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Host and environmental factors shaping gut microbiome and host-microbe interactions using multi-omics approach

장내 마이크로바이옴에 대한 숙주와 환경 요인의 영향 및 멀티오믹스를 활용한 숙주-미생물 상호작용 연구

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Host and environmental factors shaping gut microbiome and host-microbe interactions using multi-omics approach

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Host and environmental factors shaping gut microbiome and host-microbe interactions using multi-omics approach

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Abstract

Host and environmental factors shaping gut microbiome and host-microbe interactions using multi-omics approach

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The gut microbiome is a complex community of diverse microorganisms in gastrointestinal tract of host. The gut microbiome is known to play an essential role in host phenotype via complex host-microbe interactions. Furthermore, various factors including host genetics, diet and environment shape the feature of gut

microbiome. While the most of research on the gut microbiome have been carried out in humans and laboratory mice, the dynamics of the gut microbiome of domesticated and wild animals remains unlcear. Therefore, the present study aimed to investigate the effect of host and environmental factors, including host genetics, environment, and diet, on the gut microbiota of animals.

First, the present study revealed the distinct differences in the composition and the diversity of gut microbiome of Korean wild mice sharing the same habitat, namely *Micromys minutus* and *Mus musculus*. Metagenomic analysis of gut microbiome showed that *Micromys minutus* was a reservoir for *Campylobacter*, whereas *Mus musculus* did not harbor this species in their gut. The differences in the proportion of *Campylobacter* and *Lactobacillus* in the gut microbiome may explain the discrepancies in *Campylobacter* presence between the two species of wild mice.

Second, the present study revealed that environmental perturbations induce the dysbiosis of the gut microbiome and antibiotic resistance acquisition in wild migratory birds during wildlife rehabilitation, where dysbiosis of the gut microbiota was observed including decreased diversity, depletion of short-chain fatty acid producers, decreased microbial network complexity, and enrichment of zoonotic pathogens. Moreover, antibiotic resistance including tetracycline and ciprofloxacin resistance of gut microbiome significantly increased after rehabilitation, and the majority of the birds acquired multidrug resistance.

Third, the present study revealed the impact of long-term dietary intake of red ginseng dietary fiber on the canine gut microbiota, which increased the diversity and short-chain fatty acid producers and decreased the zoonotic pathogens

(including *Helicobacter*), suggesting the prebiotic potential of red ginseng dietary fiber.

Lastly, the present study revealed the effects of the cuprizone diet, which induces toxic demyelination similar to that seen in multiple sclerosis (MS). Dysbiosis of the gut microbiota was shown, including decreased diversity. Moreover, the enrichment of biomarker microbes (including *Akkermansia*) and metabolites (including branched-chain amino acids) was consistent with human MS patients.

The present study showed that the animal gut microbiome is shaped by internal and external factors, including host species, environment, and diet. Moreover, the findings of this study suggest that alteration of the gut microbiome may alter the host-microbe interactions, thereby affecting the host's health. The alteration of the gut microbiome by the various interventions in this study may provide a basis for further studies on animals' gut microbiomes, in particular, further studies exploring beneficial microbes and prebiotic diets to modulate the gut microbiota. Moreover, the present study suggests novel perspectives for the utilization of gut microbiome analysis for development of sustainable rehabilitation strategies for wild animals. In addition, the findings elucidate the relationship between the brain and the gut microbiome, which may help to overcome neurodegenerative diseases.

Keywords: Gut microbiome, Metagenomics, Multi-omics, Host-microbe interaction, Gut-Brain axis

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List of abbreviations

ANOSIM	Analysis of similarities
ASV	Amplicon sequence variant
BCAAs	Branch chain amino acids
CNS	Central nerve system
CPZ	Cuprizone
HPLC	High-performance liquid chromatography
LEfSe	Linear discriminant analysis effect size
mCCDA	Modified charcoal-cefoperazone-deoxycholate agar
MS	Multiple sclerosis
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis
QIIME	Quantitative insights into microbial ecology
SCFAs	Short chain fatty acids

Literature review

A. Factors shaping the gut microbiome

Large-scale studies based on the global population have shown that the the gut microbiome composition significantly vary among healthy individuals (Turnbaugh et al. 2007; Lloyd-Price et al. 2017). This is due to factors such as host genetics, external environmental factors, and the maternal microbiome. The host's gut microbiome is first shaped by the maternal microbiome, which is vertically transferred from the mother. During pregnancy, particularly in the third trimester, significant alterations occur in the mother's hormone homeostasis, immune and metabolic function, as well as in the maternal microbiome of various body sites such as the gut and vagina (Goltsman et al. 2018). The shift in the maternal microbiome is represented by an increase in *Enterobactericae* and *Streptococcus* species, which are major components of the infant gut microbiome (Koren et al. 2012). These facultative anaerobic microbes modify the gut environment of infants for optimized growth of anaerobic microbes by depleting oxygen in the gut. From birth to 4–6 weeks of age, the compositional and functional diversity of the microbiome in all body sites significantly increases (Koren et al. 2012).

After the vertical transfer of the gut microbiome from the mother, the gut microbiome is subsequently shaped by environmental factors such as diet, lifestyle, and medication. Particularly, diet is the predominant factor altering the gut microbiome. Long-term dietary patterns determine the enterotype of the gut microbiome; the *Bacteroides* enterotype is related with an animal protein-based diet, and the *Prevotella* enterotype is related with a carbohydrate-based diet (G. D. Wu et al. 2011). In the case of a short-term diet, the intake of animal-based foods

enriched the bile-tolerant microbes including *Alistipes*, *Bilphilophila*, and *Bacteroides* and depleted the microbes involved in the metabolism of dietary plant polysaccharides *Roseburia*, *Eubacterium*, and *Ruminococcus* (David et al. 2014). Moreover, short-term intake of a high-fiber diet increased the abundance of *Bifidobacterium* and *Lactobacillus*, which degrade microbiota-accessible carbohydrates (Oliver et al. 2021).

There is growing evidence that the host's genetics play an essential role in shaping the gut microbiome. Comparing the gut microbiome of dizygotic and monozygotic twins is the most widely used approach to discriminate the impact of host genetics on the gut microbiome in human studies (Goodrich, Waters, et al. 2014; Xie et al. 2016; Beaumont et al. 2016). Large cohort studies on human twins identified the groups of heritable bacteria in the gut microbiota, including the phyla Firmicutes Actinobacteria, Tenericutes, and Euryarchaeota, the families Christensenllaceae and Bifidobacteriaceae, and the genus *Tenericutes* (Goodrich et al. 2016). In the case of mice, previous studies revealed the effect of host genetics by comparing the gut microbiome of diverse laboratory mice strains which were maintained in the same animal facility (Snijders et al. 2016). This study showed that host genetics dominated the composition of the murine gut microbiome, while dietary interventions had a moderate impact. Moreover, studies on genetically modified mice showed that altering the host genetics not only alters the phenotype but also induces dysbiosis of the gut microbiome (Kong et al. 2020; Brandscheid et al. 2017).

Various studies have reported that the impact of environmental factors dominates the shaping of the gut microbiome in human and laboratory rodents (Gacesa et al. 2022; Lees et al. 2014). For instance, a large cohort study on the

Dutch population revealed that early-life exposure to environmental factors such as smoking, pets, and a rural environment affected the microbiome in adulthood (Gacesa et al. 2022). In the case of rodents, a study on Zucker rats showed that the cage environment and age were the most dominant factors in shaping the gut microbiome rather than genetic background (Lees et al. 2014). Moreover, it is well known that laboratory mice with an identical genetic background show differences in the intestinal microbiome depending on the feed vendor or housing facility (Long et al. 2021; Ericsson et al. 2015).

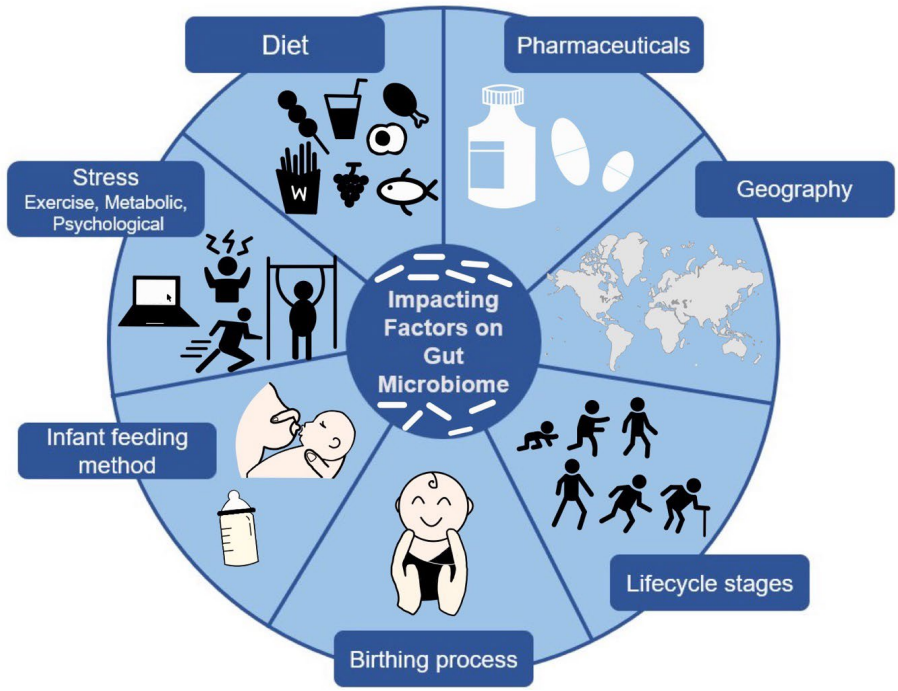


Figure 1. Host and environmental factors shaping gut microbiome

Diagram illustrates various factors shaping the composition, diversity and the function of gut microbiome. This figure is modified from Cresci et al., (2015).

B. Interaction between host health and the gut microbiome

It is well known that the gut microbiome plays an essential role in maintaining the host's health by maintaining homeostasis of the gastrointestinal tract, immune system, and central nervous system. Therefore, dysbiosis which is the imbalance of the gut microbiome can increase the risk of disease or directly induce disease.

Type 2 diabetes mellitus (T2DM), marked by insulin resistance and hyperglycemia, is one of the most frequent metabolic diseases in humans. The risk factors associated with T2DM include host genetics, obesity, age, and lifestyle, and the gut microbiome is also known to be a putative risk factor. A metagenome-wide study on a large cohort showed that the functional properties and taxonomic composition of the gut microbiome in healthy group and patients with T2DM had distinct features, including an abundance of microbes involved in butyrate production and mucin degradation (Qin et al. 2012). Moreover, the gut microbiome of individuals with diabetes showed lower diversity and short-chain fatty acid (SCFA) production (Menni et al. 2020). These studies indicated a dysbiosis of the gut microbiome associated with T2DM. Therefore, recent studies on diabetes evaluated the therapeutic potential of augmenting the gut microbiome through the administration of prebiotics or probiotics (Paul et al. 2022).

The gut microbiome and the brain affect each other through endocrine, immune, and neural pathways (Cryan and Dinan 2012). For instance, the secretion of cortisol which is modulated by the hypothalamus-pituitary-adrenal axis, alters the gut permeability and barrier function by affecting immune cells, which may influence the gut microbiome (Mudd et al. 2017). Conversely, the gut microbiome

affects brain function by altering the circulating cytokines and producing neuroactive metabolites (Grenham et al. 2011b). Dysbiosis of the gut microbiome may affect the development of wide range of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and MS (Cryan et al. 2020). Previous studies showed that the gut microbiome of autism patients had distinct features from healthy controls, including a low Bacteroidetes:Firmicutes ratio, a high abundance of *Clostridium*, *Lactobacillus*, and *Desulfovibrio*, and a low abundance of *Bifidobacterium*, *Prevotella*, and *Akkermansia* (Finegold et al. 2010; D.-W. Kang et al. 2013; Tomova et al. 2015). A study using germ-free mice revealed the role of the gut microbiome in autism, showing that transplantation of the gut microbiota from autism patients into mice induced autism-like symptoms (Sharon et al. 2019). Moreover, a multi-omics study on the gut microbiome of autism patients revealed that the microbial metabolite p-cresol induced autism symptoms in mice (Bermudez-Martin et al. 2021). As the gut microbiome is associated with autism, recent studies are targeting microbiome-based therapies for autism by augmenting the gut microbiome through fecal microbiota transplantation from healthy individuals. Notably, a human clinical trial showed that microbiota transfer therapy improved autism symptoms (D.-W. Kang et al. 2017).

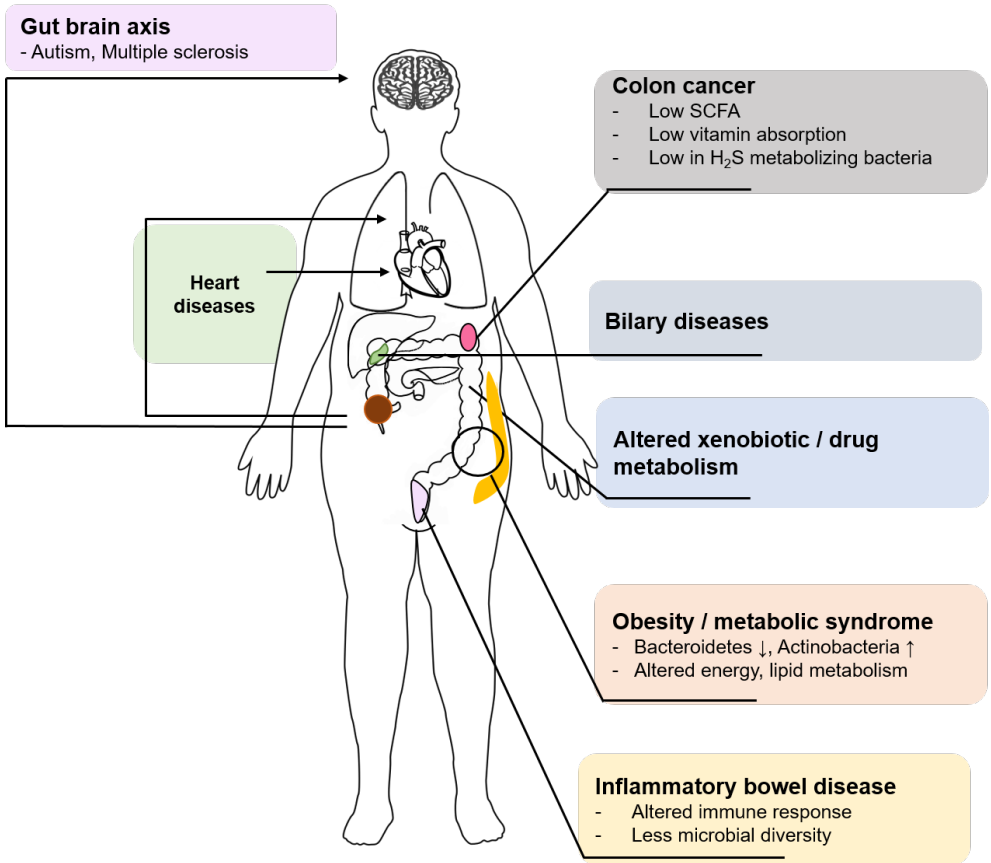


Figure 2. Systemic diseases associated with gut microbiome

Various systemic diseases are affected by dysbiosis of gut microbiome. Metabolic and immunological pathways involved in diseases that are directly influenced by the gut microbiota is demonstrated. This figure is modified from Kinross et al., (2011).

C. Metagenomic analysis of the gut microbiome

While the human gut microbiome comprises more than 1,000 species, the majority of gut microbes are not culturable because the complex gut environment is not fully reproducible in the laboratory environment (Vartoukian, Palmer, and Wade 2010; Forster et al. 2016). Traditional studies on the gut microbiota were restricted to culturable isolates or targeting certain species, which may not fully represent the complex microbial community (Jespers et al. 2012; Greub 2012). Recent developments in next-generation sequencing (NGS) have enabled in-depth studies of the microbiome, including taxonomy profiling, phylogenetic analysis, diversity analysis, and functional research. The most common NGS method used for microbiome analysis is 16S rRNA sequencing which targets the hypervariable region of 16S ribosomal RNA of bacteria (Janda and Abbott 2007). While the specific methods and principles vary between NGS platforms such as Illumina, Pacificbio, and Nanopore sequencing, hypervariable region-specific primers are selected for the polymerase chain reaction (PCR) to generate amplicons in all platforms (Ji and Nielsen 2015; Maghini et al. 2021). Raw NGS data of 16S rRNA amplicons are classified into operational taxonomic units (OTUs), which are clusters of sequence reads with more than a certain threshold of similarity (generally > 97%), and amplicon sequence variants (ASVs), which are sequence variants distinguished by single nucleotide (Schloss and Handelsman 2005; Callahan et al. 2016a). Both clustering methods enable an analysis of taxonomy composition, diversity, and functional prediction of the gut microbiome (Schloss et al. 2009; Bolyen, Rideout, Dillon, Bokulich, Abnet, Al-Ghalith, et al. 2019).

The major focus of analysis of the gut microbiome is not only resolving the taxonomic classification but also identifying functional properties. In the past, most studies on the gut microbiome were conducted with amplicon sequencing because of its relatively low cost and simple bioinformatics methods (Shin et al. 2016; Huse et al. 2012). Advances in bioinformatics and sequencing technology, however, have enabled whole metagenome shotgun sequencing for the deeper analysis of the metagenome, including de novo assembly of novel microbial genomes, and the identification of the functional and metabolic repertories of whole microbial communities (Sedlar, Kupkova, and Provaznik 2017). Whole metagenome sequencing produces short sequence reads originating from various genomes, including hosts and microorganisms; therefore, it requires more complicated bioinformatics methods for clustering the microbial genome. There are currently two major types of methods for assembly, namely the reference-based method and the de novo method (Lapidus and Korobeynikov 2021). The reference-based method clusters the short sequence reads into an assembled genome by performing aligning algorithms against a specific microbial reference database and discarding host genome contamination against a host genome reference database (Camacho et al. 2009; Langmead et al. 2009; H. Li and Durbin 2010). On the other hand, the de novo method clusters short reads to an assembled genome without a reference database using composition-based, abundance-based, and hybrid algorithms (Sedlar, Kupkova, and Provaznik 2017).

