 cecal content samples of *A. agrarius* using iSeq 100 produced an average number of assigned read counts of 27,697 ± 14,281 SD. The relative abundances of bacterial taxa in the cecal microbiomes of individual wild rodents are shown in Fig. 2. At the family level, all samples were dominated by the presence of Muribaculaceae (9.32–57.13% of the relative abundance, with an average of 26.71%). The second most abundant bacterial family was Lachnospiraceae (3.95–61.59% of the relative abundance, with an average of 16.83%), which was also detected in all samples. Bacterial OTUs at all the taxonomic levels are provided in Supplementary Table 2. *Helicobacter rodentium* and *Helicobacter aurati* were detected in 100% (48/48) and 72.9% (38/48) of samples, respectively. *Helicobacter fennelliae* was found in one sample.

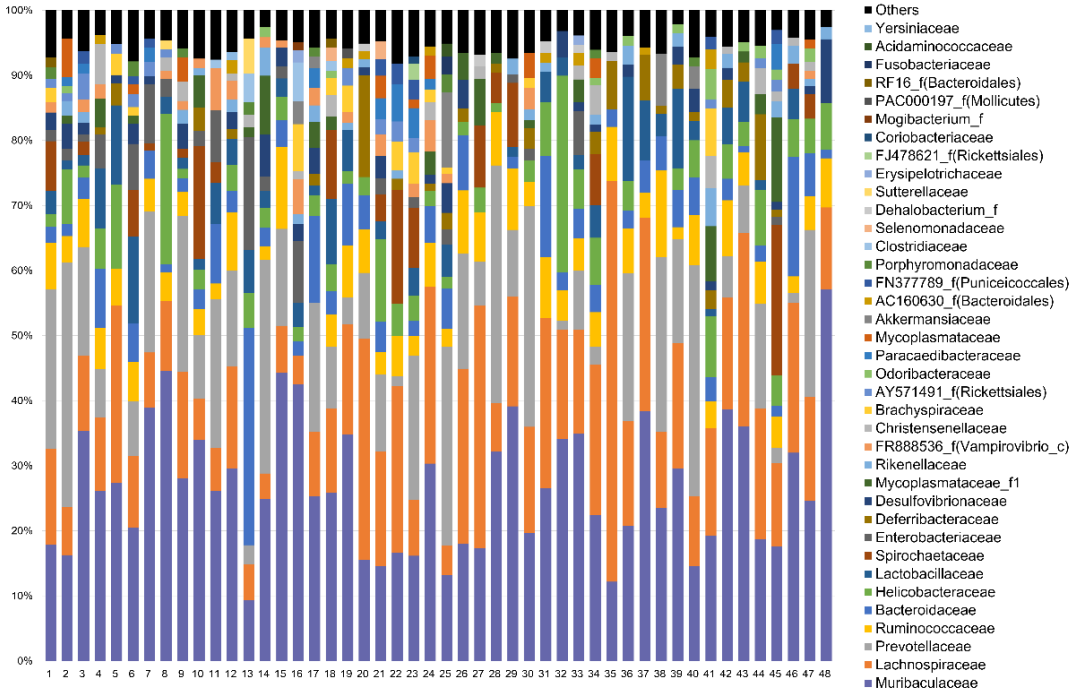
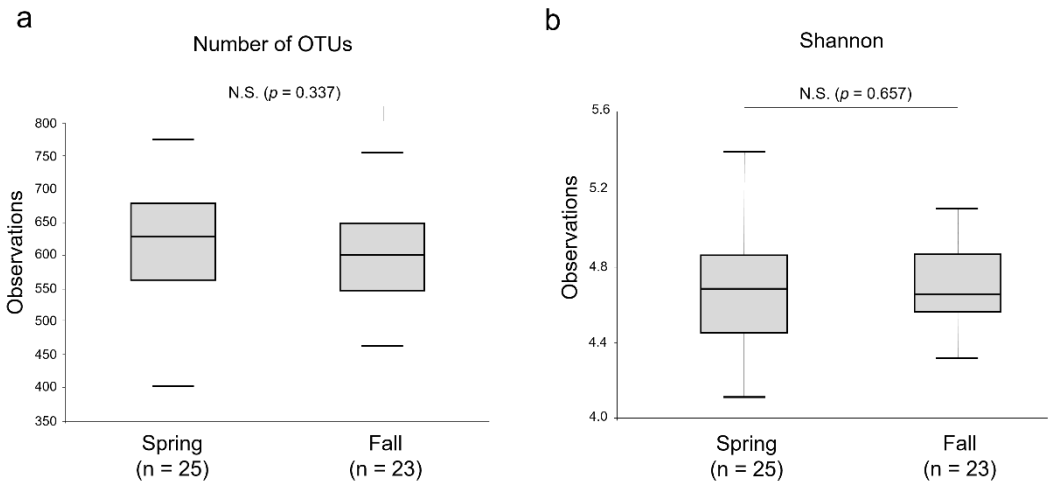
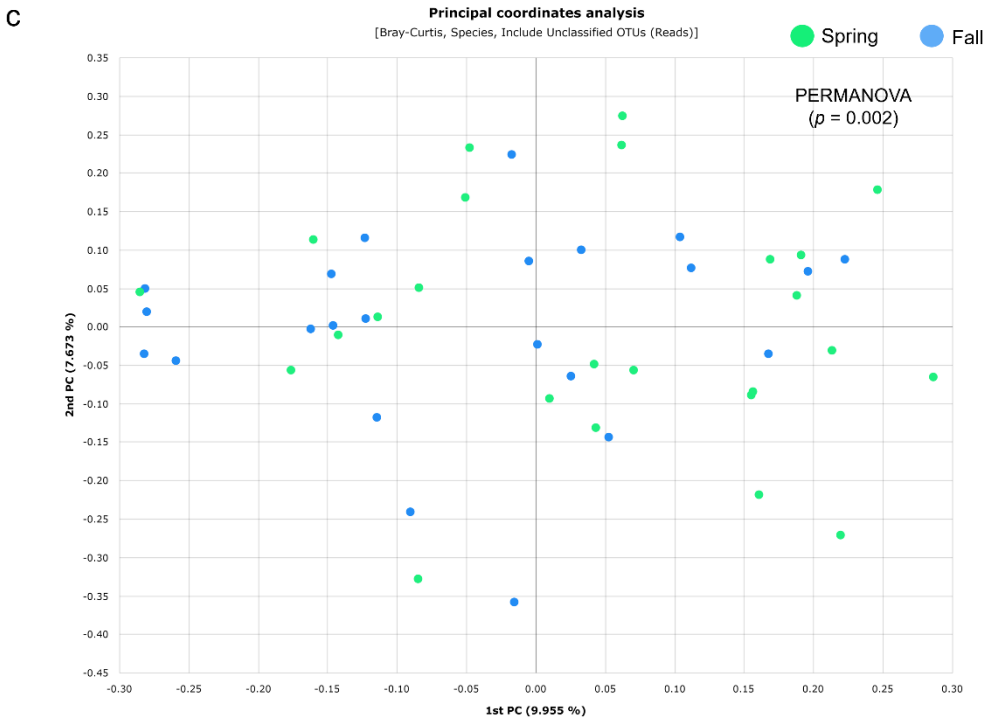