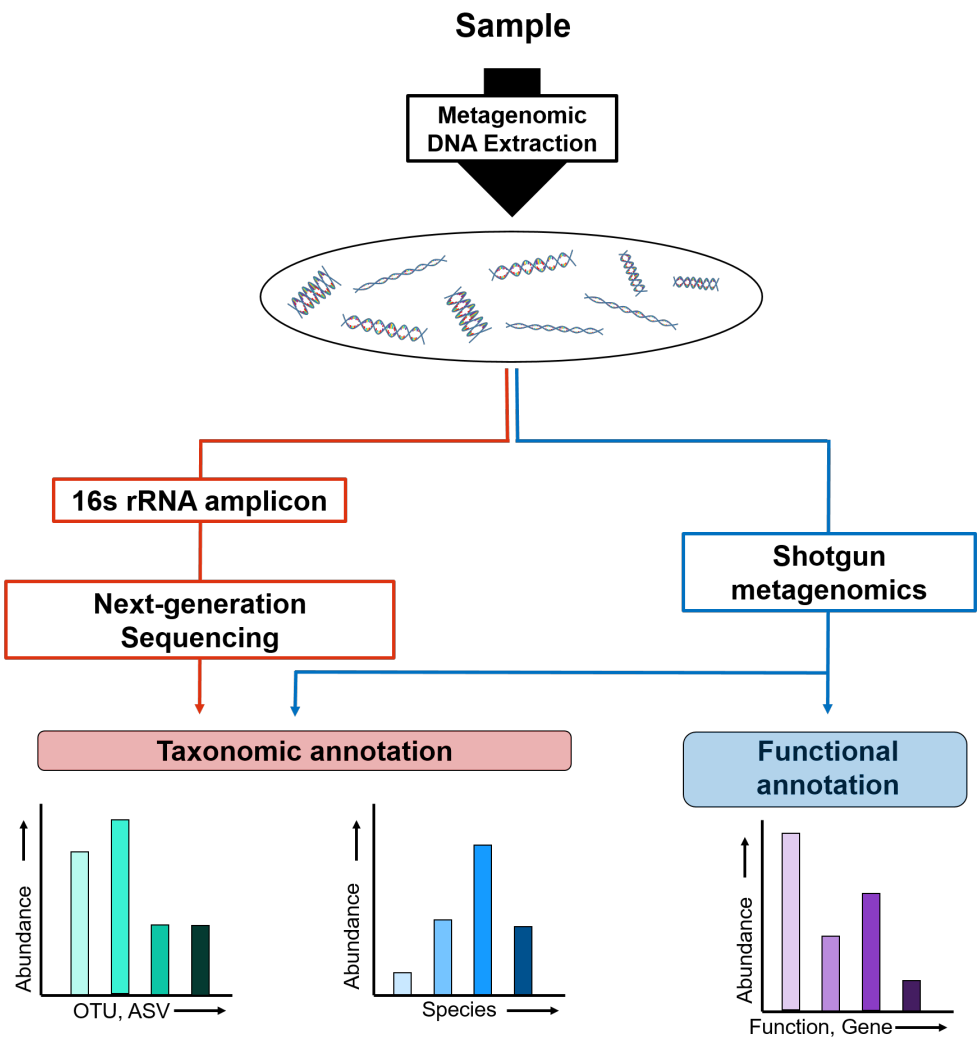


Figure 3. General overview of analysis methods of gut microbiome including 16S rRNA sequencing and shotgun metagenomics

This figure is modified from Boers et al., (2019).

D. The gut microbiome and antibiotic resistance

Humans and animals are major reservoirs of antibiotic-resistant microbes because the gut microbiota harbors such microbes as well as antibiotic-resistant genes (Salyers, Gupta, and Wang 2004; Yongfei Hu et al. 2016). The healthy gut microbiome is a stable, diversified community that offers key advantages to the host, such as protection against infections and nutritional absorption. Antibiotic perturbation alters the taxonomic composition, function, and diversity of the gut microbiome. This dysbiosis may induce the expansion of antibiotic-resistant microbes and antibiotic-resistant genes, consequently leading to antibiotic-resistant pathogen invasion into the other organs, such as the bloodstream and urinary tract. Therefore, improving the understanding of how the dysbiosis of the gut microbiome induces the enrichment of antimicrobial resistance in the gut is needed.

Advances in metagenomics have enabled the comprehensive research of antibiotic resistance in the gut microbiome. Whole metagenome shotgun sequencing may provide information on the abundance, type, function, and origin of antibiotic resistance genes with high resolution. Recent studies using metagenomics have shown that the gut microbiome harbors more abundant antibiotic resistance genes than other environmental sources (Forsberg et al. 2012; Yongfei Hu et al. 2013). Moreover, there is growing evidence that even commensal gut microbes, which are not antibiotic targets, are involved in the propagation and evolution of antibiotic resistance genes (McInnes et al. 2020). Furthermore, a recent study suggested that antibiotic resistance genes originate from the maternal gut microbiome and are transferred to infants during birth (Yassour et al. 2018).

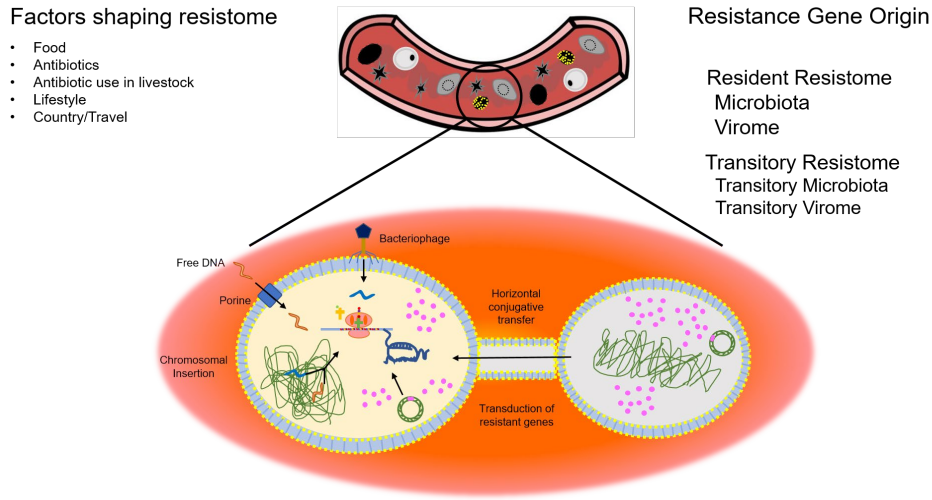


Figure 4. Factors shaping the resistome and mechanisms of antibiotic resistance gene transfer in the microbiota

This figure is modified from Baron et al., (2018).

Chapter I

Metagenomic Analysis of the Gut Microbiota of Wild Mice, a Newly Identified Reservoir of *Campylobacter*

* This chapter is reproduced from Song, Hyokeun, et al. "Metagenomic analysis of the gut microbiota of wild mice, a newly identified reservoir of *Campylobacter*." *Frontiers in cellular and infection microbiology* 10 (2021): 596149.

Abstract

Campylobacter, the most common etiologic agent of zoonotic gastroenteritis in humans, is present in many reservoirs including livestock animals, wildlife, soil, and water. Previously, the author reported a novel *C. jejuni* strain SCJK02 (MLST ST-8388) from the gut of wild mice (*Micromys minutus*) using culture-dependent methods. However, due to fastidious growth conditions and the presence of viable but non-culturable *Campylobacter* spp., it is unclear whether *M. minutus* is a *Campylobacter* reservoir. This study aimed to: (1) determine the distribution and proportion of *Campylobacter* spp. in the gut microbiota of wild mice using culture-independent methods and (2) investigate the gut microbiota of wild mice and the relationship of *Campylobacter* spp. with other gut microbes. The gut microbiota of 38 wild mice captured from perilla fields in Korea and without any clinical symptoms (18 *M. minutus* and 20 *Mus musculus*) were analyzed. Metagenomic analysis showed that 77.8% (14 of 18) of the captured *M. minutus* harbored *Campylobacter* spp. (0.24–32.92%) in the gut metagenome, whereas none of the captured *M. musculus* carried *Campylobacter* spp. in their guts. Notably, 75% (6 of 8) of *M. minutus* determined to be *Campylobacter*-negative using culture-dependent methods showed a high proportion of *Campylobacter* through metagenome analysis. The results of metagenome analysis and the absence of clinical symptoms suggest that *Campylobacter* may be a component of the normal gut flora of wild *M. minutus*. Furthermore, linear discriminant analysis (LDA) showed that *Campylobacter* was the most enriched genus in the gut microbiota of *M. minutus* (LDA score, 5.37), whereas *Lactobacillus* was the most enriched genus in *M. musculus* (LDA score, -5.96). The differences in the presence of

Campylobacter between the two species of wild mice may be attributed to the differential abundance of *Campylobacter* and *Lactobacillus* in their respective gut microbiota. In conclusion, the results indicate that wild *M. minutus* may serve as a potential *Campylobacter* reservoir. This study presents the first metagenomics analysis of the *M. minutus* gut microbiota to explore its possible role as an environmental *Campylobacter* reservoir and provides a basis for future studies using culture-independent methods to determine the role of environmental reservoirs in *Campylobacter* transmission.

1.1 Introduction

Campylobacter is one of the most common etiologic agents of zoonotic gastroenteritis in humans (Kaakoush et al. 2015). Although the most common cause of *Campylobacter* infection is the intake or handling of contaminated poultry, environmental sources such as wildlife, soil, and water are also important infection routes (Whiley et al. 2013; Hofreuter 2014; Skarp, Hänninen, and Rautelin 2016). As an environmental reservoir, wildlife is an emerging source of *Campylobacter* infection via the direct transmission of *Campylobacter* to humans or indirectly via the wildlife-livestock-human cycle (J. Kim et al. 2020). While the majority of studies on *Campylobacter* reservoirs in wildlife have been conducted on wild birds, several studies on other hosts, such as deer, boars, and reptiles, have also been conducted (French et al. 2009; Díaz-Sánchez et al. 2013; Carbonero et al. 2014; Patrick et al. 2013). Wild mice are distributed in a wide range of habitats globally and often transmit diverse zoonotic pathogens to humans and livestock, serving as a link between wildlife and the urban community (Razzauti et al. 2015); however, *Campylobacter* in wild mice is not well understood. One study reported *Campylobacter* strains isolated from wild rodents, suggesting wild rodents as a risk factor of *Campylobacter* infection in livestock (Meerburg et al. 2006).

Most studies on *Campylobacter* in wildlife have been conducted using culture-dependent methods, such as the isolation and characterization of bacterial strain (French et al. 2009; Díaz-Sánchez et al. 2013; Patrick et al. 2013; Carbonero et al. 2014). Previously, the author reported a novel *C. jejuni* strain SCJK02 (MLST ST-8388) isolated from fecal samples of wild mice (*Micromys minutus*) (J. Kim et al. 2020). In the previous study, *Campylobacter* was isolated from 63% of

M. minutus, whereas none was isolated from *Mus musculus*. Considering the limitations of culture-dependent methods, such as fastidious growth conditions and the presence of viable but non-culturable *Campylobacter* spp. (Mihaljevic et al. 2007; Jackson et al. 2009), it is likely that *Campylobacter* was not detected, even if it was present. Therefore, it is essential to apply culture-independent methods together with traditional culture-dependent methods to precisely determine the presence of *Campylobacter* in a host.

The role of the gut microbiota in *Campylobacter*-mediated infection has been reported in several studies (Z. Li et al. 2018; Xiaolun Sun et al. 2018). In humans, the microbiota of poultry workers infected with *Campylobacter* and those resistant to colonization of *Campylobacter* show significant differences in the abundance of certain genera (Dicksved et al. 2014). In laboratory mice, elevated levels of intestinal *Escherichia coli* reduce colonization resistance to *Campylobacter* (Haag et al. 2012), and the gut microbiota composition affects the extraintestinal dissemination of *Campylobacter* (O'Loughlin et al. 2015). In poultry, neonatal chickens transplanted with mature microbiota show a reduced transmission potential of *Campylobacter* (Gilroy et al. 2018). Thus, the infection risk of *Campylobacter* is affected by the gut microbiota of the host through diverse microbe-microbe interactions. Since the gut microbiota of *M. minutus* has not yet been investigated, studies are needed to improve the prediction and prevention of the transmission of *Campylobacter* from wildlife to humans.

This study was conducted to: (1) determine the distribution and proportion of *Campylobacter* spp. in the gut microbiota of wild mice using culture-independent methods and (2) investigate the core microbiota of wild mice and the relationship of *Campylobacter* spp. with other gut microbes. The gut microbiota of

38 wild mice without clinical symptoms (18 *M. minutus* and 20 *M. musculus*) and captured for 2 years from perilla fields in Korea at the end of winter torpor were analyzed. This study is the first to investigate the gut microbiota of *M. minutus* using metagenomics to explore its possible role as an environmental *Campylobacter* reservoir.

1.2 Material and Methods

Study Design and Sample Collection

The Institutional Animal Care and Use Committee of Hallym University (approval number Hallym2017-5, Hallym 2018-6) approved this study. Two species of wild mice *(M. minutus* and *M. musculus*) were captured for 2 years from the perilla fields of Chuncheon in Korea at the end of their winter torpor. Information on the wild mice used in this study is included in the supplementary material (Table 1). All captured mice were transferred to the lab facility immediately. Fresh fecal samples from the mice were collected in single cages and stored at -80°C.

In the previous study, *Campylobacter* was isolated from mice fecal samples using two different culture methods (J. Kim et al. 2020). Briefly, homogenized fecal samples (in phosphate-buffered saline – PBS) were directly spread onto modified cefoperazone–deoxycholate agar plates (mCCDA; Oxoid Ltd., Hampshire, United Kingdom) containing the CCDA-selective supplement (Oxoid, Ltd.) and plates were incubated at 42°C for 2 days under microaerobic conditions. Next, *Campylobacter*-like colonies were inoculated into Müller–Hinton agar plates (Oxoid Ltd.) and then tested by *Campylobacter* genus-specific polymerase chain reaction (PCR) (Gehua Wang et al. 2002). All *Campylobacter*-positive colonies were identified as *C. jejuni* by species-specific PCR (Gehua Wang et al. 2002). Additionally, fecal samples that were *Campylobacter*-negative subjected to enrichment in Bolton broth (Oxoid, Ltd.) containing the Bolton broth selective supplement (Oxoid, Ltd., Hampshire, United Kingdom) for 2 days at 42°C under microaerobic conditions. Thereafter, the presence of *C. jejuni* was

investigated as above. Of note, results showed that *Campylobacter* was culture-positive in 63.6% of *M. minutus*, and culture-negative in all *M. musculus*.

Here, to investigate the differences in the gut microbiota of *Campylobacter* culture-positive and culture-negative *M. minutus*, 10 fecal samples from culture-positive *M. minutus* and 8 fecal samples from culture-negative *M. minutus* were used for microbial community analysis. Additionally, to investigate the difference between the gut microbiota of the two wild mice species, 20 fecal samples from *M. musculus* (all *Campylobacter* culture-negative) were used for microbial community analysis.

DNA Extraction and 16S rRNA Sequencing

Metagenomic DNA extraction from fecal samples was performed using the Fast DNA Soil kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The V3–V4 regions of the 16S rRNA gene were amplified using the following primers: 341F and 805R. PicoGreen was used to pool and normalize the amplified products. All sequencing processes were performed using an Illumina Miseq (San Diego, CA, USA) platform at Macrogen, Inc. (Seoul, Korea).

Bioinformatics and Statistical Analyses

The bioinformatics analysis of the sequence data was performed using QIIME 2 (version 2019.10) software package (Bolyen, Rideout, Dillon, Bokulich, Abnet, Caporaso, et al. 2019) and *MicrobiomeAnalystR* in R package (Dhariwal et al. 2017). An amplicon sequence variant (ASV) table was generated by filtering,

dereplicating, and denoising the raw sequence data using DADA2 (Callahan et al. 2016b). A phylogenetic tree of representative sequences was generated using MAFFT (Katoh and Standley 2013). Taxonomy assignment of the ASV table was conducted at the phylum and genus levels using a naïve Bayes classifier implemented in the q2-feature-classifier (Bokulich et al. 2018) against the SILVA database, version 132 (Quast et al. 2012). ASVs that were classified into the genus *Campylobacter* were further identified at the species-level. For downstream analysis, the sequencing data were normalized via rarefication to the minimum library size.

The alpha diversity of the microbial community was measured using the phyloseq package with two metrics, including the number of observed ASVs, which accounts for richness, and the Simpson's and Shannon's indexes, which account for richness and evenness (McMurdie and Holmes 2013). Differences in alpha diversity between wild mice groups were evaluated using the Mann-Whitney U test. Beta diversity was measured based on Bray-Curtis dissimilarity, and the differences in beta diversity between wild mice groups were evaluated using the analysis of group similarities (ANOSIM) test. Sample core microbiota were defined as those with a minimum abundance of 0.01% and a prevalence of 50% as the cut-off values. Differential abundance analysis of microbiota was performed using Linear Discriminant Analysis Effect Size (LEFSe), implemented in *MicrobiomeAnalystR* in the R package (Seata et al., 2011). The author considered a *p* value lower than 0.05 to indicate significance. Statistical analyses were performed using SPSS 25 (SPSS, Inc., Chicago, IL, USA) and R version 3.6.3.

1.3 Results

Taxonomic composition of the gut microbiota of wild mice

To determine the distribution and proportion of *Campylobacter* in the gut microbiota of wild mice, fecal microbiota from 18 *M. minutus* (10 culture-positive, 8 culture-negative) and 20 *M. musculus* (all culture-negative) were compared. No ASV was classified into the genus *Campylobacter* in the gut microbiota of *M. musculus*. The taxonomic composition of the gut microbiota of individual *M. minutus* at the phylum and genus levels is shown in Figure 5A and 5B. *Campylobacter* was present (0.24–32.92%) in the gut microbiota of 14 of 18 *M. minutus* (77.8%) but not in any of the *M. musculus*. The relative abundance of *Campylobacter* in the culture-positive and -negative groups of *M. minutus* showed no significant difference according to the Mann-Whitney U test ($p > 0.05$) (Figure 5C). Of note, all ASVs classified into the genus *Campylobacter* were identified as *C. jejuni* at the species-level.

The microbiota of all *M. minutus* samples comprised nine main bacterial phyla including Firmicutes, Bacteroidetes, Epsilonbacteraeota, Proteobacteria, Actinobacteria, Patescibacteria, Deferribacteres, Spirochaetes, and Tenericutes. Firmicutes (45.47%) was the most dominant phylum, followed by Bacteroidetes (38.61%) and Epsilonbacteraeota (7.34%). At the genus level, *Bacteroides* (23.79%) was the most dominant genus, followed by *Lactobacillus* (18.92%), uncultured *Muribaculaceae* (5.96%), *Lachnospiraceae NK4A136 group* (4.67%), uncultured *Lachnospiraceae* (4.65%) *Campylobacter* (4.03%), and *Helicobacter* (3.30%). The microbiota of *M. musculus* comprised seven main bacterial phyla, including Firmicutes, Bacteroidetes, Epsilonbacteraeota, Actinobacteria,

Proteobacteria, Patescibacteria, and Deferribacteres. Firmicutes (62.02%) was the most dominant phyla, followed by Bacteroidetes (32.70%) and Epsilonbacteraeota (2.00%). At the genus level, *Lactobacillus* (36.44%) was the most dominant genus, followed by *Bacteroides* (12.99%), uncultured *Muribaculaceae* (5.39%), and *Alistipes* (4.17%) (Figure 5D). The taxonomic composition of the gut microbiota of individual *M. musculus* is shown in Figure 6.

Members of the core microbiota of *M. minutus* at the phylum level were identified as Firmicutes, Bacteroidetes, Epsilonbacteraeota, Proteobacteria, and Actinobacteria (Figure 7A and 7C). Members of the core microbiota of *M. minutus* at the genus level were identified as *Bacteroides*, *Lactobacillus*, uncultured *Muribaculaceae*, *Lachnospiraceae NK4A136 group*, uncultured *Lachnospiraceae*, *Helicobacter*, *Campylobacter*, uncultured *Desulfovibrionaceae*, and *Alistipes* (Figure 7B and 7D).

Differences in the gut microbiota of *Micromys minutus* according to the culture results of *Campylobacter*

When the two culture groups of *M. minutus* were compared using the Mann-Whitney test, no significant differences ($p > 0.05$) were observed in the number of observed ASVs, the Simpson's index and the Shannon's index (Figure 8A).

The beta diversity as per the principle coordinate analysis based on Bray-Curtis dissimilarity showed distinct clustering of the gut microbiota of *M. minutus* according to the *Campylobacter* culture results (Figure 8B). An ANOSIM test revealed a significant difference in the gut microbiota between the *Campylobacter*

culture-positive and -negative groups of *M. minutus* (R: 0.23253, $p < 0.05$). Of note, no significant differences in the beta diversity of the *M. minutus* groups were detected for other factors, such as gender and habitat ($p > 0.05$).

To identify the bacterial taxa with significantly different abundances between wild mice groups, LEFSe was performed. When the *Campylobacter* culture-positive and negative groups of *M. minutus* were compared at the phylum level, Actinobacteria (LDA score -4.89, $p < 0.05$) was the most enriched phylum in the microbiota of *Campylobacter* culture-positive *M. minutus*, followed by Patescibacteria (LDA score -4.4, $p < 0.05$). At the genus level, *Lactobacillus* (LDA score 6.23, $p < 0.05$) was the most enriched genus in the microbiota of *Campylobacter* culture-negative *M. minutus*, whereas *Desulfovibrio* (LDA score -4.5, $p < 0.05$), *Candidatus Saccharimonas* (LDA score -4.4, $p < 0.05$), and *Streptococcus* (LDA score -3.73, $p < 0.05$) were enriched in *Campylobacter* culture-positive *M. minutus* (Figure 8C).

Difference in the gut microbiota between two species of wild mice

When the alpha diversity of two species of wild mice (*M. minutus* and *M. musculus*) was compared using the Mann-Whitney test, no significant differences ($p > 0.05$) were observed in the alpha diversity metrics, including the number of observed ASVs, the Simpson's index and the Shannon's index (Figure 9A).

The beta diversity as per the principle coordinate analysis based on Bray-Curtis dissimilarity showed distinct clustering of the gut microbiota of wild mice according to species (Figure 9B). An ANOSIM test revealed a significant difference in the gut microbiota between *M. minutus* and *M. musculus* (R: 0.57627, $p < 0.001$).

When the two species of wild mice (*M. minutus* and *M. musculus*) were compared, the abundance of eight phyla, including Firmicutes, Verrucomicrobia, Deferribacteres, Spirochaetes, Patescibacteria, Actinobacteria, Proteobacteria and Epsilonbacteraeota were found to be significantly different ($p < 0.05$) based on LEFSe. Firmicutes (LDA score -5.92) was the most enriched phylum in the gut microbiota of *M. musculus*, whereas Epsilonbacteraeota (LDA score 5.43) was the most enriched phylum in the gut microbiota of *M. minutus,* followed by Proteobacteria (LDA score 5.19), Actinobacteria (LDA score 4.69), Patescibacteria (LDA score 4.3), Spirochaetes (LDA score 4.2), Deferribacteres (LDA score 3.96), and Verrucomicrobia (LDA score 3.35). At the genus level, the abundance of all 35 genera was significantly different ($p < 0.05$). *Campylobacter* (LDA score 5.3) was the most enriched genus in *M. minutus*, whereas *Lactobacillus* (LDA score -5.94) was the most enriched genus in *M. musculus* (Figure 9C, Table 2).

1.4 Discussion

Previously, the author reported a novel *C. jejuni* strain isolated from wild *M. minutus* using a culture-dependent method (J. Kim et al. 2020). However, the incrimination of *M. minutus* as a reservoir based on culture-dependent methods alone remained unclear because of difficulties in the isolation of *Campylobacter* owing to the fastidious growth conditions required (i.e., microaerophilic) and the presence of viable but non-culturable *Campylobacter* (Mihaljevic et al. 2007; Jackson et al. 2009). Moreover, numerous studies have highlighted the role of a reservoir's microbiota composition in the transmission of a wide range of zoonotic pathogens (Jones et al. 2008; Stecher, Berry, and Loy 2013; Razzauti et al. 2015). However, most studies on the microbiota of wild mice have focused on that of wild *M. musculus*, belonging to the same species as the laboratory mouse, and no study has investigated the microbiota of *M. minutus* (Weldon et al. 2015; Rosshart et al. 2017; 2019). Therefore, it is essential to investigate the gut microbiota of *M. minutus* using a culture-independent method to predict the role of *M. minutus* in *Campylobacter* transmission.

The current study revealed that Firmicutes and Bacteroidetes are the most dominant phyla in the gut microbiota of *M. minutus*; in fact, these are the dominant phyla in a wide range of wild rodents (Debebe et al. 2017; Lavrinienko et al. 2018) and are involved in nutrition metabolism and the immune response of the host (Tremaroli and Bäckhed 2012). Members of Firmicutes play key roles in the degradation of polysaccharides (Flint et al. 2012); thus, the high abundance of Firmicutes in the gut may be related to the food sources and habitats of *M. minutus* (Hata 2011). At the genus level, *Bacteroides* and *Lactobacillus* were the

predominant genera, accounting for nearly half of the microbiota composition. The high abundance of *Bacteroides* and *Lactobacillus* is consistent with the results of another study on omnivorous mammals, including wild mice (*Apodemus sylvaticus*), bears, squirrels, and lemurs (Maurice et al. 2015). The next dominant genera were uncultured *Muribaculaceae*, which is a major component of the mouse gut microbiota and a member of the family *Muribaculaceae*, which was previously known as the S24-7 group (Lagkouvardos et al. 2019), and *Lachnospiraceae NK4A136 group*, a short-chain fatty acid-producing bacteria in the gut (S. Hu et al. 2019). Therefore, the components of the gut microbiota of *M. minutus* appear to be comparable to those of the gut microbiota of wild rodents reported in previous studies.

Notably, *Campylobacter* was the sixth most abundant genus in the microbiota of all *M. minutus* and varied among samples; this high abundance is inconsistent with previous studies on the microbiota of wild mice (Weldon et al. 2015; Maurice et al. 2015; Rosshart et al. 2017; 2019). Moreover, most *M. minutus* harbored *Campylobacter* in their gut metagenome. Of note, this high prevalence of *Campylobacter* in the gut microbiota is similar to that in poultry, which is known to harbor *Campylobacter* as part of the normal gut flora (O'Sullivan et al. 2000; Sahin, Morishita, and Zhang 2002; Humphrey 2006). Moreover, the concept of core microbiota considers not only the abundance but also the prevalence to identify microbial communities that exist persistently (Shade et al. 2012; Astudillo-García et al. 2017); thus, *Campylobacter* appears to be a member of the core microbiota of the gut of *M. minutus*. Furthermore, when laboratory mice are infected with *Campylobacter*, clinical signs of campylobacteriosis, such as a ruffled coat, hunched posture, lethargy, and diarrhea are observed (Stanfield,

McCardell, and Madden 1987; Mansfield et al. 2008; F. Liu et al. 2018). Therefore, if the high abundance and prevalence of *Campylobacter* in the gut microbiota of *M. minutus* were due to an external infection, there would have been clinical signs of campylobacteriosis in *M. minutus*; however, no clinical signs were observed in any captured mice. Considering the results of metagenome analysis and the absence of clinical signs, *Campylobacter* may exist as a normal component of the gut microbiota of *M. minutus*.

The core microbiota of *M. minutus* contained taxa that, in previous studies, were shown to be members of the microbiota of wild mice (*A. sylvaticus*) and laboratory mice, such as *Alistipes* (Maurice et al. 2015) and uncultured *Desulfovibrionaceae* (C. Zhang et al. 2010). Notably, *Helicobacter*, which can infect humans and other hosts (Bagheri et al. 2015; Tohidpour 2016) is also a member of the core microbiota of *M. minutus*. Previous studies suggested wild mice (*M. musculus molossinus* and *A. sylvaticus*) as a reservoir of diverse *Helicobacter* strains according to culture-dependent (Won et al. 2002) and culture-independent methods (Maurice et al. 2015); however, the possibility of *M. minutus* as a potential reservoir of other zoonotic pathogens has not been studied. Future studies using culture-dependent methods for further analyses, such as the isolation and characterization of pathogens, are needed to explore the potential of wild mice as a reservoir of other zoonotic pathogens.

Metagenomic analysis results showed that most of the captured *M. minutus* harbored *Campylobacter* in the gut metagenome, regardless of their culture status. Notably, most *M. minutus* that were determined to be *Campylobacter*-negative by culture-dependent methods harbored high proportions of *Campylobacter* in the gut metagenome, indicating that culture-dependent

methods alone cannot reliably indicate whether *Campylobacter* is present in the gut. This may be attributed to difficulties in the isolation of *Campylobacter* (as mentioned above) or the cultivation of *Campylobacter* may have been affected by components of the gut microbiota, such as competing flora that inhibit the growth of *Campylobacter* (Jasson et al. 2009; Hazeleger, Jacobs-Reitsma, and Besten 2016). Moreover, the difference in the microbiota composition between the culture-positive and -negative groups may have affected the isolation of *Campylobacter*. Beta diversity analysis, which showed that the microbiota of *M. minutus* was clustered by the *Campylobacter* culture results rather than by other factors such as gender or habitat, supported this possibility. Differential abundance analysis showed that *Lactobacillus* was the only significantly enriched genus in the culture-negative group compared to that in the culture-positive group. Previous studies revealed that the growth of *Campylobacter* in co-cultures of *Campylobacter* and *Lactobacillus* was significantly lower than that in a single culture of *Campylobacter*, indicating that *Lactobacillus* acts as an antagonist to reduce the level of *Campylobacter* in culture (Gang Wang et al. 2014; Taha-Abdelaziz et al. 2019). These results support the possibility that the relatively high abundance of *Lactobacillus* in the culture-negative group affected the isolation of *Campylobacter* during the culture procedures. As studies on the characteristics of *Lactobacillus* strains isolated from wild mice are lacking, further studies are needed to better understand the antagonistic activities of wild mice-derived *Lactobacillus* strains on *Campylobacter*.

The presence of *Campylobacter* in the gut of the two species of wild mice was also very distinctly different by species. Most *M. minutus* harbored *Campylobacter* in their gut, whereas none of the *M. musculus* harbored

Campylobacter in their gut. Notably, the presence of *Campylobacter* differed remarkably, despite the fact that the two species of mice were captured in adjacent areas. These results suggest that the different microbiota composition of the two species of wild mice may affect the colonization of *Campylobacter* in the gut. Recent studies showed that components of the gut microbiota provide colonization resistance to *Campylobacter* by competing for nutrition, by modulating the host immune response, and through direct antagonism (Neish 2009; O'Loughlin et al. 2015; Kampmann et al. 2016); thus, the components of the microbiota in wild *M. musculus* may have prevented the colonization of *Campylobacter* in their gut. Differential abundance analysis to identify significantly enriched taxa in *M. musculus* showed that *Lactobacillus* was the most enriched genus in *M. musculus*. Diverse *Lactobacillus* strains are known to reduce the colonization of *Campylobacter* in the gut (Alemka, Corcionivoschi, and Bourke 2012; Sicard et al. 2017); thus, highly abundant *Lactobacillus* may have played a role as a prophylactic agent against *Campylobacter* in the gut of *M. musculus*. Further studies are needed to demonstrate the interaction of the gut microbiota and colonization of *Campylobacter* in wild mice.

1.5 Conclusion

This study is the first to investigate the gut microbiota of *M. minutus* using metagenomics to explore its possible role as an environmental *Campylobacter* reservoir. This culture-independent approach indicated that wild *M. minutus* may serve as a reservoir of *Campylobacter*. Metagenomic analysis results revealed that most *M. minutus* harbored high proportions of *Campylobacter* in the gut microbiota regardless of culture status, indicating the necessity of using a culture-independent method together with traditional culture-dependent methods to precisely determine the presence of *Campylobacter*. Considering the high abundance and prevalence of *Campylobacter* in the gut microbiota, and the absence of clinical symptoms, *Campylobacter* may be a component of the normal gut flora of wild *M. minutus.* These findings provide a basis for future studies on the role of environmental reservoirs in the transmission cycle of *Campylobacter* using culture-independent methods.

Table 1. Information of wild mice used in this study.

Sample	Gender	Sampling Month	Sampling Year	Location	Species	*Campylobacter* culture result
M1	Male	4	2019	Dang-Rim	*Micromys minutus*	negative
M2	Female	4	2019	Madang-kyo	*Micromys minutus*	negative
M3	Male	4	2019	Madang-kyo	*Micromys minutus*	negative
M4	Male	4	2019	Dongsan-ri	*Micromys minutus*	negative
M5	Male	4	2019	Dongsan-ri	*Micromys minutus*	negative
M6	Male	4	2019	Dongsan-ri	*Micromys minutus*	negative
M7	Male	4	2019	Dongsan-ri	*Micromys minutus*	negative
M8	Male	4	2019	Dongsan-ri	*Micromys minutus*	negative
M9	Male	4	2019	Dang-Rim	*Micromys minutus*	positive
M10	Male	4	2019	Pal-Mi-gil	*Micromys minutus*	positive
M11	Male	4	2019	Pal-Mi-gil	*Micromys minutus*	positive
M12	Male	4	2019	Dongsan-ri	*Micromys minutus*	positive
M13	Male	4	2019	Dongsan-ri	*Micromys minutus*	positive
M14	Female	4	2019	Dongsan-ri	*Micromys minutus*	positive
M15	Female	4	2019	Dongsan-ri	*Micromys minutus*	positive
M16	Female	4	2019	Dongsan-ri	*Micromys minutus*	positive
M17	Female	4	2019	Dongsan-ri	*Micromys minutus*	positive
M18	Female	4	2019	Dongsan-ri	*Micromys minutus*	positive
MM1	Male	4	2017	Gunja-ri	*Mus musculus*	negative
MM2	Female	4	2017	Gunja-ri	*Mus musculus*	negative
MM3	Male	4	2017	Gunja-ri	*Mus musculus*	negative
MM4	Male	4	2017	Gunja-ri	*Mus musculus*	negative
MM5	Female	3	2017	Dongchon-ro	*Mus musculus*	negative
MM6	Female	3	2017	Gunja-ri	*Mus musculus*	negative
MM7	Baby	4	2017	Gunja-ri	*Mus musculus*	negative
MM8	Baby	4	2017	Gunja-ri	*Mus musculus*	negative

MM9	Baby	4	2017	Gunja-ri	*Mus musculus*	negative
MM10	Baby	4	2017	Gunja-ri	*Mus musculus*	negative
MM11	Male	4	2017	Yupo-ri	*Mus musculus*	negative
MM12	Female	4	2017	Yupo-ri	*Mus musculus*	negative
MM13	Female	4	2017	Boksa	*Mus musculus*	negative
MM14	Male	4	2017	Boksa	*Mus musculus*	negative
MM15	Male	4	2017	Yupo-ri	*Mus musculus*	negative
MM16	Female	4	2017	Yupo-ri	*Mus musculus*	negative
MM17	Male	4	2017	Joyang	*Mus musculus*	negative
MM18	Female	4	2017	Joyang	*Mus musculus*	negative
MM19	Male	4	2017	Boksa	*Mus musculus*	negative
MM20	Female	4	2017	Boksa	*Mus musculus*	negative

Table 2. Genera showing significant difference between wild *Micromys minutus* and *Mus musculus* in LEfSe analysis

Genus	p value	LDA score
Campylobacter	2.23E-06	5.3
Lachnospira	0.01192	4.83
Candidatus Arthromitus	1.64E-05	4.72
Rikenella	7.01E-06	4.53
Ruminiclostridium 5	0.00342	4.35
Desulfovibrio	0.019884	4.32
Candidatus Saccharimonas	0.013334	4.3
ASF356	3.77E-05	4.26
Millionella	6.65E-07	4.2
Brachyspira	0.000161	4.2
Lachnospiraceae UCG 001	0.005724	4.11
Eubacterium brachy group	0.00606	4.11
Mycoplasma	0.000161	4.06
Odoribacter	0.000475	3.98
Mucispirillum	0.009778	3.96
Ruminiclostridium 6	0.000161	3.95
Anaerotruncus	0.044704	3.95
GCA 900066575;Ambiguous taxa	0.001033	3.89
Butyricicoccus	0.037967	3.43
Candidatus Stoquefichus	0.028165	2.95
Anaeroplasma	0.048213	-3.21
Ruminococcaceae UCG 010	0.012864	-3.37
Escherichia Shigella	0.04817	-3.37
Ruminococcaceae UCG 013	0.048213	-3.39
Angelakisella	0.04817	-3.5
Intestinimonas	0.002221	-3.96
Tyzzerella	0.00186	-3.98
Gemella	5.25E-05	-4.15
Eubacterium coprostanoligenes group	0.000302	-4.18
Alistipes	2.05E-05	-4.26
Ruminiclostridium	0.009903	-4.59
Parabacteroides	0.014464	-4.59
Rikenellaceae RC9 gut group	6.65E-05	-4.61
Prevotellaceae UCG 001	0.003149	-4.76
Lactobacillus	0.017882	-5.94