Figure 2. Relative abundance of bacterial taxa in the cecal microbiome of wild *Apodemus agrarius*. Abundance was determined at the family level for each sample (n=48).

3. Bacterial microbiome differences based on the season

The number of OTUs was not different between the mice caught in spring (n = 25, median = 941) and those caught in fall (n = 23, median = 808, p = 0.337, Fig. 3a). Shannon index was not different between the mice caught in spring (median = 4.70) and those caught in fall (median = 4.68, p = 0.657, Fig. 3b). However, PCoA and PERMANOVA showed that the gut bacterial composition of mice caught in the spring and fall was significantly different (PERMANOVA: F = 1.805, R2 = 0.042, p = 0.005, PERMDISP: F = 0.04, R2 = 0.0009, p = 0.83, Fig. 3c). *Escherichia coli* (LDA score = 4.057) and *Lactobacillus murinus* (LDA score = 3.529) were more abundant in the mice collected in the spring, but *Helicobacter rodentium* (LDA score = 3.773) was less abundant in mice collected in the spring than in those collected in the fall (Fig. 3d).





d

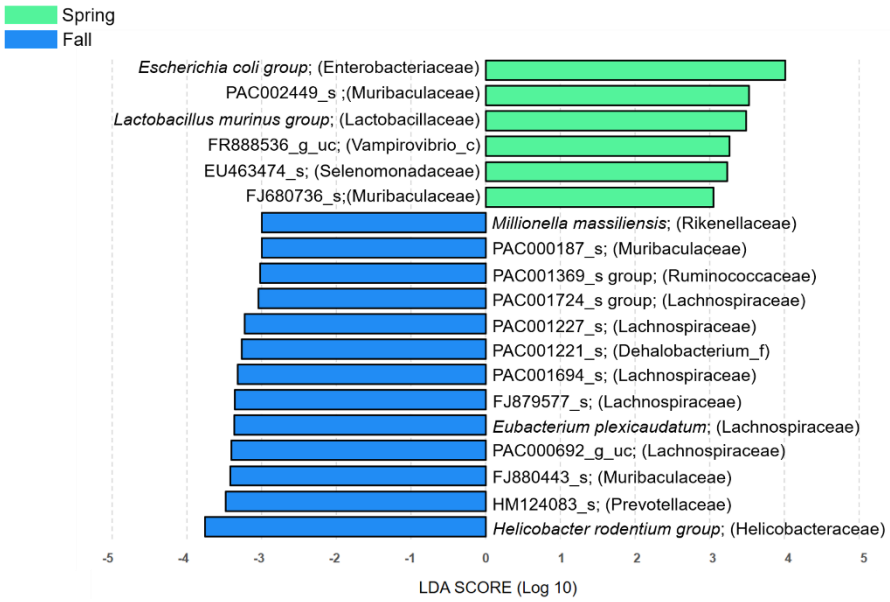
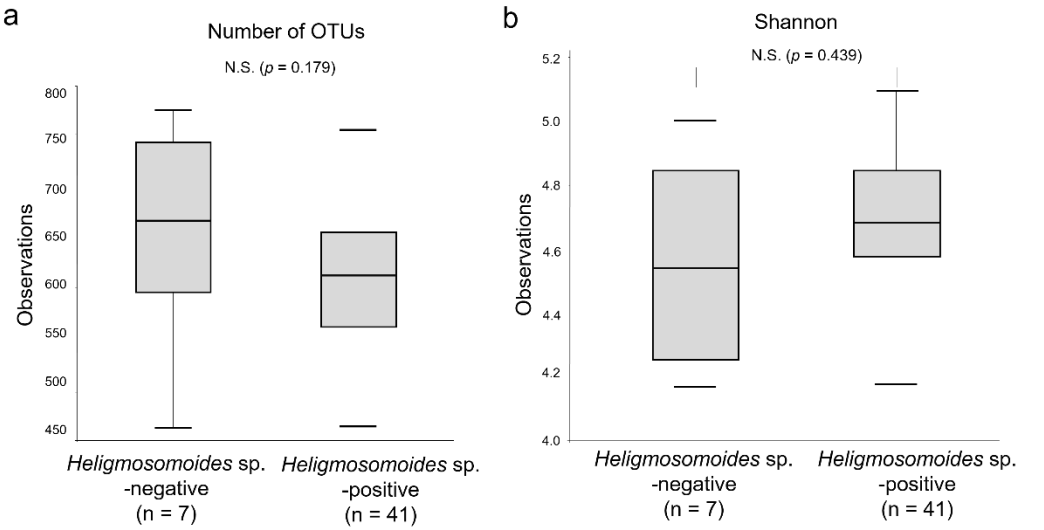
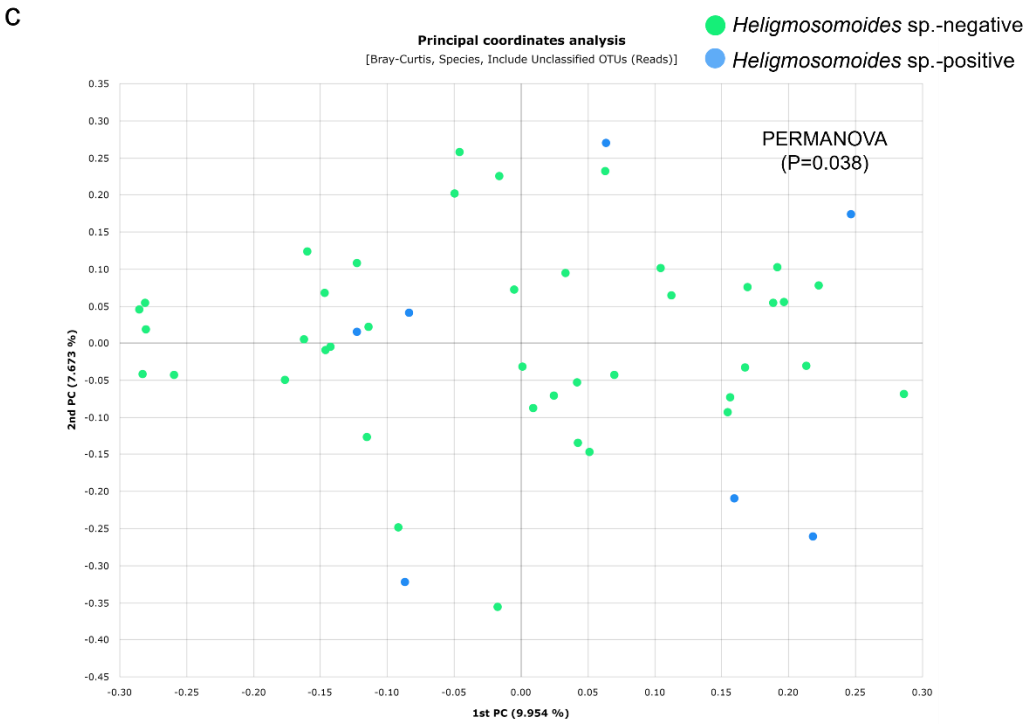