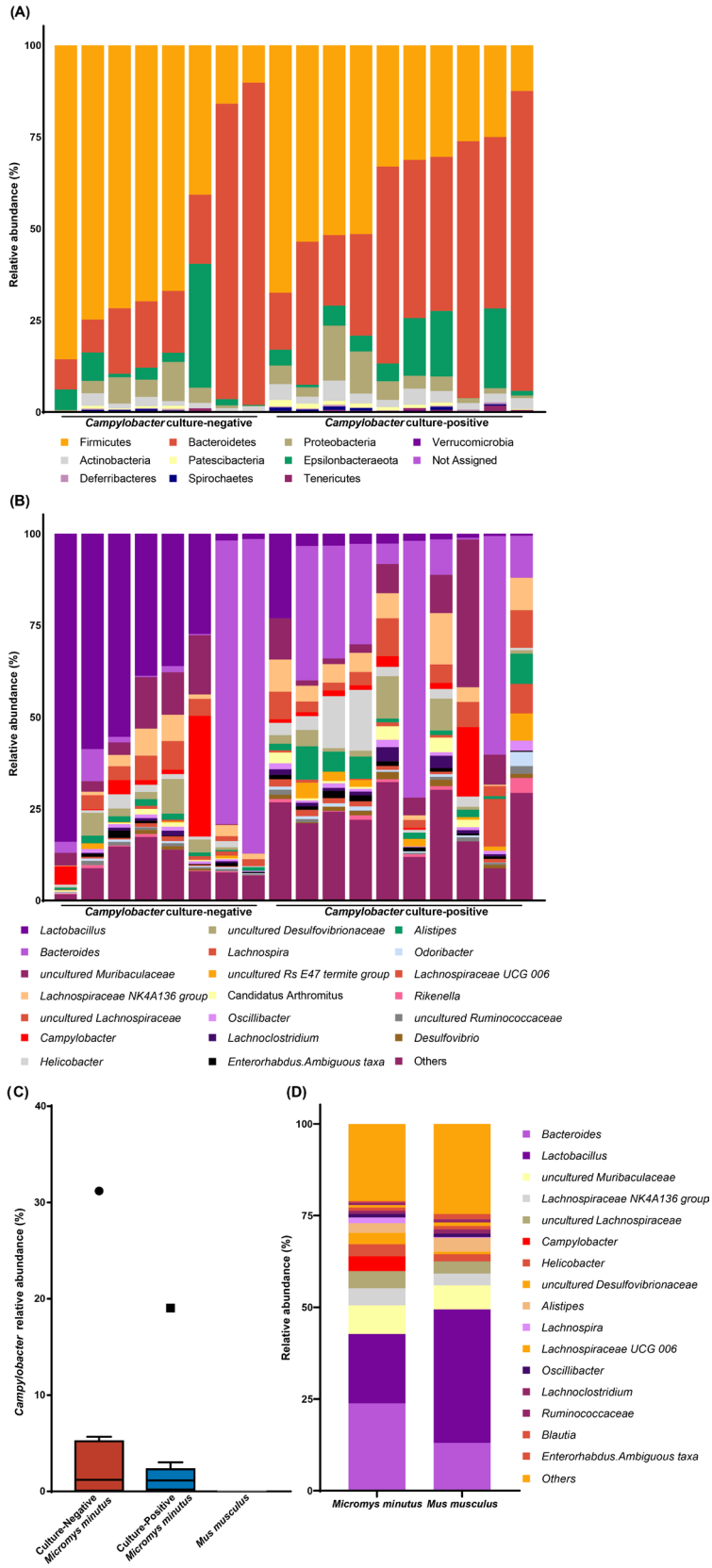

(A)
Relative abundance (%)
100
75
50
25
0
Campylobacter culture-negative
Campylobacter culture-positive
Firmicutes
Bacteroidetes
Proteobacteria
Verrucomicrobia
Actinobacteria
Patescibacteria
Epsilonbacteraeota
Not Assigned
Deferribacteres
Spirochaetes
Tenericutes
(B)
Relative abundance (%)
100
75
50
25
0
Campylobacter culture-negative
Campylobacter culture-positive
Lactobacillus
uncultured Desulfovibrionaceae
Alistipes
Bacteroides
Lachnospira
Odoribacter
uncultured Muribaculaceae
uncultured Rs E47 termite group
Lachnospiraceae UCG 006
Lachnospiraceae NK4A136 group
Candidatus Arthromitus
Rikenella
uncultured Lachnospiraceae
Oscillibacter
uncultured Ruminococcaceae
Campylobacter
Lachnoclostridium
Desulfovibrio
Helicobacter
Enterorhabdus.Ambiguous taxa
Others
(C)
Campylobacter relative abundance (%)
40
30
20
10
0
Culture-Negative Micromys minutus
Culture-Positive Micromys minutus
Mus musculus
(D)
Relative abundance (%)
100
75
50
25
0
Micromys minutus
Mus musculus
Bacteroides
Lactobacillus
uncultured Muribaculaceae
Lachnospiraceae NK4A136 group
uncultured Lachnospiraceae
Campylobacter
Helicobacter
uncultured Desulfovibrionaceae
Alistipes
Lachnospira
Lachnospiraceae UCG 006
Oscillibacter
Lachnoclostridium
Ruminococcaceae
Blautia
Enterorhabdus.Ambiguous taxa
Others

Figure 5. Taxonomic composition of the gut microbiota of wild mice. Taxonomy bar plot of the gut microbiota of *Micromys minutus* at the (A) phylum and (B) genus levels. (C) The relative abundance of *Campylobacter* in the gut microbiota of *Micromys minutus* and *Mus musculus*. The blue and orange boxes represent the relative abundance of *Campylobacter* in the *Campylobacter* culture-positive and culture-negative *M. minutus* groups. Circle (●) and square (▪) represent the maximum point of relative abundance of *Campylobacter*, respectively. (D) Taxonomic composition of gut microbiota of two species of wild mice (*Micromys minutus* and *Mus musculus*) at the genus level.

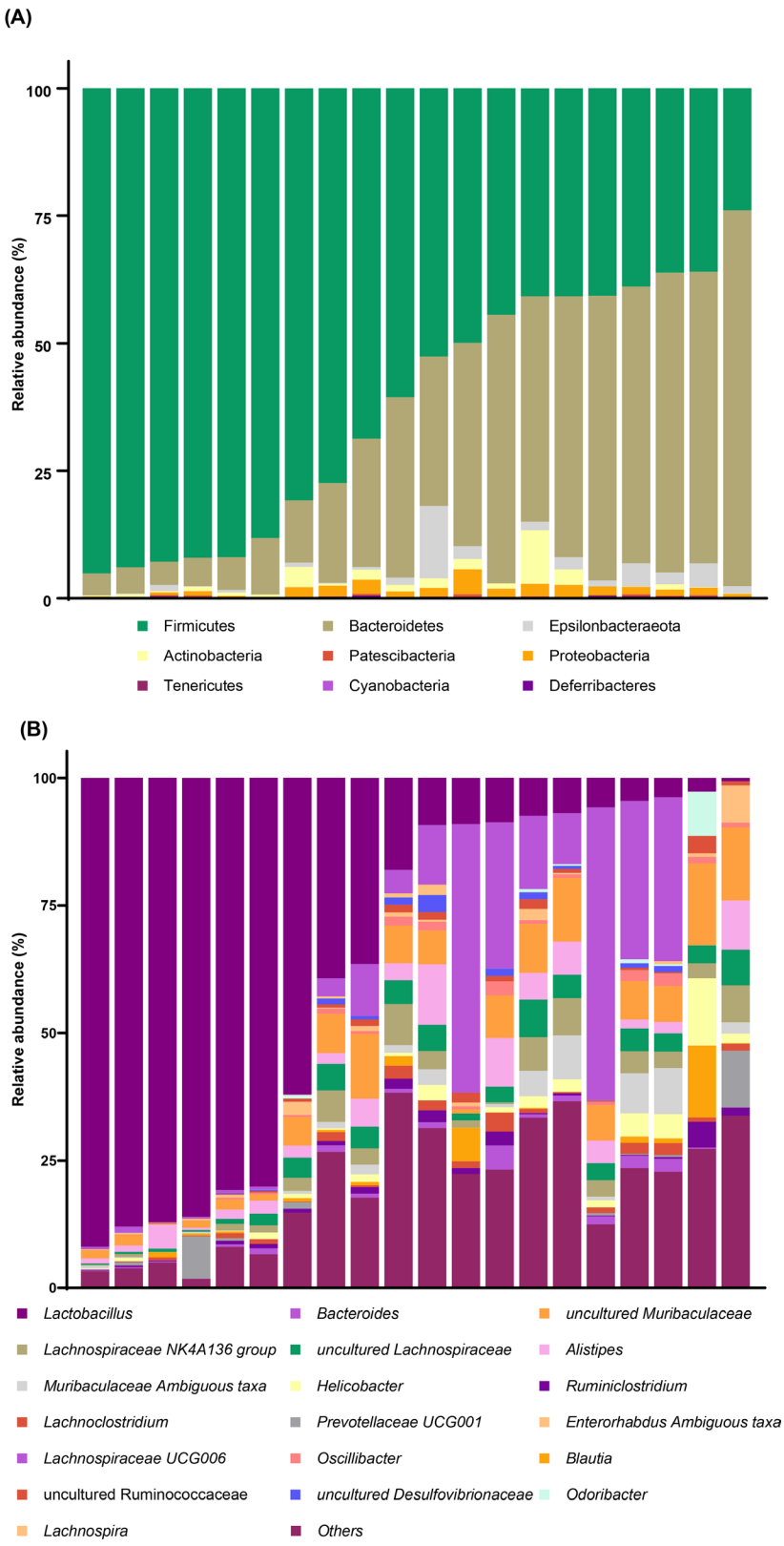

(A)
100
75
50
25
0
Relative abundance (%)
Firmicutes
Bacteroidetes
Epsilonbacteraeota
Actinobacteria
Patescibacteria
Proteobacteria
Tenericutes
Cyanobacteria
Deferribacteres
(B)
100
75
50
25
0
Relative abundance (%)
Lactobacillus
Bacteroides
uncultured Muribaculaceae
Lachnospiraceae NK4A136 group
uncultured Lachnospiraceae
Alistipes
Muribaculaceae Ambiguous taxa
Helicobacter
Ruminiclostridium
Lachnoclostridium
Prevotellaceae UCG001
Enterorhabdus Ambiguous taxa
Lachnospiraceae UCG006
Oscillibacter
Blautia
uncultured Ruminococcaceae
uncultured Desulfovibrionaceae
Odoribacter
Lachnospira
Others

Figure 6. Taxonomic composition of the gut microbiota of wild *Mus musculus.*

(A) taxonomy bar plot of wild *M. musculus* at the phylum and (B) and genus levels.

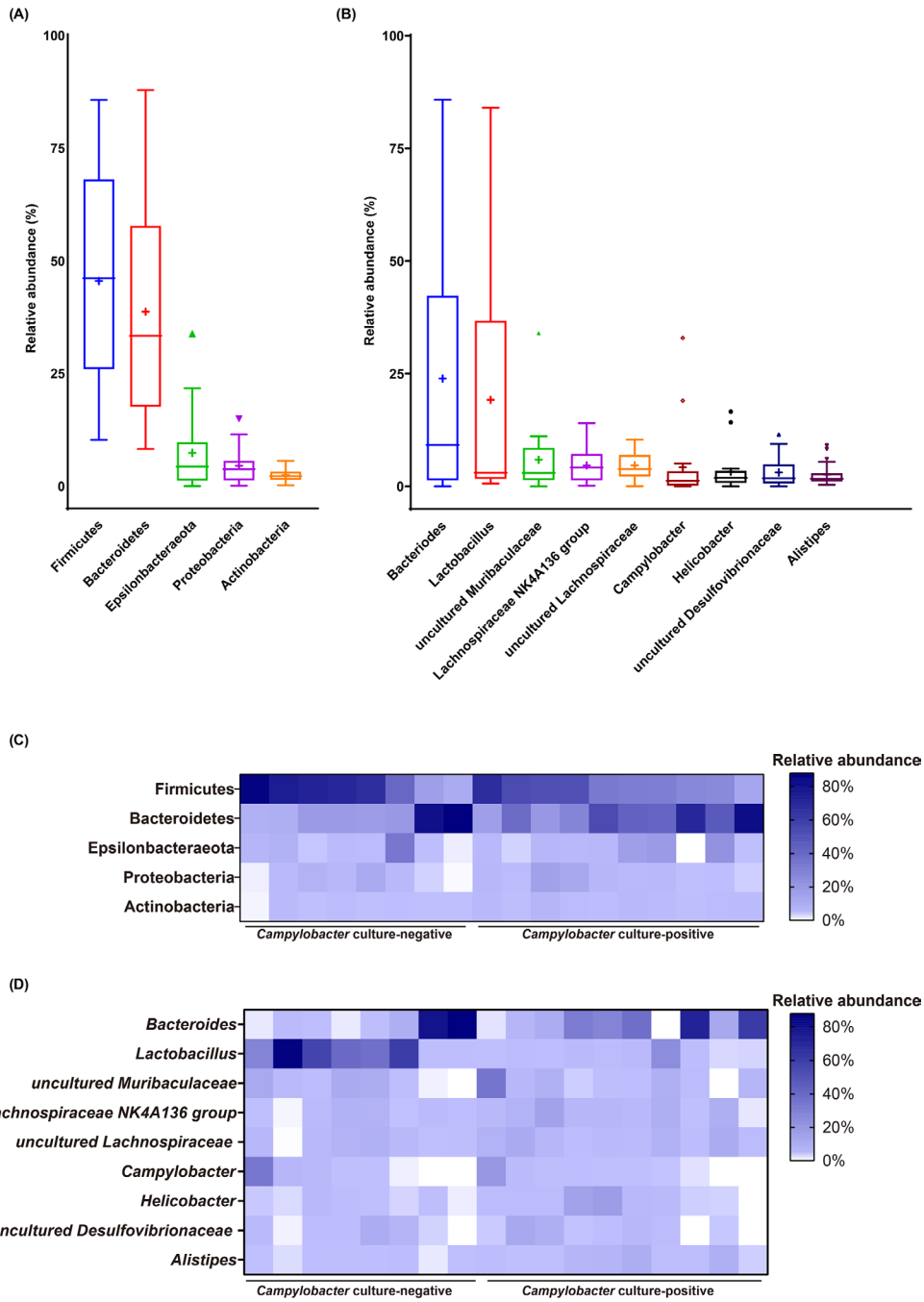

(A)
Relative abundance (%)
100
75
50
25
0
Firmicutes
Bacteroidetes
Epsilonbacteraeota
Proteobacteria
Actinobacteria
(B)
Relative abundance (%)
100
75
50
25
0
Bacteriodes
Lactobacillus
uncultured Muribaculaceae
Lachnospiraceae NK4A136 group
uncultured Lachnospiraceae
Campylobacter
Helicobacter
uncultured Desulfovibrionaceae
Alistipes
(C)
Relative abundance
80%
60%
40%
20%
0%
Firmicutes
Bacteroidetes
Epsilonbacteraeota
Proteobacteria
Actinobacteria
Campylobacter culture-negative
Campylobacter culture-positive
(D)
Relative abundance
80%
60%
40%
20%
0%
Bacteroides
Lactobacillus
uncultured Muribaculaceae
Lachnospiraceae NK4A136 group
uncultured Lachnospiraceae
Campylobacter
Helicobacter
uncultured Desulfovibrionaceae
Alistipes
Campylobacter culture-negative
Campylobacter culture-positive

Figure 7. Core gut microbiota of *Micromys minutus*. Box plots showing the relative abundance of the members of the core microbiota at the (A) phylum and (B) genus levels. Plus sign (+) represents the mean value. Heatmaps showing the relative abundance of core microbiota (C) at the phylum and (D) genus levels in individual M. minutus samples. The X-axis represents the individual samples of M. minutus. The Y-axis represents the core taxa. The color scale represents the relative abundance of core taxa in individual samples.

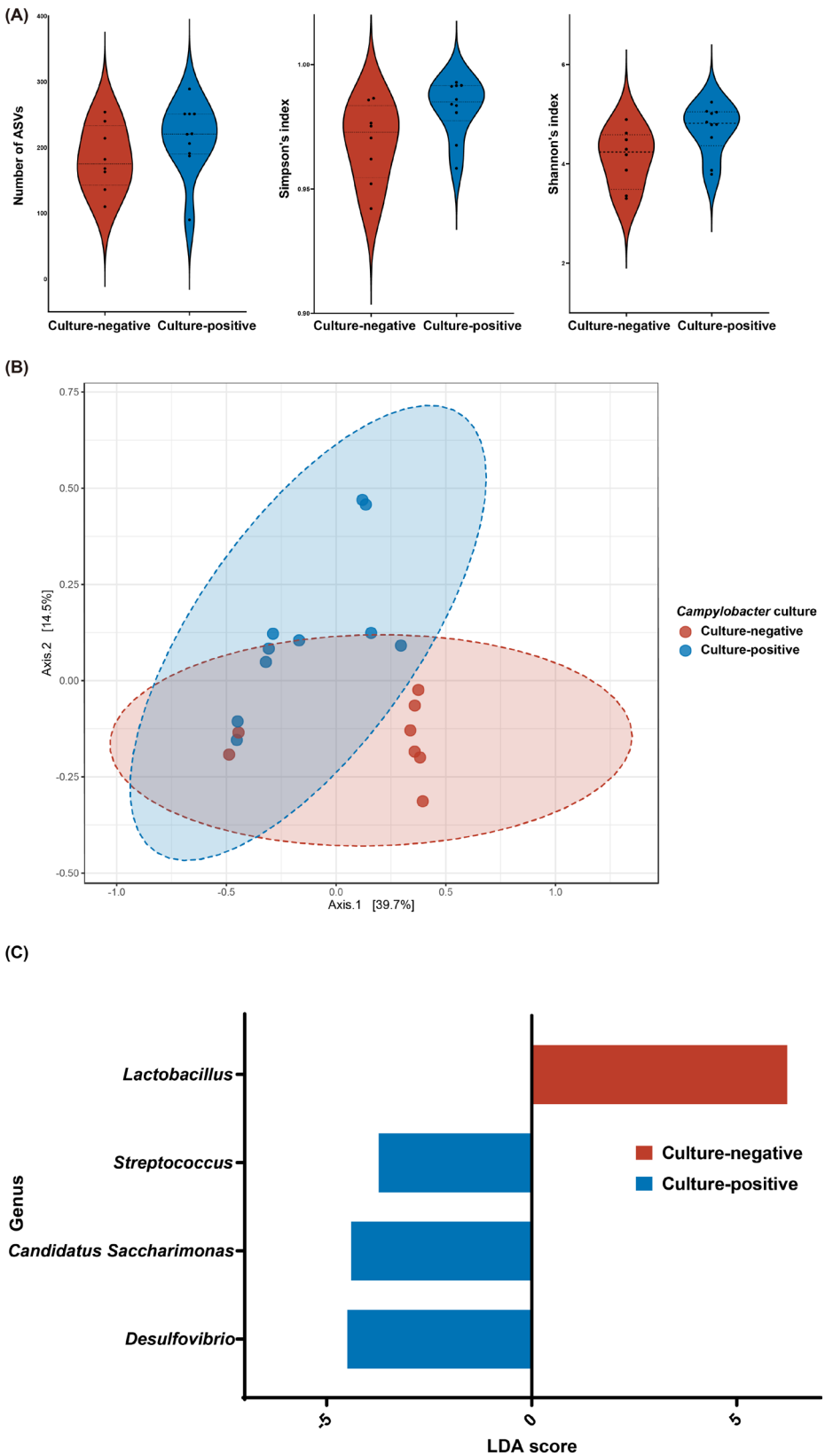
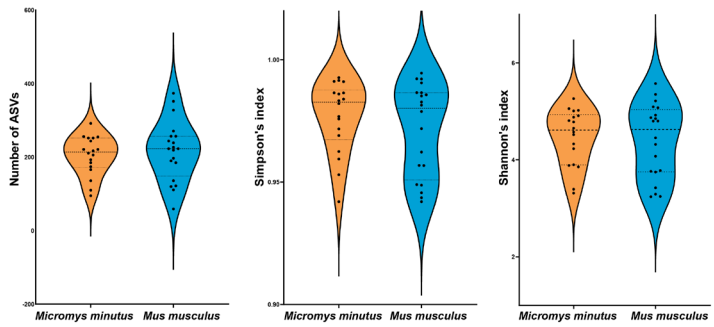
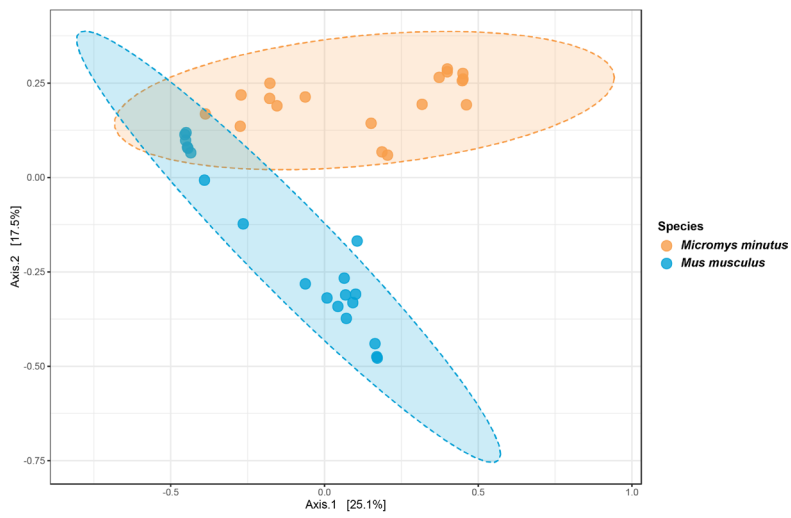

(A)
Number of ASVs
Simpson's index
Shannon's index
Culture-negative
Culture-positive
(B)
Axis.2 [14.5%]
Axis.1 [39.7%]
Campylobacter culture
Culture-negative
Culture-positive
(C)
Genus
Lactobacillus
Streptococcus
Candidatus Saccharimonas
Desulfovibrio
Culture-negative
Culture-positive
LDA score

Figure 8. Differences in the gut microbiota of *Micromys minutus* according to *Campylobacter* culture status. (A) Alpha diversity of the gut microbiota of two groups of *Micromys minutus*. The distribution of the number of observed amplicon sequence variants, the Simpson's index and the Shannon's index of each group is shown in the box plot. The blue box denotes the *Campylobacter* culture-positive group, and the red box denotes the *Campylobacter* culture-negative group. (B) Principle coordinate analysis plot of Bray-Curtis dissimilarity between the gut microbiota of the *Campylobacter* culture-negative and -positive groups of *M. minutus*. Ellipses indicate 95% confidence intervals. (C) Histograms of the linear discriminant analysis scores for genera with differential abundance identified using linear discriminant analysis effect size in a culture-positive (blue) and culture-negative (red) group of *Micromys minutus*.

(A)


Number of ASVs
Simpson's index
Shannon's index
Micromys minutus
Mus musculus

(B)


Axis.2 [17.5%]
Axis.1 [25.1%]
Species
Micromys minutus
Mus musculus

(C)

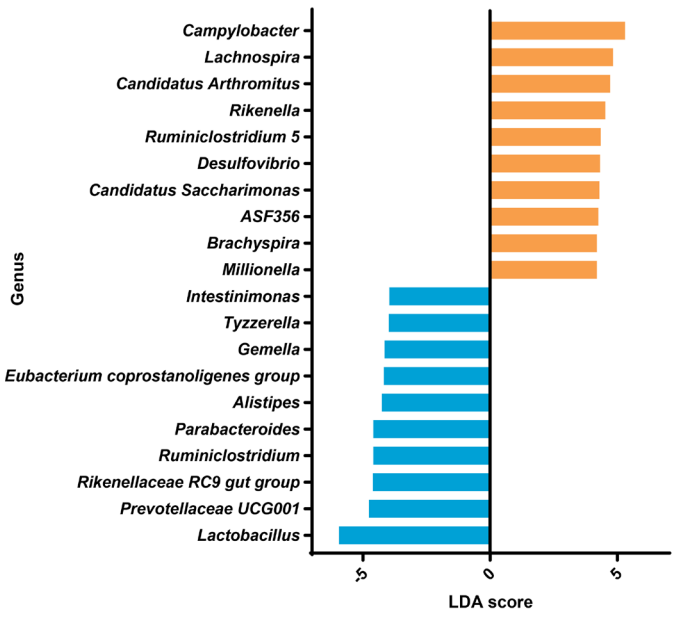

Genus
Campylobacter
Lachnospira
Candidatus Arthromitus
Rikenella
Ruminiclostridium 5
Desulfovibrio
Candidatus Saccharimonas
ASF356
Brachyspira
Millionella
Intestinimonas
Tyzzerella
Gemella
Eubacterium coprostanoligenes group
Alistipes
Parabacteroides
Ruminiclostridium
Rikenellaceae RC9 gut group
Prevotellaceae UCG001
Lactobacillus
LDA score

Figure 9. Differences in the gut microbiota of two species of wild mice. (A) Alpha diversity of the gut microbiota of two species of wild mice. The distribution of the number of observed amplicon sequence variants, the Simpson's index and the Shannon's index of each group is shown in the box plot. (B) Principle coordinate analysis plot of Bray-Curtis dissimilarity between the gut microbiota of *Micromys minutus* (orange) and *Mus musculus* (blue). Ellipses indicate 95% confidence intervals. (C) Histograms of the linear discriminant analysis scores for genera with differential abundance identified using linear discriminant analysis effect size in *M. minutus* (orange) and *M. musculus* (blue).

Chapter II

Environmental Perturbations during the Rehabilitation of Wild Migratory Birds Induce Gut Microbiome Alteration and Antibiotic Resistance Acquisition

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Abstract

Wild migratory birds are essential for sustaining healthy ecosystems, but the effects of a rehabilitation period on their gut microbiomes are still unclear. Here, the present study performed longitudinal sampling, 16S rRNA sequencing and antibiotic resistance monitoring of the gut microbiome of six species of wild migratory birds protected as natural monuments in South Korea subject to short- or long-term rehabilitation periods. Overall, gut microbiome diversity was significantly decreased in the early stages of rehabilitation and it did not recover to a level comparable to that of wild birds. Moreover, while the abundance of short-chain fatty acid-producing bacteria decreased, that of zoonotic pathogens increased indicating rehabilitation-induced dysbiosis. The metabolic pathways involved in the degradation of aromatic pollutants were significantly downregulated, suggesting the depletion of pollutant-degrading microorganisms. Antibiotic resistance of *Escherichia coli* significantly increased during rehabilitation, particularly ciprofloxacin and tetracycline resistance, and seven of the rehabilitated wild birds acquired multi-drug resistance. The diet and habitat changes experienced by wild migratory birds during rehabilitation may have induced the observed gut microbiome dysbiosis and acquisition of antibiotic resistance. These rehabilitation-induced alterations might affect the adaptability of wild birds to their natural environments and contribute to the spread of antibiotic resistance after their release.

Keywords: Rehabilitation, Microbiota, Dysbiosis, Wild birds, Antibiotic resistance

2.1 Introduction

Wild migratory birds play an essential role in sustaining a healthy ecosystem. For instance, wild migratory birds contribute to shaping the distribution of global biodiversity, as they are involved in the long-distance dispersal of various organisms, including seeds and microorganisms (Viana et al. 2016). Moreover, as they may frequently interact with humans, they are sensitive to human activities (Grant, Todd, and Pennycott 2007; Magle et al. 2012). Owing to their high position in the food chain and sensitivity to both natural and anthropogenic environmental changes, wild migratory birds are recognized as highly effective indicators of biodiversity (Gregory and Strien 2010). However, migratory birds are facing an ever-growing anthropogenic threat due to the increasing modifications of their natural habitats and global climate change (Hutchins et al. 2018). Consequently, several migratory bird species have been classified as endangered by the International Union for Conservation of Nature (IUCN), or as natural monuments (Kirby et al. 2008; J. H. Kang et al. 2016; Hutchins et al. 2018). Therefore, an improved understanding of the impact of human activities on wild migratory birds is required for the development of sustainable conservation strategies.

The gut microbiome is a symbiotic community of microorganisms, including bacteria, fungi, and viruses, and their genomes (Goodrich, Di Rienzi, et al. 2014; Kundu et al. 2017; Paez-Espino et al. 2016). It is widely recognized that the gut microbiome is closely associated with host fitness, including its genetics, digestion, immune response, metabolic functions, and pathogen resistance, via complex host-microorganism interactions (Ley et al. 2006; J. L. Sonnenburg and Bäckhed 2016; S. G. Kim et al. 2019; Song et al. 2021). The gut microbiome of

birds is likely to differ from that of mammals because birds have a unique digestive, reproductive, and immune system (Videvall et al. 2018). However, similar to the gut microbiome of other animals, that of birds also consists of beneficial, commensal, and pathogenic microorganisms, and it is shaped by various factors, including host genetics, diet, behavior, and environment. As most studies on the gut microbiome of wild birds have been conducted in natural populations (J. Cao et al. 2020; Hird et al. 2018; Fuentes-Castillo et al. 2019; Y. Lin et al. 2020), the effect of human activities on the dynamics of the gut microbiome of wild birds has not yet been fully understood.

Wildlife rehabilitation is a human-related activity in which a variety of wild animal species are bred or grown and then reintroduced into their natural habitats; this is mostly performed to prevent species extinction and for biodiversity monitoring (Mullineaux 2014). In the rehabilitation center, the environmental factors that shape the gut microbiome of wild birds, such as diet and habitat, differ from those in the natural habitat. As the gut microbiome is associated with host fitness, changes in the gut microbiome induced during the rehabilitation process may have profound effects on the birds' adaptability to the wild environment after they are released. Indeed, recent studies have reported a shift in the gut microbiome of wild animals due to rehabilitation (Ahasan et al. 2018; Samuelson et al. 2020; Bloodgood et al. 2020; Yan et al. 2021). However, most studies on the effects of rehabilitation on the dynamics of the gut microbiome have been conducted on mammals and reptiles; hence, the impact of rehabilitation on wild birds remains unclear.

It is essential to improve the understanding of the dynamics of the gut microbiome of wild birds in response to rehabilitation to establish a sustainable

conservation strategy for these species. Thus, in the present study, the author investigated the effect of rehabilitation on the gut microbiome of wild migratory birds. The present study performed longitudinal sampling, 16S rRNA sequencing analysis of the gut microbiome, and antibiotic resistance monitoring on six species of wild migratory birds that are protected as natural monuments in South Korea. Individuals of *Falco tinnunculus*, *Falco subbuteo*, *Otus bakkamoena*, *Otus scops*, *Ninox scutulata,* and *Accipiter gentilis* kept in a rehabilitation center were evaluated from the wild state (immediately after rescue) to the release state (immediately before release). The present study revealed the dynamics of the gut microbiome, including changes in the taxonomic composition, diversity, bacterial network, and potential metabolic pathways, as well as changes in antibiotic resistance, which may affect host fitness after release to the natural habitat. This study provides information for developing sustainable rehabilitation strategies for wild birds.

2.2 Materials and methods

Rehabilitation and gut microbiome sampling of wild migratory birds

Orphaned wild migratory birds were rescued in Seoul, South Korea and immediately transferred to the Seoul Wildlife Rehabilitation Center. They were kept in individual cages, following the rehabilitation manual of the Seoul Wildlife Center. One-day-old chicks (sourced from the poultry industry) and captive-bred quails were provided to birds that were alert and had the ability to feed themselves. The health status and behavior of the rescued wild birds were monitored daily by veterinary staff at the Seoul Wildlife Center. Injured and convalescent wild birds, birds showing abnormal clinical signs, and juvenile birds not capable of self-feeding were excluded from the study. Thus, all birds used in the present study were clinically healthy. When birds were physically and behaviorally able to forage and breed in the wild, and therefore considered ready for release by veterinarians, they were released into a native forest or park. Wild birds that were rehabilitated for less than 4 weeks were considered to have undergone short-term rehabilitation, while those that were rehabilitated for four weeks or more, were considered to have undergone long-term rehabilitation. Information regarding the wild birds used in the present study is provided in Table 3.

Fecal samples of the wild birds were collected by veterinarians, immediately transferred to the laboratory, and processed for gut microbiome 16S rRNA sequencing analysis and antibiotic resistance monitoring. Sample collection was performed in the wild state, within the first two weeks of rehabilitation, and then at four-week intervals until release. Fifty fecal samples were used for DNA extraction and microbiome 16S rRNA sequencing analysis (Figure 10).

DNA extraction, library preparation, and 16S rRNA sequencing

Fecal DNA extraction and sequencing were performed as previously described (Song et al. 2021). Briefly, DNA was extracted from fecal samples using a Fast DNA Soil kit (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. Sequencing of the *16S rRNA* V3-V4 hypervariable gene region was performed using the primers 341F and 805R from Illumina Inc. (San Diego, CA, USA). PicoGreen was used for pooling and normalizing the amplified products. All sequencing procedures were conducted using the Illumina MiSeq platform at Macrogen, Inc. (Seoul, Korea).

Bioinformatics and statistical analyses

Bioinformatics of the sequence data was performed using the QIIME2 (version 2021.02) software package (Bolyen, Rideout, Dillon, Bokulich, Abnet, Caporaso, et al. 2019). Raw sequence data were filtered, dereplicated, and denoised to generate ASV tables using DADA2 as implemented in QIIME2 (Callahan et al. 2016a). A phylogenetic tree of the ASVs was generated using MAFFT (https://mafft.cbrc.jp/alignment/software/). The taxonomy profile of ASVs was generated using the q2-feature-classifier implemented in QIIME2 against the SILVA database (version 138, Ref NR99) (Bokulich et al. 2018). Sequence data were normalized using the rarefaction to the minimum library size method for downstream analysis.

Downstream analysis of sequence data was performed using the MicrobiomeAnalyst R package (Dhariwal et al. 2017). The alpha diversity of the microbiome was measured using the number of observed ASVs and Shannon's

index. The significance of differences in alpha diversity was evaluated using the Mann-Whitney U test for inter-group comparisons and the Wilcoxon test for paired samples. The beta diversity of the microbiome was measured using the unweighted UniFrac distance, followed by PERMANOVA to evaluate significant differences in beta diversity. Differential abundance analysis of the gut microbiome and its metabolic pathways was performed using edgeR (Robinson, McCarthy, and Smyth 2009). A co-occurrence network was constructed using network analysis for metagenomic abundance profiles (NAMAP) based on Pearson's correlations, using the MetagenoNets tool (Yadav, Ghosh, and Mande 2016; Nagpal et al. 2020) with $r > 0.7$ and $p < 0.05$ as the cut-off values for significant correlations. Metabolic pathways were analyzed using the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) (Douglas et al. 2020) and the MetaCyc database (https://metacyc.org).

Isolation of *Escherichia coli* for antibiotic susceptibility tests

To isolate *E. coli* for monitoring antibiotic resistance in wild birds during rehabilitation, fecal swabs from 17 wild birds in the wild state and release state were inoculated into 2 mL of *E. coli* broth (Oxoid, Basingstoke, UK) and enriched overnight at 37°C. After enrichment, 100 μL of the culture broth was spread on MacConkey agar (Oxoid) and incubated at 37°C for 24 h. Cultures were then streaked on eosin methylene blue agar (BD, Sparks, MD, USA) and colonies exhibiting the culture characteristics of *E. coli* were pure-cultured and confirmed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. As a result, *E. coli* was isolated from 15 birds and further analyzed for antibiotic susceptibility.

Disk diffusion susceptibility tests (Kirby-Bauer method) were conducted for eight antibiotics: amoxicillin/clavulanic acid (20/10 μg), ampicillin (10 μg), cefotaxime (30 μg), imipenem (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), colistin (10 μg), and cefoxitin (30 μg). Antibiotic susceptibility results were interpreted following the Clinical and Laboratory Standards Institute guidelines.

2.3 Results

Taxonomic composition of the gut microbiome of wild birds in wild and release states

The overall scheme of sampling and study design is shown in Figure 10. To understand how the gut microbiome changes during rehabilitation, the author first analyzed its taxonomic composition both in the wild and release states. The taxonomic composition of the gut microbiome at the phylum and genus levels is shown in Figure 11.

Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, Fusobacteriota, and Patescibacteria were the six dominant phyla in both the wild and release states (accounting for 97.72–100% and 93.00–100% of total abundance, respectively). In the wild state, Firmicutes was the most enriched phylum across all samples (average, 50.79%), followed by Proteobacteria (average, 24.33%) and Bacteroidetes (average, 10.70%). In contrast, in the release state, Proteobacteria was the most enriched phylum across all samples (average, 51.16%), followed by Firmicutes and Bacteroidetes (averages of 30.00% and 9.41%, respectively).

The dominant genera differed between the wild and release states. For instance, *Ralstonia* and *Enterococcus* were the most enriched genera in the wild state (averages of 15.00% and 13.88%, respectively), followed by *Staphylococcus* and *Bacteroides* (averages of 9.92% and 9.26%, respectively). In the release state, *Escherichia-Shigella* and *Ralstonia* were the most enriched genera (averages of 25.27% and 21.11%, respectively), followed by *Bacteroides* and *Enterococcus* (averages of 9.40% and 6.42%, respectively).

Rehabilitation induces the rapid and irreversible decrease of the gut microbiome alpha diversity

The present study investigated the dynamics of alpha diversity during rehabilitation to determine whether dysbiosis of the gut microbiome occurred. The present study analyzed the shift in the alpha diversity of the gut microbiome based on two indices: the number of amplicon sequence variants (ASVs) and Shannon's index. Both showed significantly lower values ($p < 0.05$) in release birds than in wild birds (Figure 12A, B). Notably, the Wilcoxon test for paired samples showed that the values of both alpha-diversity indices decreased significantly ($p < 0.05$) during long- and short-term rehabilitation (Figure 12C, D). However, the decrease in the values of both alpha-diversity indices was not significantly different ($p > 0.05$) between the short- and long-term rehabilitation groups (Figure 12E, F). Longitudinal analysis revealed that the alpha diversity of the gut microbiome significantly decreased ($p < 0.05$) in the first two weeks of rehabilitation, and it did not recover to the wild state level during long-term rehabilitation (Figure 12G, H).

Shifts in the gut microbiome beta diversity during rehabilitation

Shifts in beta diversity were evaluated using principal coordinate analysis (PcoA) based on the unweighted UniFrac distance followed by permutational multivariate analysis of variance (PERMANOVA). There was a significant difference in the gut microbiome composition between the wild and release states ($p < 0.05$). Moreover, samples taken from wild birds immediately before their release were clustered, regardless of the period of rehabilitation (Figure 13A).

To explore the specific components of the gut microbiome that

contributed to this shift, and thus to the disruption of the microbiome composition and function (dysbiosis), the present study performed differential abundance analysis between the wild and release states. Proteobacteria was the only phylum significantly enriched in the release state compared to the wild state (adjusted $p < 0.05$). Twelve genera (Figure 13B), namely *Brachybacterium*, *Alistipes*, *Fournierella*, *Parabacteroides*, the *Ruminococcus torques* group, the *Eubacterium coprostanoligenes* group, *CHCKI001*, *Blautia*, *Sutterella*, *Rubrobacter*, *Reyranella*, and *ASF356*, were significantly decreased in the release state compared to the wild state (adjusted $p < 0.05$). Contrastingly, only five genera (*Atopostipes*, *Escherichia-Shigella*, *Campylobacter*, *Lactobacillus*, and *Peptoclostridium*) were significantly enriched in the release state compared to the wild state (adjusted $p < 0.05$).