Figure 3. Alpha and beta diversities of the cecal microbiome of wild *Apodemus agrarius* by collection season. (a) Number of operational taxonomic units (OTUs) and (b) Shannon index between spring (n = 25) and fall groups (n = 23). (c) Principal coordinates analysis (PCoA) representing the cecal microbiome composition. (d) Linear discriminant analysis effect size analysis of differentially abundant bacterial taxa among the two groups. Only taxa meeting a significant (>3) linear discriminant analysis threshold are shown.

4. Bacterial microbiome differences based on parasitic infection status

No difference in the number of OTUs was found between *Heligmosomoides* sp.-infected (n = 41, median = 582) and uninfected (n = 7, median = 619) individuals (p = 0.179, Fig. 4a). Shannon index showed no difference between *Heligmosomoides* sp. infected (median = 4.68) and uninfected (median = 4.54) individuals (p = 0.439, Fig. 4b). PCoA and PERMANOVA showed that the gut bacterial composition was significantly different based on the *Heligmosomoides* sp. infection status (PERMANOVA: F = 1.408, R2 = 0.029, p = 0.019, PERMDISP: F = 0.822, R2 = 0.0176, p = 0.683, Fig. 4c). Interestingly, *Lactobacillus gasseri* (LDA score = 3.667) and *Lactobacillus intestinalis* (LDA score = 3.492) were more abundant in the *Heligmosomoides* sp.-positive group than that in the negative group (Fig. 4d). Then, we tested the impact of *Heligmosomoides* sp. and *Giardia* sp. co-infection. Mono-infection with *Heligmosomoides* sp. did not alter Shannon index (p = 0.874, Fig. S1a). However, the pair-wise PERMANOVA (Bray-Curtis distance) indicated significantly different microbial compositions between the *Heligmosomoides* sp. mono-infected and uninfected groups, and between the co-infected (*Heligmosomoides* sp. and *Giardia* sp.) and uninfected groups (p = 0.012, p = 0.013, respectively). PERMANOVA did not indicate a significant difference between the mono-infected and co-infected groups (p = 0.251, Fig. 5).