Shifts in the gut microbiome ecological interactions during rehabilitation

To elucidate the shifts in the ecological interactions among gut microorganisms during rehabilitation, co-occurrence networks were constructed for the wild and release states. Seventy-nine and 62 genera (nodes) were considered significant ($p < 0.05$, $r > 0.7$) in the wild and release state networks, respectively (Figure 14A). Moreover, 1814 correlations (edges) were observed in the wild state network, whereas 1208 edges were observed in the release state network. The networks of the gut microbiome in the wild and release states shared 52 nodes and 483 edges (Figure 14B). The numbers of unique edges and nodes in the network of the gut microbiome in the wild state were 27 and 1331, respectively, while the network of the gut microbiome in the release state comprised ten unique edges and

725 unique nodes. Compared to the wild state, ecological interactions between the gut microorganisms were attenuated in the release state, as shown by the decrease in average degree values from 45.35 to 38.35. The corresponding correlograms for the networks of the gut microbiome in the wild and release states are shown in Figure 14C. Detailed information on the nodes and edges is provided in Tables 4.

Shifts in the gut microbiome metabolic pathways during rehabilitation

The impact of rehabilitation on the metabolic pathways of the gut microbiome was analyzed using PICURSt2 software (Figure 15A, B). Differential abundance analysis showed that six metabolic pathways, including aromatic compound degradation, nucleoside and nucleotide degradation, glycan biosynthesis, fatty acid and lipid degradation, and cell structure biosynthesis, were significantly enriched (adjusted $p < 0.05$) in the wild state. On the other hand, 11 metabolic pathways, including carbohydrate biosynthesis, cofactor, carrier, and vitamin biosynthesis, lipopolysaccharide biosynthesis, glycan biosynthesis, amino acid degradation, fatty acid and lipid biosynthesis, amine and polyamine degradation, and carbohydrate biosynthesis, were significantly enriched (adjusted $p < 0.05$) in the release state.

Wild birds acquire antibiotic resistance during rehabilitation

To explore if antibiotic resistance of the gut microbiome of wild birds shifted during rehabilitation, the author isolated *Escherichia coli* from fecal samples. The 30 *E. coli* strains isolated from 15 of the 17 wild birds (one isolate each per bird for the wild and release states) were then tested for antibiotic

susceptibility. In the wild state, *E. coli* showed the highest resistance rate to ampicillin (46.67%), followed by tetracycline (33.33%), amoxicillin (13.33%), and ciprofloxacin (6.67%). In the release state, *E. coli* showed the highest resistance rate to ampicillin and tetracycline (both at 66.66%), followed by ciprofloxacin (60.00%), amoxicillin (26.67%), and cefotaxime (6.67%). *E. coli* showed no resistance to colistin, imipenem, and cefoxitin in both the wild and release states (Figure 16A). Antibiotic-resistant scores, determined by the number of antibiotic resistance phenotypes, were significantly increased (Wilcoxon test, $p < 0.05$) in the release state compared to that in the wild state (Figure 16B). Notably, seven (87.5%) of the eight *E. coli* strains with no antibiotic resistance in the wild state acquired multi-drug resistance in the release state (Figure 16C).

2.4 Discussion

As the host gut microbiome is closely associated with host fitness, it is essential to better understand the influence of wildlife rehabilitation procedures on the dynamics of the gut microbiome to establish sustainable rehabilitation strategies. The present study aimed to explore the impact of rehabilitation on the gut microbiome of wild migratory birds, as it may affect their adaptability after being released into natural habitats. The present study hypothesized that environmental stress during rehabilitation may induce gut microbiome dysbiosis and increase antibiotic resistance in wild migratory birds. Therefore, the present study investigated the dynamics of the gut microbiome of wild migratory birds using longitudinal sampling, 16S rRNA sequencing and antibiotic resistance monitoring.

In the present study, the overall taxonomic composition of the gut microbiome of birds in the wild and release states differed at both the phylum and genus levels. The phylum Firmicutes, which is involved in the metabolism of carbohydrates, polysaccharides, and fatty acids (Kundu et al. 2017; Mahowald et al. 2009) was the most dominant in the wild state, coherent with previous findings (J. Cao et al. 2020; Teyssier et al. 2018). However, during rehabilitation, the phylum Proteobacteria increased significantly, and it constituted a dominant proportion of the gut microbiome of wild birds in the release state. Indeed, a high abundance of Proteobacteria is an indicator of gut microbiome dysbiosis and epithelial dysfunction (Litvak et al. 2017). The present results, therefore, support that rehabilitation can lead to gut microbiome dysbiosis.

In the present study, the dynamics of alpha diversity demonstrated rapid

and irreversible dysbiosis during rehabilitation. This dysbiosis of the gut microbiome during rehabilitation may be due to alterations in the diet and/or habitat of the wild birds at the rehabilitation center. The wild bird species investigated in the present study primarily hunt and feed on different vertebrates and invertebrates, ranging from insects to large animals, thereby having a highly diverse diet (Navarro-López and Fargallo 2015). However, during rehabilitation, they were only fed chicks, which is far from representing their diets in the wild. Dietary modifications in rehabilitation centers excluding the diverse components from the wild environment may therefore deplete certain microorganisms by purging necessary nutrients, resulting in the decreased species richness of the gut microbiome. Previous studies have reported that diet alteration induces a rapid shift in the composition and diversity of the gut microbiome, which is not recoverable even after the reintroduction of the original diet (David et al. 2014; Carmody et al. 2015; E. D. Sonnenburg et al. 2016). In the present study, wild birds were kept in cages at the rehabilitation center, wherein the conditions differed from those in their wild habitat. This may have contributed to the shift of the gut microbiome during rehabilitation, as birds in their wild habitats are exposed to diverse microorganisms and environmental factors, all of which are involved in shaping the gut microbiome. Consistent with the present results, previous studies have shown that habitat changes significantly alter the gut microbiome of wild animals (Y. Wu et al. 2018; Lees et al. 2014). Overall, the present study indicates that environmental stresses during rehabilitation, including alterations in diet and habitat, may have induced dysbiosis of the gut microbiome of wild migratory birds.

Notably, most of the microbes that significantly decreased after rehabilitation were SCFA-producers, such as *Parabacteroides* and *Blautia*. SCFAs

are generated by the fermentation of carbohydrates and are essential for gut integrity and host health (Cani et al. 2019). A decrease in SCFA-producing bacteria in the gut microbiome is associated with various physiological and metabolic disorders due to the loss of gut integrity (Dalile et al. 2019). Thus, the present results suggest that the decrease in the SCFA-producing bacteria in the gut microbiome during rehabilitation may negatively affect the fitness of wild migratory birds after their release into the wild environment. Dietary modifications are the major factors associated with a decrease in SCFA-producing bacteria in the gut (Nogal, Valdes, and Menni 2021). For birds, insects are major dietary sources of SCFA-producing bacteria (Borrelli et al. 2017; Biasato et al. 2018; Józefiak et al. 2020). As the birds used in the present study were only fed chicks during rehabilitation, the observed decrease in SCFA-producing bacteria may be due to the lack of diet variability, which resulted in a lack of nutrients for the growth of these bacteria.

Bacteria that were enriched in the release state were mainly zoonotic pathogens, such as *Campylobacter* and *Peptoclostridium*, indicating that pathogenic species were able to colonize the gut of wild migratory birds during rehabilitation. If an external infection was the source of this increase in zoonotic pathogens, clinical signs, such as lethargy, diarrhea, or behavioral changes, would have been observed; however, none of the birds enrolled in the present study showed obvious signs of infection. Considering the absence of clinical signs and the results of the 16S rRNA sequencing analysis, these pathogens may have colonized the gut of wild migratory birds during rehabilitation as common members of the gut microbiome. The present result is consistent with that of a previous study, which showed that several zoonotic pathogens, such as

Campylobacter, increased in the gut microbiome of wild animals during rehabilitation (Ahasan et al. 2018). This may be due to 1) a decrease in SCFA-producing bacteria and/or 2) a modified diet during rehabilitation. SCFA-producing bacteria are known to inhibit colonization by pathogens, such as *Campylobacter* and *Peptoclostridium,* by directly regulating microorganism-microorganism interactions and indirectly regulating host-microorganism interactions (Luethy et al. 2017; Hayashi et al. 2021). Thus, the decrease in SCFA producers may have played a key role in the overgrowth of zoonotic pathogens in the guts of wild migratory birds. Moreover, chicks and quails commonly harbor *Campylobacter* and *Peptoclostridium* in their gut microbiome (Elokil et al. 2020; Fu et al. 2018). As wild birds in the present study were fed on whole carcasses of chicks and quails during rehabilitation, the components of the gut microbiomes of these species, including *Campylobacter and Peptoclostridium,* may have been transmitted to the wild migratory birds. The enrichment of zoonotic pathogens in the gut microbiomes of wild birds after rehabilitation may increase their potential to be transmitted to humans and other animals, with migratory birds serving as the link between wildlife and human communities after being released into the wild.

The present study demonstrated that the complexity of microbial interactions, which can be inferred by the number of nodes and edges, decreased during rehabilitation. This may be due to the decreased species richness induced by rehabilitation, as shown by the alpha- and beta-diversity analyses of the gut microbiome. Because several species were depleted, several nodes with significant correlations were lost. A previous study showed that environmental stress reduces the complexity of the microbiome network, supporting the present findings (Hernandez et al. 2021). Notably, the present study showed that the unique nodes

observed in the wild state mainly included SCFA-producers. This is consistent with the present differential abundance analysis results, which showed that SCFA producers were depleted during rehabilitation along with the complex microorganism-microorganism interactions they mediate. As microbial interactions mediated by SCFA-producers are associated with a wide range of host fitness factors, including immune and metabolic functions (Dugas et al. 2018; X. Cao et al. 2021), this depletion of the microbiome network may negatively affect wild birds after their release.

The metabolic pathways that were the most affected during rehabilitation were those involved in the degradation of aromatic compounds. Aromatic compounds are the most widespread and abundant pollutants in the natural environment and diet of wild birds (Xu et al. 2013; Seo, Keum, and Li 2009). Wild birds, particularly birds of prey, have the potential to accumulate high concentrations of aromatic compounds in wild environments and therefore harbor microorganisms that are able to biodegrade these compounds in their gut (Custer et al. 2017; Fernie et al. 2018; Salgado-Flores et al. 2019). Thus, the enriched metabolic pathways involved in aromatic compound degradation in the wild state may reflect a strategy used by wild migratory birds to survive in the wild environments. However, the present results showed that rehabilitation downregulated the metabolic pathways involved in aromatic compound degradation, which may be due to the alterations in diet and habitat that shifted the gut microbiome diversity and composition. These results indicate that the fitness of wild migratory birds to degrade aromatic pollutants and aromatic compound-rich diets might be decreased when these birds are released into their natural habitats. Collectively, this rehabilitation-induced shift in the metabolic pathways of the gut

microbiome may affect the adaptation to wild migratory environments after release.

Wild birds are known as potentially important sources of antibiotic resistance dissemination in the environment (Y. Lin et al. 2020). *E. coli* is a well-established antibiotic resistance indicator to evaluate the anthropogenic impact on the environment (Anjum et al. 2021). Therefore, the present isolated *E. coli* from fecal samples of wild birds and examined the shift in its antibiotic resistance during rehabilitation. Notably, the present results showed that antibiotic resistance was significantly increased during rehabilitation. Since none of the birds used in the present study were treated with antibiotics during rehabilitation, the increased antibiotic resistance observed is unlikely to have resulted from antibiotic exposure. Antibiotics associated with increased resistance included ciprofloxacin, ampicillin, amoxicillin, and tetracycline, which are the most frequently used drugs in veterinary clinics (Stewart and Allen 2019). In a clinical environment frequently exposed to these antibiotics, environmental microbes acquire antibiotic resistance and opportunistically infect hosts (Chng et al. 2020; Hassoun-Kheir et al. 2020). Therefore, it can be inferred that the microorganisms of wild birds acquired antibiotic resistance from the rehabilitation environment via the colonization of antibiotic-resistant environmental microorganisms. However, wild birds did not acquire resistance to colistin and imipenem which are drugs of last resort and are thus rarely used in the clinical environment (Nordmann and Poirel 2014; Osei Sekyere et al. 2016). Environmental microorganisms in the rehabilitation environment were, therefore, less exposed to colistin and imipenem, and the microorganisms of wild birds did not acquire resistance to these antibiotics.

Dietary modifications during rehabilitation may also have contributed to the acquisition of antibiotic resistance in wild birds. Indeed, 1-day-old chicks are a

major source of in-farm-transmitted antibiotic resistance owing to their high antibiotic resistance levels (Moreno et al. 2019; AbdelRahman et al. 2020). The remaining antibiotic-resistant microorganisms in the guts of chicks could have colonized the guts of wild birds during rehabilitation resulting in increased antibiotic resistance. Collectively, the present findings indicate that wild birds may acquire antibiotic resistance during rehabilitation and thus serve as the source of antibiotic resistance spread in the environment after they are released.

2.5 Conclusions

The present study showed that wildlife rehabilitation induces alterations in the gut microbiome and the acquisition of antibiotic resistance in wild migratory birds. The diversity of the gut microbiome significantly decreased during rehabilitation, and it did not recover to the level observed in the wild state, indicating the possibility of rehabilitation-induced dysbiosis. Moreover, zoonotic pathogens, including *Peptoclostridium* and *Campylobacter*, were enriched whereas SCFA-producing bacteria were depleted in the gut of wild migratory birds at the end of the rehabilitation period, and the ecological network of the gut microbiome showed decreased complexity. Metabolic pathways involved in the degradation of aromatic compounds were significantly downregulated, indicating that the ability of the gut microbiome to degrade these pollutants might have been compromised. Overall, these results indicate that the rehabilitation-induced dysbiosis of the gut microbiome of wild migratory birds may affect their adaptation to the wild environment after release. Moreover, wild birds may serve as a potential source of the dissemination of antibiotic resistance when released into the wild environments because they may acquire antibiotic resistance during rehabilitation. Therefore, more attention should be devoted to studying the dynamics of the gut microbiome of wild migratory birds during rehabilitation for achieving sustainable rehabilitation strategies.

Table 3. Information on the wild birds used in the present study.

Sample-id	Date of rescue	Sampling time point					Species	Rescue spot	Food
		Wild	2 week	4 week	8 week	12 week			
WB01	2020-07-14	TRUE	TRUE	TRUE	FALSE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB02	2020-08-18	TRUE	TRUE	TRUE	TRUE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB03	2020-03-23	TRUE	TRUE	FALSE	FALSE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB04	2020-06-19	TRUE	TRUE	TRUE	FALSE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB05	2020-07-01	TRUE	TRUE	TRUE	TRUE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB06	2020-05-13	TRUE	TRUE	TRUE	FALSE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB07	2020-07-14	TRUE	TRUE	TRUE	FALSE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB08	2020-06-26	TRUE	TRUE	FALSE	FALSE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB09	2020-07-07	TRUE	TRUE	FALSE	FALSE	FALSE	Falco tinnuculus	Seoul	Chick and quail
WB10	2019-12-04	TRUE	TRUE	TRUE	TRUE	TRUE	Falco tinnuculus	Seoul	Chick and quail
WB11	2020-04-29	TRUE	TRUE	FALSE	FALSE	FALSE	Otus bakkamoena	Gwang ju	Chick and quail
WB12	2020-04-29	TRUE	TRUE	FALSE	FALSE	FALSE	Otus bakkamoena	Seoul	Chick and quail
WB13	2020-04-29	TRUE	TRUE	TRUE	FALSE	FALSE	Otus scops	Seoul	Chick and quail
WB14	2020-04-29	TRUE	TRUE	FALSE	FALSE	FALSE	Ninox scutulata	Seoul	Chick and quail
WB15	2020-05-27	TRUE	TRUE	TRUE	TRUE	FALSE	Ninox scutulata	Seoul	Chick and quail
WB16	2020-08-26	TRUE	TRUE	TRUE	TRUE	FALSE	Falco Subbuteo	Seoul	Chick and quail
WB17	2020-08-03	TRUE	TRUE	FALSE	FALSE	FALSE	Accipiter gentilis	Seoul	Chick and quail

Table 4. Nodes of the ecological network of gut microbiome in the wild and release states.

Wild	Release	Shared
Akkermansia	Alkanindiges	0319_6G20
Alistipes	Arthrobacter	ASF356
BD7_11	Atopostipes	Acinetobacter
Brachybacterium	Kurthia	Allorhizobium_Neorhizobium_Pararhizobium_Rhizobium
Butyricimonas	Monoglobus	Anaerosporobacter
CHKCI001	Muribaculaceae	Bacteroides
Candidatus_Kaiserbacteria	Novosphingobium	Bdellovibrio
Caulobacter	Obscuribacteraceae	Bilophila
Colidextribacter	Peptoclostridium	Blautia
Coprobacter	Proteus	Bradyrhizobium
Cupriavidus		Brevundimonas
Enterobacter		Burkholderia_Caballeronia_Paraburkholderia
Eubacterium coprostanoligenes_group		Butyricicoccus
GCA_900066575		Campylobacter
Gaiella		Candidatus_Adlerbacteria
Gastranaerophilales		Candidatus_Arthromitus
Oscillibacter		Candidatus_Nomurabacteria
Phascolarctobacterium		Chloroplast
Rikenella		Clostridium_sensu_stricto_1
Rikenellaceae_RC9_gut_group		Collinsella
Rothia		Corynebacterium
Ruminococcus_torques_group		Cutibacterium
Slackia		Deinococcus
Sphaerochaeta		Dyella
Sulfuritalea		Enhydrobacter
Sutterella		Enterococcus
TRA3_20		Escherichia_Shigella
		Faecalitalea
		Flavonifractor
		Fournierella
		Fusobacterium
		Incertae_Sedis
		Lachnoclostridium
		Lactobacillus

Lactococcus
Mesorhizobium
Methylobacterium_Methylorubrum
Olsenella
Parabacteroides
Pelomonas
Pseudomonas
Psychrobacter
Ralstonia
Reyranella
Rhodococcus
Rubrobacter
Sellimonas
Sphingomonas
Staphylococcus
Subgroup_2
Varibaculum
Variovorax

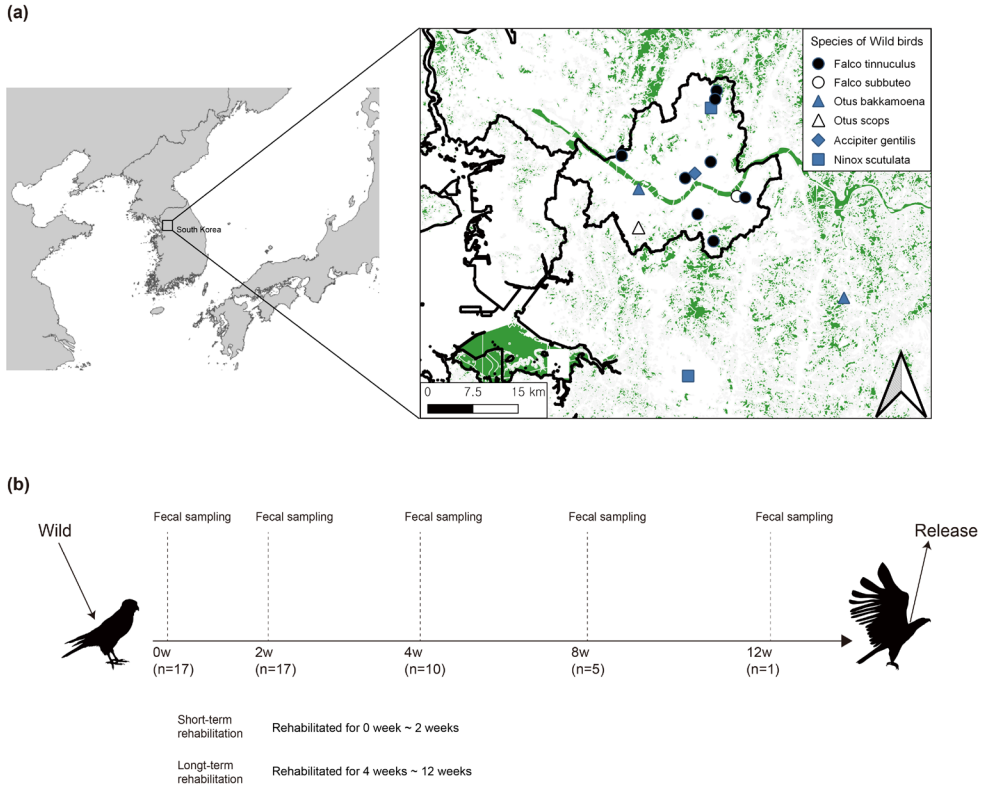


Figure 10. Information on wild migratory birds and the study design. (A) Rescue spots of the 17 wild migratory birds that were transferred to the Seoul Wildlife Center and used in the present study. The map was produced using the Quantum Geographical Information System version 3.16.16 (http://qgis.org) based on GPS coordinates. (B) Graphical representation of the study design and sample collection times.