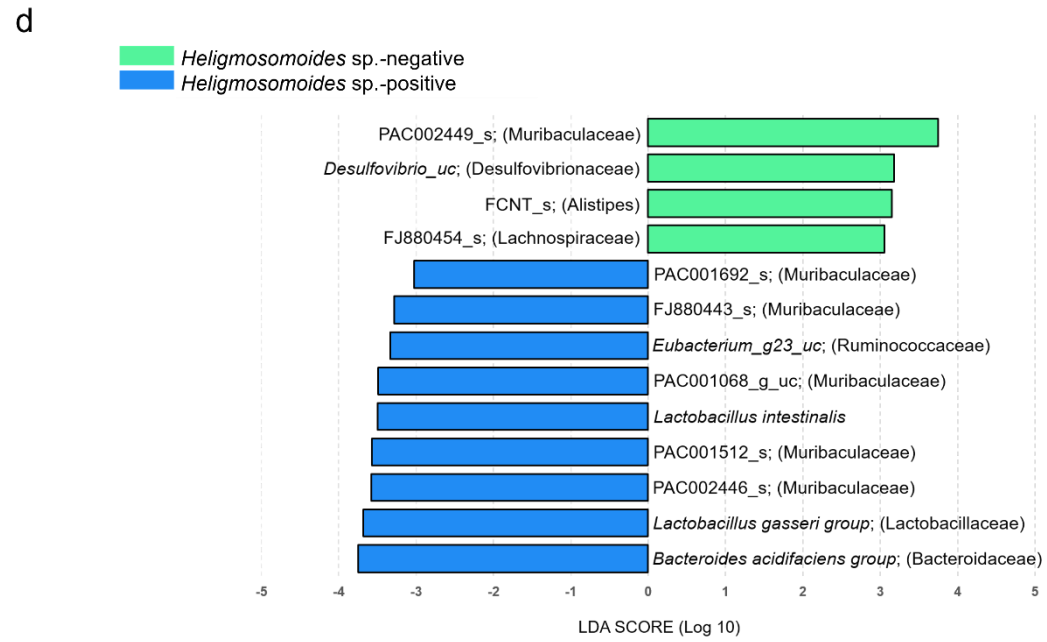
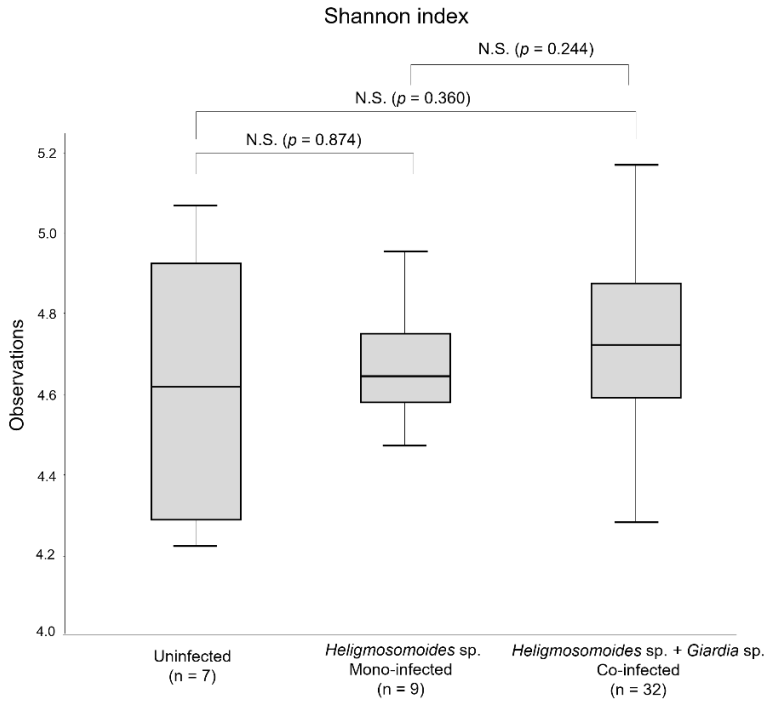