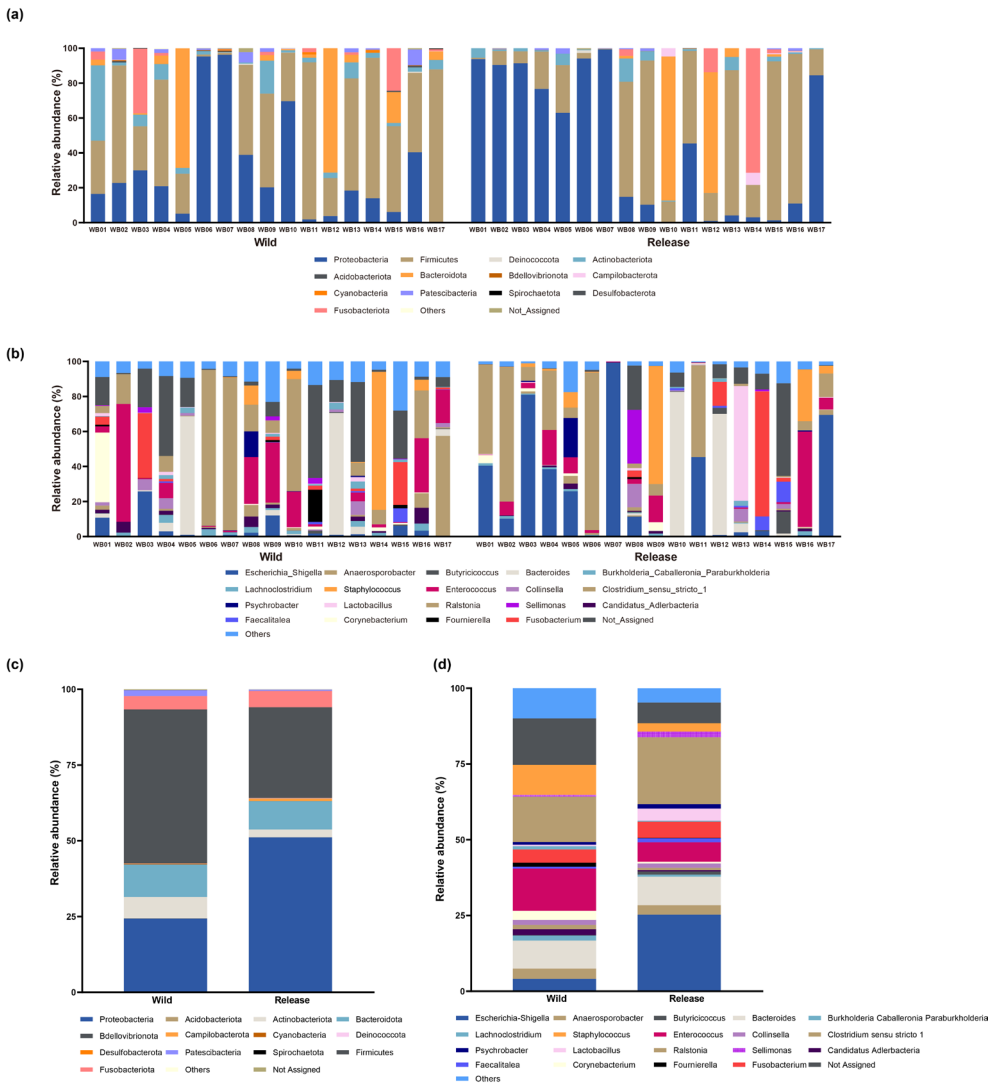


Figure 11. Gut microbiome taxonomic composition in the wild and release states of the wild migratory birds at the phylum level (A) and genus level (B). Only the top 20 genera are shown. (C and D) Merged bar plots show the taxonomy composition in the wild and release states at the phylum level (C) and the genus level (D).

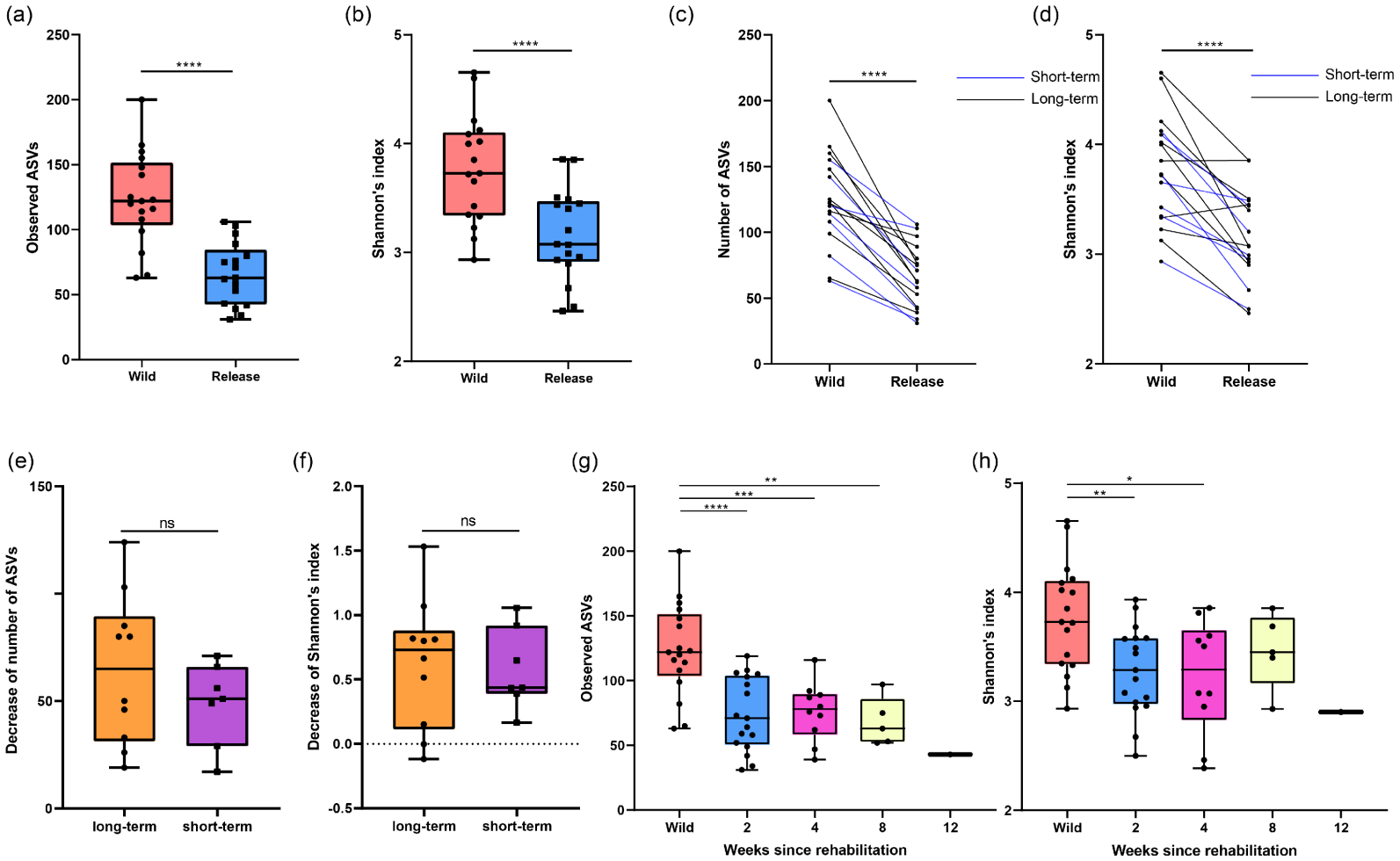

(a)
Observed ASVs
****
Wild
Release
(b)
Shannon's index
****
Wild
Release
(c)
Number of ASVs
****
Short-term
Long-term
Wild
Release
(d)
Shannon's index
****
Short-term
Long-term
Wild
Release
(e)
Decrease of number of ASVs
ns
long-term
short-term
(f)
Decrease of Shannon's index
ns
long-term
short-term
(g)
Observed ASVs
**
***
****
Wild
2
4
8
12
Weeks since rehabilitation
(h)
Shannon's index
*
**
Wild
2
4
8
12
Weeks since rehabilitation

Figure 12. Decreased alpha diversity of the gut microbiome of wild migratory birds after short- and long-term rehabilitation. (A and B) Box plots show the decrease in the number of observed ASVs (A) and Shannon's index (B) in the wild and release states. (C and D) Dot plots show the paired sample analysis of the number of observed ASVs (C) and Shannon's index (D) in the wild and release states. (E and F) Box plots show the decrease in the number of ASVs (E) and Shannon's index (F) in long-term and short-term rehabilitation groups. (G and H) Box plots show the longitudinal dynamics of the number of observed ASVs (G) and Shannon's index (H) throughout the rehabilitation period.

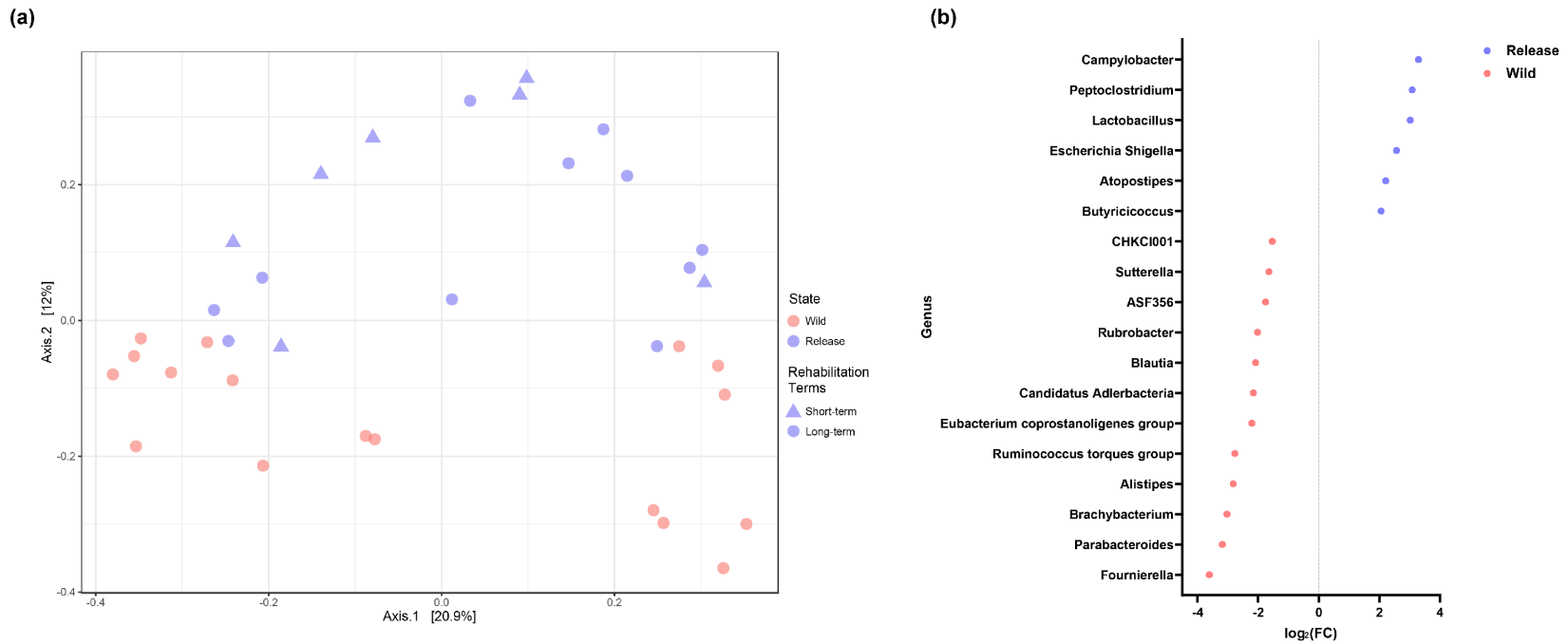

(a)
Axis.2 [12%]
Axis.1 [20.9%]
0.2
0.0
-0.2
-0.4
-0.4
-0.2
0.0
0.2
State
Wild
Release
Rehabilitation Terms
Short-term
Long-term
(b)
Genus
Campylobacter
Peptoclostridium
Lactobacillus
Escherichia Shigella
Atopostipes
Butyricicoccus
CHKCl001
Sutterella
ASF356
Rubrobacter
Blautia
Candidatus Adlerbacteria
Eubacterium coprostanoligenes group
Ruminococcus torques group
Alistipes
Brachybacterium
Parabacteroides
Fournierella
-4
-2
0
2
4
log2(FC)
Release
Wild

Figure 13. Shifts in the beta diversity of the gut microbiome of wild migratory birds during rehabilitation. (A) Principal coordinates analysis based on unweighted UniFrac distance. Birds in the wild and release states are clustered in different sections of the PCoA plot. (B) Differential abundance analysis of the gut microbiome of birds in the wild and release states.

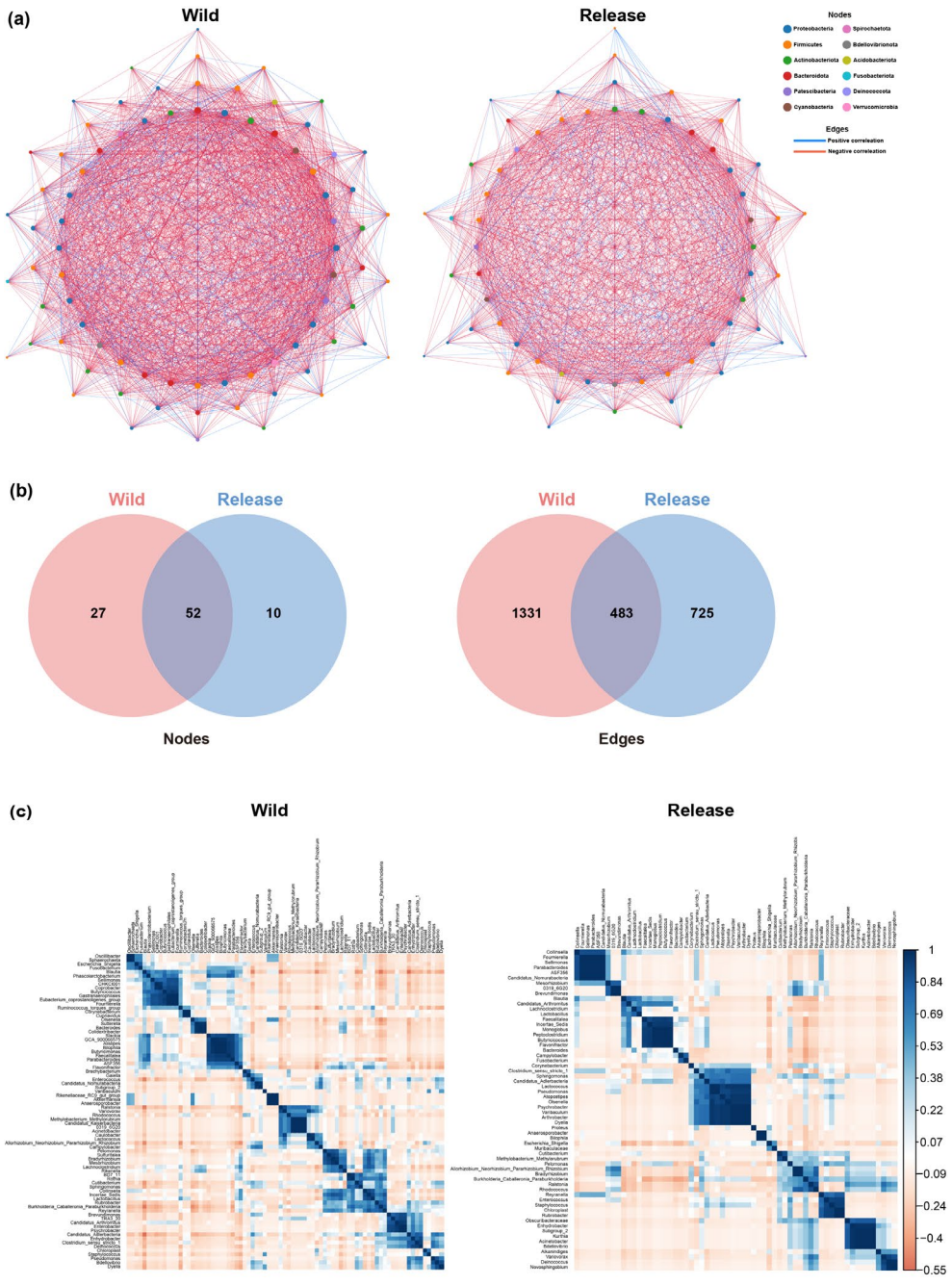

(a)
Wild
Release
Nodes
Proteobacteria
Firmicutes
Actinobacteriota
Bacteroidota
Patescibacteria
Cyanobacteria
Spirochaetota
Bdellovibrionota
Acidobacteriota
Fusobacteriota
Deinococcota
Verrucomicrobia
Edges
Positive correlation
Negative correlation
(b)
Wild
Release
27
52
10
Nodes
Wild
Release
1331
483
725
Edges
(c)
Wild
Release
1
0.84
0.69
0.53
0.38
0.22
0.07
-0.09
-0.24
-0.4
-0.55

Figure 14. Shifts in the gut microbiome ecological interactions due to rehabilitation. (A) Co-occurrence networks of the gut microbiome in the wild and release states at the genus level. Networks were constructed using NAMAP with Pearson's correlation. Statistically significant associations using $P < 0.05$ and $r > 0.7$ as cutoff values and 100 bootstrapping iterations are shown. The colors of nodes indicate the phylum each genus belongs to, and the sizes of the nodes represent their degree (number of edges). Blue lines indicate a positive correlation and red lines indicate a negative correlation. (B) Venn diagrams show the shared and unique nodes and edges of the co-occurrence networks in the wild and release states. (C) Corresponding correlograms for the networks of the gut microbiome in the wild (left) and release (right) states.