Figure 4. Alpha and beta diversities of wild *Apodemus agrarius* cecal microbiomes by *Heligmosomoides* sp. infection status. (a) Number of operational taxonomic units (OTUs) and (b) Shannon index between *Heligmosomoides* sp.-negative (n = 7) and *Heligmosomoides* sp.-positive mice (n = 24). (c) Principal coordinates analysis (PCoA) representing the cecal microbiome composition. (d) Linear discriminant analysis effect size analysis of differentially abundant bacterial taxa among the two groups. Only taxa meeting a significant (>3) linear discriminant analysis threshold are shown.

a



b

All sets

p-value
* (p = 0.012)

Sets
3

MTPs
48

No. of permutations
999

Pair-wise

		pseudo-F	p-value	q-value
Heligmosomoides sp. (-) *Giardia* sp. (-)	*Heligmosomoides* sp. (+) *Giardia* sp. (-)	1.519	0.012	0.0195
	Heligmosomoides sp. (+) *Giardia* sp. (+)	1.565	0.013	0.0195
Heligmosomoides sp. (+) *Giardia* sp. (-)	*Heligmosomoides* sp. (+) *Giardia* sp. (+)	1.123	0.251	0.251

Figure 5. Alpha and beta diversity of *Apodemus agrarius* cecal microbiomes based on *Heligmosomoides* sp. and *Giardia* sp. infection status. (a) Shannon index of *Heligmosomoides* sp.– *Giardia* sp. were both negative (n = 7), *Heligmosomoides* sp. single positive (n = 9), and *Heligmosomoides* sp.– *Giardia* sp. both positive mice (n = 32). (b) PERMANOVA test results representing the differences in cecal microbiome composition.

IV. DISCUSSION

Wild rodents are likely to play roles as vital reservoirs of zoonotic pathogens, including bacteria, parasites, and fungi [51, 52]. Pathogens can be spread to humans via direct contact or ingesting food and water contaminated with rodent feces [53]. In this study, we comprehensively investigated the presence of prokaryotic and eukaryotic organisms in the gut of *A. agrarius* using metabarcoding and analyzed interactions among them.

Using the screening method described herein, the Illumina iSeq 100 system, we detected potential prokaryotic and eukaryotic pathogens. The metabarcoding method has many advantages over conventional methods, such as PCR and microscopic and culture-based screening. The metabarcoding technique could be applied when screening a massive sample researchers. Furthermore, this technique could be used to detect veiled pathogens because of its untargeted nature and ability to investigate non-culturable organisms, which were problematic to investigate using conventional screening methods.

Notably, this study identified various Helicobacter strains in the rodent feces. Many studies have demonstrated that wild rodents can be reservoirs of various Helicobacter strains [54-56]. *Helicobacter fennelliae* was identified in one sample in this study, which is known to cause gastroenteritis in immunocompromised humans [57]. Thus, the current study showed that *A. agrarius* is a repository of various Helicobacter strains, some of which may be pathogenic to humans. *Serratia marcescens*, known as an opportunistic pathogen, was also detected in 17 samples in the present study, and this species can cause severe symptoms in patients, such as sepsis, pneumonia, and meningitis [58]. *L. interrogans*, *O. tsutsugamushi*, and *A. phagocytophilum* are known as infectious

pathogens and were previously reported to be present in the spleen, kidney, and blood of *A. agrarius* at prevalence of 4.92%, 17.6%, and 19.1%, respectively, but these species were not detected in the current study [7].

Similar to a previous study conducted in the UK, we found a lower relative abundance of Lactobacillus in the fall compared to that in the spring, whereas Helicobacter had a higher relative abundance in the fall [54].

Unlike bacterial community studies using the 16S rRNA gene, metabarcoding analysis targeting eukaryotic communities is still in its early stage. We identified the infection status of parasites and fungi in the rodent gut through 18S rRNA gene amplicon sequencing in this study. A previous study that analyzed the feces of seven *Rattus norvegicus* and two *R. rattus* demonstrated the powerfulness of the metabarcoding method by comparing microscopy results [59]. In that previous study, all kinds of helminths, such as *Ascaridia* and *Hymenolepis*, found using microscopy were also detected by the Illumina-based metabarcoding method [59]. In our study, we used the NCBI database as it contains a greater range of data than the SILVA database used in that previous study. For example, *Heligmosomoides* sp. was found in the NCBI database and not in the SILVA database.

The samples used in the current study included many parasites in wild mice that have been reported in previous papers [52, 60-65]. In this study, *Isospora* sp., *Cryptosporidium* sp., and *Blastocystis* sp. were found, which might include zoonotic agents. *Cryptosporidium parvum*, for example, is a zoonotic pathogen that causes diarrhea in humans [65]. Furthermore, potential fungal pathogenic agents were found in this study. Among these agents, *Mucor* sp. and *Rhizopus* sp. are major fungi that can cause mucormycosis in humans [66, 67]. In addition, *Cladosporium* sp., *Periconia* sp., *Candida*

sp., and *Kazachstania* sp were found herein, which were reported in the human infection cases [68-70].

Interestingly, *Hymenolepis* sp. was only detected in spring. In a previous study, it was found that temperature and humidity during the summer and fall seasons could be advantageous for *Hymenolepis* sp. infection in wild rodents [62]. In the present study, *Syphacia* sp. was detected in only 25% (12/48) of samples. A previous study reported that *Syphacia* sp. could be found in 14.0% of wild rodents. Albeit rare, *Syphacia* sp. can infect humans and is a zoonotic parasite [71, 72]. *Heligmosomoides* sp. was the most prevalent infectious helminth in this study (85%). A previous report suggested that intestinal helminth infections are more prevalent in *Heligmosomoides* sp.-infected wild mice than that in an uninfected group. [63] We detected *Raillietina* sp. in 8.3% of samples. This tapeworm was reported to have the highest prevalence among potential zoonotic helminths infecting wild rodents in the Indochinese Peninsula [64]. Additionally, this study found *Strongyloides* sp. in three samples. *Strongyloides ratti* is a skin-penetrating nematode and normally used as a laboratory model for *Strongyloides stercoralis*. *Oscheius* sp. and *Panagrolaimus* sp. have not been reported in wild rodents yet. *Oscheius* spp. was previously identified as an entomopathogenic nematode [73]. *Panagrolaimus* spp. is a free-living nematode that feeds on bacteria, and it has been isolated from soil [74].

Plagiorchis sp. was detected in three samples. Parasitic trematodes of the genus *Plagiorchis* have reported to have zoonotic potential. *Plagiorchis muris* and *Plagiorchis elegans* have been known to cause intestinal infections in wild mice [75]. In addition, *Plagiorchis* sp. has been reported to cause intestinal infections in human patients in Japan and Korea [76]. In 2007 and 2014, *P. muris* was reported in *A. agrarius* in Korea (5.3%

and 14.8%, respectively) [4, 77]. In total, 717 *P. elegans* specimens were collected from the small intestines of 27 of 117 wood mouse (*Apodemus sylvaticus*) samples in the UK [78].

There was no difference between the alpha-diversity in *Heligmosomoides* sp.-infected and uninfected groups herein. This result agrees with those of Kreisinger et al. regarding the impact of helminths infection on microbial compositions [19]. In particular, higher *L. gasseri* and *L. intestinalis* abundances were observed in the *Heligmosomoides* sp.-infected group. A recent study demonstrated that *Heligmosomoides* sp. helminth infection alters the intestinal microbiota [79]. In addition, the results of other studies confirmed that the prevalence of Lactobacillus is increased in laboratory mice infected with various intestinal helminths [80–82].

In addition, when we analyzed the impact of *Heligmosomoides* sp. and *Giardia* sp. co-infection, the co-infection showed no significantly different effects (neutral effect) compared to that of *Heligmosomoides* sp. mono-infection (Fig. S1).

Interestingly, we were able to detect *Heligmosomoides* sp. genes in the ceca despite this parasite typically residing within the small intestine. This is because metabarcoding analysis can detect and identify gene sequences from the small amounts of parasitic cells, tissues, and eggs in the ceca.

In this study, parasitic worms or eggs were not collected and identified under a microscope. In addition, since this study was conducted using iSeq 100, which covers short sequence lengths, there is a limitation in resolution of accurate identification of the parasite species. For example, the 18S V9 regions of *Heligmosomoides* sp. and *Nippostrongylus brasiliensis* had one base-pair difference although all samples had higher identity to *Heligmosomoides* sp. Metabarcoding using various primers that target

different regions of 18S rRNA gene such as V4 and V9 may produce more accurate metabarcoding information [83].

This study did not distinguish the fungi that reside within animal guts from those that are non-residents which are ingested in the diet. Further research on this topic is needed to facilitate a more precise understanding of the causes and consequences of variations in wild animal gut fungi and parasites compositions [84].

We were unable to detect blood pathogens in this study due to the nature of cecal samples. Future studies will attempt to detect such pathogens from other organ tissues.

V. CONCLUSION

In this study, we screened bacteria, fungi, protozoa, and helminths in the gut of *A. agrarius* using 16S and 18S rDNA-targeted high-throughput sequencing and identified potential zoonotic pathogens such as *Cryptosporidium* sp. and *Hymenolepis* sp.. In addition, the bacterial composition was found to be changed based on the season and specific parasitic infection status of collected mice. This approach, with some improvements, will enable us to analyze a large number of samples in a high throughput manner and could be the next standard applied to investigate bacterial and parasitic infections.

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ABSTRACT(IN KOREAN)

메타바코딩 분석을 이용한 등줄쥐 장내 세균 및 기생충 군집분석

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김 수 림

등줄쥐는 (*Apodemus agrarius*) 한국에서 흔히 발견되는 야생 설치류로 인간에게 신증후군출혈열, 렙토스피라증과 같은 설치류매개 감염병을 일으킬 수 있는 것으로 알려져있다. 본 연구에서는 iSeq 100 차세대염기서열 분석 장비를 이용하여 야생 등줄쥐의 (*Apodemus agrarius*) 분변의 세균과 기생충 군집분석을 시행하고 병원체를 스크리닝 하였다. 군집분석의 타겟 유전자는 세균의 16S rRNA gene V4와 기생충의 18S rRNA gene V9이었다. 본 연구에 사용된 등줄쥐는 강원도 강릉과 원주에서 5월, 6월, 10월 채집하였으며 총 48개의 분변 DNA를 확보하였고 박테리아, 원생동물, 진균 및 기생충의 염기서열을 분석하였다. 원생동물에서 *Tritrichomonas* sp. 가 모든 샘플에서 확인되었으며 *Monocercomonas* sp. (95.8%; 46/48)이 두 번째로 가장 많았고, *Giardia* sp. (75%, 36/48)가 그 뒤를 이었다. 기생충의 경우, *Heligmosomoides* sp. 가 샘플의 85.4%(41/48)에서 발견되었고, *Hymenolepis* sp. (10.4%; 5/48), *Syphacia* sp.

(25%; 12/48), *Raillietina* sp. (8.3%; 4/48) 및 *Strongyloides* sp. (6.3%; 3/48) 등을 확인하였다. 16S rRNA 유전자 분석에서 모든 샘플은 Muribaculaceae의 존재가 지배적이었고, Lachnospiraceae가 그 뒤를 이었다. 또한, 미생물 조성은 계절(p=0.005)과 성별(p=0.001)에 따라 변화하였다. 선형 판별 분석 효과 크기 분석 (Linear discriminant analysis)에서는 봄철 채집된 생쥐에서 가을철 채집된 생쥐에 비해 *Escherichia coli*와 *Lactobacillus murinus*가 더 풍부하게 관찰되었다. 또한, *Heligmosomoides* sp. 감염 상태에 따라 미생물 조성이 변화하였다 (p=0.019). 추가적으로, *Lactobacillus gasseri*와 *Lactobacillus intenseis*는 음성군보다 *Heligmosomoides* sp. 양성군에서 더 풍부하였다. 이 연구는 수집된 쥐의 계절, 성별 및 특정 기생충 감염 상태에 따라 박테리아의 양이 변한다는 것을 보여주었다. 이러한 결과는 인수공통전염병 모니터링에서 NGS 기술의 장점을 강조한다. 본 연구결과는 메타바코딩 분석을 통한 선제적 감염 매개 동물 스크리닝 시스템 구축 개발에 중요한 자료가 될 것이다.

핵심되는 말 : 메타바코딩, 16S rDNA, 18S rDNA, *Apodemus agrarius*, 마이크로바이옴, 기생충

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