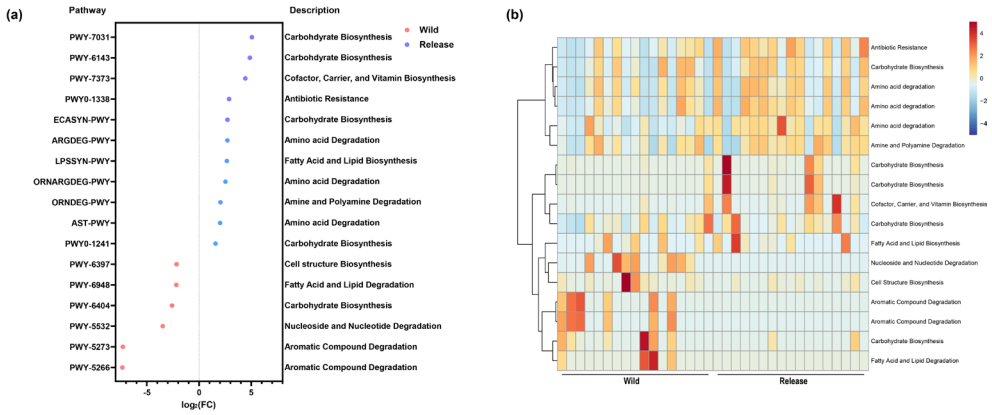


Figure 15. Shifts in the gut microbiome metabolic pathways due to rehabilitation. (A) Differential abundance analysis of potential metabolic pathways in the wild and release states. (B) Heatmap of metabolic pathways differing significantly between the wild and release states.

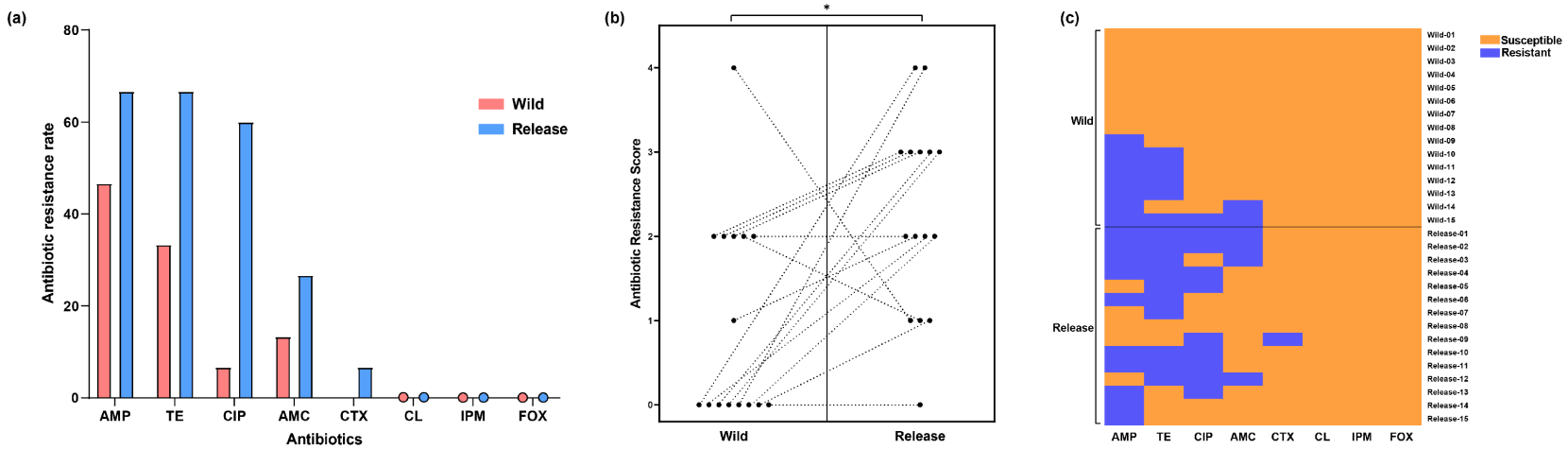

(a)
Antibiotic resistance rate
80
60
40
20
0
AMP
TE
CIP
AMC
CTX
CL
IPM
FOX
Antibiotics
Wild
Release
(b)
*
Antibiotic Resistance Score
4
3
2
1
0
Wild
Release
(c)
Wild
Release
Wild-01
Wild-02
Wild-03
Wild-04
Wild-05
Wild-06
Wild-07
Wild-08
Wild-09
Wild-10
Wild-11
Wild-12
Wild-13
Wild-14
Wild-15
Release-01
Release-02
Release-03
Release-04
Release-05
Release-06
Release-07
Release-08
Release-09
Release-10
Release-11
Release-12
Release-13
Release-14
Release-15
AMP
TE
CIP
AMC
CTX
CL
IPM
FOX
Susceptible
Resistant

Figure 16. Shifts in antibiotic resistance owing to rehabilitation. (A) Antibiotic resistance rates to the eight types of antibiotics used in the present study. (B) Dot plots show the paired sample analysis of antibiotic resistance scores in the wild and release states. (C) Heatmap of the antibiotic resistance in the wild and release states.

Chapter III

Red ginseng dietary fiber shows prebiotic potential by modulating gut microbiota in dogs

Abstract

Red ginseng improves human health by modulating the gut microbiota. However, the effect of red ginseng dietary fiber on the canine gut microbiota remains unclear. A total of 37 healthy dogs were enrolled in the study and randomly assigned into mid-dose (8 g/5 kg) and low-dose (3 g/5 kg) groups. The dogs were fed a normal diet supplemented with red ginseng dietary fiber for 8 weeks, and their gut microbiota was analyzed by 16S rRNA sequencing. After long-term dietary intake of ginseng dietary fiber, the alpha diversity of the gut microbiota significantly increased in the mid-dose group. Differential abundance analysis showed that short-chain fatty acid producers, including *Akkermansia*, *Leuconostoc*, and *Turicibacter* were significantly enriched after intake. Zoonotic pathogens, including *Helicobacter,* significantly decreased in the low-dose group. The complexity of ecological interactions between components of the gut microbiota was altered by the intake of red ginseng dietary fiber. The present study revealed the impact of long-term dietary intake of red ginseng dietary fiber on the canine gut microbiota, suggesting the prebiotic potential of red ginseng dietary fiber. This study may provide a basis for future research on the development of red ginseng dietary fiber feed for companion dogs.

Keywords

Dog, Gut microbiota, Metagenomics, Nanopore sequencing, Red ginseng dietary fiber

3.1 Introduction

Red ginseng is a well-known plant and is widely used in traditional medicine worldwide (Ratan et al. 2021). Red ginseng comprises beneficial components, including ginsenosides and saponins, that improve health by conferring anti-inflammatory, anticancer, and anti-obesity potential (Min, Cho, and Yi 2021; Yoon et al. 2021; Xin Sun et al. 2022). When these active ingredients are extracted from red ginseng during manufacturing, a large quantity of ginseng residue is produced. Red ginseng residue consists of beneficial compounds, mainly soluble and insoluble dietary fibers, in addition to proteins, amino acids, mineral elements, and other components (Han et al. 2020). The beneficial effects of dietary fibers have been revealed in various studies that have shown improvements in metabolic and immune functions (Slavin 2008). However, most studies on red ginseng components have focused on the effects of ginseng extract, whereas the effects of fibers originating from red ginseng residue remains unclear.

The gut microbiota is a complex community of microbes, including bacteria, viruses, archaea, and fungi residing in the gastrointestinal tract of the host (Knight et al. 2018). Complex interactions between the host and gut microbiota regulate host fitness, including metabolism, immune response, digestion, and pathogen resistance (Yichen Hu et al. 2022; Ishizaka et al. 2021; Lu et al. 2021; Song et al. 2021; Kundu et al. 2017). Diverse factors, including host genetics, diet, and environment, shape the gut microbiota (Song et al. 2022; Goodrich, Di Rienzi, et al. 2014). Among them, diet is suggested to be the most important driving factor of the gut microbiota (David et al. 2014). As the gut microbiota plays major role in host health, dietary interventions, such as a high-fiber diet, low-sugar diet, and diet

supplemented with prebiotics, improve host health by modulating the gut microbiota (Kuo 2013; Saulnier et al. 2013; Tian et al. 2015). Although a wide range of studies on the response of gut microbiota to dietary intervention have been conducted, the effect of red ginseng residue on the gut microbiota remains unclear.

Dogs are companion animals that live in close relationships with humans and share lifestyles and environments. As dogs are exposed to environmental factors comparable to those experienced by humans that induce lifestyle-related disorders, they frequently suffer from similar diseases and show similar responses to treatment (Vázquez-Baeza et al. 2016). Because of this characteristic that set them apart from conventional laboratory animals, dogs have been widely used as sentinel for human health and disorders (Shi et al. 2019; Hayward et al. 2016). Moreover, they are frequently exposed to external factors, such as diet and environment, that shape the gut microbiota to a level comparable to that of humans. Therefore, analyzing the canine gut microbiota is important because of its high similarity to that of humans. Compared to other animals, such as laboratory mice and pigs, the canine gut microbiota has the most similar genetic contents to that of humans, and it responds similarly to dietary intervention as does the human gut microbiota (Coelho et al. 2018). Therefore, improving understanding of the dynamics of canine gut microbiota in response to dietary interventions is essential considering the translational value of dogs for human studies.

Here, the current study investigated the effects of long-term dietary intake of red ginseng residue on the gut microbiota of healthy companion dogs. The present study conducted longitudinal sampling, metagenomic analysis, and health status monitoring of healthy companion dogs. The dogs were fed low-dose or mid-dose red ginseng residue supplemented to a normal canine diet for 8 weeks. The

present study revealed alterations in the gut microbiota in response to the intake of red ginseng dietary fiber, including alterations in the taxonomic composition, diversity, and ecological network of the gut microbes, which are associated with host health. This study may provide a basis for developing prebiotics using red ginseng residue for dogs.

3.2 Material and Methods

Ginseng materials

Red ginseng feed was processed using water-soluble ingredients extracted from 6-year-old Korea ginseng. During processing, pulverization was carried out to increase swelling during molding, and then it is formulated through extrusion. The formulated product was dried and coated with palatability enhancer liquid and powder under vacuum. Details of the compounds in red ginseng residue used in this study are shown in Table 5.

Study design and sample collection

Clinically healthy and privately owned dogs were enrolled in the present study, and written consent was acquired from the owners after thoroughly explaining the study. This study was reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-210115-2). The following exclusion criteria were set: (1) Abnormalities in health screening, including physical examination, blood analysis, urinalysis, and abdominal radiology ultrasonography; (2) use of antibiotics in the last 2 weeks; (3) dogs less than 2 years of age; and (4) pregnant dogs. The exclusion criteria were to avoid factors that could influence the gut microbiota and to accurately assess the influence of red ginseng dietary fiber. Thirty-seven dogs were included in the study, and they were randomly assigned into mid-dose intake (8 g/5 kg per day, 20 dogs) and low-dose intake (3 g/5 kg per day, 17 dogs) groups. The owners were told to feed the dogs with the provided red ginseng dietary fiber daily, in addition to the regular diet, for the following 8 weeks. After every 4 weeks, the health status of the

participants was examined by experienced veterinarians. The assessment included history, physical examination, and blood collection for a complete blood count and biochemical profile. Fecal samples were collected monthly and transferred to the laboratory for metagenomic analysis.

Fecal DNA extraction, library preparation, and 16S rRNA sequencing

DNA was extracted from fecal samples of dogs using a Qiagen Power Fecal DNA Pro kit. From 10 ng of extracted DNA, Full-length bacterial 16S rRNA amplicons were generated by polymerase chain reaction using barcode primers provided in the 16S Barcoding Kit. The amplicons from each sample were purified and cleaned using AMPure XP beads. Purified amplicons were pooled at equivalent concentrations. Pooled barcoded libraries were loaded into flow cells (version R9.4) and long-read 16S rRNA sequencing was conducted using a MinION platform.

Bioinformatic analysis

Quality control of the raw sequence data was conducted by trimming barcode sequences and discarding reads with lengths below 1400 bp. Preprocessed sequence data were base-called using MinKNOW with the super-accuracy base-calling option. The taxonomy profile and feature table of sequence data were generated using Kraken2 (Wood, Lu, and Langmead 2019) using the Greengenes database (version 13.5).

Downstream analysis was performed using R packages (McMurdie and Holmes 2013; Dhariwal et al. 2017; Chong et al. 2020). The alpha diversity of the microbiota was evaluated using 2 indices, including the number of observed

species and Fisher's index. Wilcoxon test for paired samples was used to evaluate the significance of differences in the alpha diversity. Differential abundance analysis of gut microbiota was performed using edgeR (Robinson, McCarthy, and Smyth 2009). The ecological network of gut microbiota was constructed with network analysis for metagenomic abundance profiles (NAMAP) based on Pearson's correlations using the MetagenoNets (Yadav, Ghosh, and Mande 2016; Nagpal et al. 2020) with $r > 0.7$ and $p < 0.05$ as the cut-off for significance.

3.3 Results

General characteristics of participant dogs

General characteristics of the participant dogs are presented in Table 6. The dogs showed insignificant changes in their clinical status during the study period. All participants were clinically healthy according to physical examination and laboratory analysis. No owner reported noticeable side effects or clinical signs during the intake of RGR fibers.

Taxonomic composition of the canine gut microbiota

To assess the effects of red ginseng dietary fiber, the present study first investigated the taxonomic composition of the canine gut microbiota at the phylum and genus levels (Figure 17). Before the dietary intake of red ginseng dietary fiber (0 week, baseline), Firmicutes, Proteobacteria, Bacteroidetes, and Fusobacteria were the most dominant phyla in both low-dose and mid-dose groups (average 99.80% and 99.97%, respectively) (Figure 17A). In the low-dose group, the abundance of Firmicutes decreased (average 79.75% to 74.56%) and those of 3 phyla, Bacteroidetes (average 4.38% to 6.89%), Fusobacteria (4.32% to 5.12%), and Proteobacteria (11.34% to 13.36%), increased after 4 weeks. Verrucomicrobia, which was absent at the baseline, colonized the gut microbiota with low abundance. After 8 weeks of low-dose intake, the abundance of Firmicutes (average 79.75% to 79.49%), Fusobacteria (average 4.32% to 2.65%), and Proteobacteria (11.34% to 10.86%) decreased compared to that at the baseline. In contrast, the abundance of Bacteroidetes increased (average 4.38% to 6.97%) compared to that at the baseline. In the mid-dose group, the abundance of Firmicutes increased (average 75.43% to

80.23%) and those of 3 phyla, Bacteroidetes (average 6.43% to 4.09%), Fusobacteria (7.45% to 7.19%), and Proteobacteria (10.66% to 8.42%), decreased after 4 weeks. After 8 weeks of intake, the abundance of Firmicutes (average 75.43% to 74.37%) and Fusobacteria (average 4.32% to 2.65%) decreased, and that of Proteobacteria (10.66% to 11.70%) and Bacteroidetes increased (average 4.09% to 6.45%) compared to that at the baseline.

At the genus level, before dietary intake of red ginseng dietary fiber, *Blautia* (average 17.96% and 17.94%, respectively) and *Ruminococcus* (average 20.87% and 14.83%, respectively) were the most dominant genera in both low-dose and mid-dose groups (Figure 17B). In the low-dose group, the abundance of both *Blautia* (average 17.96% to 17.62%) and *Ruminococcus* (average 17.94% to 14.68%) decreased compared to that at the baseline after 4 weeks. After 8 weeks, the abundance of *Blautia* (average 17.96% to 15.45%) and *Ruminococcus* (average 17.9% to 14.96%) decreased compared to that at the baseline. In the mid-dose group, the abundance of *Blautia* (average 20.87% to 18.21%) and *Ruminococcus* (average 14.83% to 13.19%) decreased after 4 weeks compared to that at the baseline. After 8 weeks, the abundance of both *Blautia* (average 20.87% to 18.57%) and *Ruminococcus* (average 14.83% to 14.18%) decreased compared to that at the baseline.

Shifts of the diversity of canine gut microbiota

The present study analyzed the alteration of diversity of the gut microbiota based on the number of observed species and Fisher's index. As shown in Figure 18A, B, both indices of the alpha diversity did not show significant differences between the time points (Wilcoxon test, $p > 0.05$) in the low-dose group.

However, as shown in Figure 18C and D, both indices of the alpha diversity increased after 4 weeks in the mid-dose group ($p < 0.05$). No significant difference was observed between weeks 0 and 8 in the mid-dose group ($p > 0.05$).

Differential abundance analysis of the canine gut microbiota

The present study analyzed specific components of the gut microbiota, which differed significantly by the intake of red ginseng dietary fiber (Figure 19). In the low-dose group, *Parabacteroides* and *Akkermansia* were significantly enriched (FDR < 0.05) after 4 weeks; *Parabacteroides* and *Haemophilus* were significantly enriched (FDR < 0.05) after 8 weeks. In the mid-dose group, 4 genera including *Sarcina*, *Proteinclasticum*, *Leuconostoc*, and *Turicibacter,* were significantly enriched (FDR < 0.05) after 4 weeks; *Epulopiscium* and *Sarcina* were significantly enriched, and 5 genera including *Helicobacter*, *Succinivibrio*, *Peptococcus*, *Candidatus Arthromitus,* and *Phascolarctobacterium* were significantly decreased after 8 weeks.

Red ginseng dietary fiber alters microbial interactions of the canine gut microbiota

To elucidate the shift in ecological interactions between the gut microbes according to the intake of red ginseng dietary fiber, the present study constructed co-occurrence networks for the time points in mid-dose and low-dose groups. In the low-dose group, 128 genera (nodes) and 768 correlations (edges) were significant ($p < 0.05$, $r > 0.7$) at 0 week. After 4 weeks of intake of red ginseng dietary fiber, 129 nodes and 920 edges were observed; after 8 weeks, 153 and 879 nodes were observed (Figure 20A). The network of canine gut microbiota in the

low-dose group shared 111 nodes and 342 edges among time points. The numbers of unique nodes and edges were 7 and 286 at 0 week, 7 and 399 after 4 weeks, and 27 and 432 after 8 weeks, respectively.

In the mid-dose group, 140 nodes and 1213 edges were significant ($p < 0.05$, $r > 0.7$) at 0 week. After 4 weeks, 141 nodes and 1077 nodes were observed; after 8 weeks, 150 nodes and 1025 edges were observed (Figure 20B). The network of canine gut microbiota in the mid-dose group shared 116 nodes and 470 edges among time points. The numbers of unique nodes and edges were 12 and 490 at 0 week, 11 and 371 after 4 weeks, and 14 and 384 after 8 weeks.

3.4 Discussion

The canine gut microbiota is drawing concern for its translational value as it has highly similar genetic contents and responds similarly to dietary interventions, compared to those of humans (Coelho et al. 2018). In this study, the present study investigated the impact of red ginseng dietary fiber on canine gut microbiota to explore its prebiotic potential. The present study is noteworthy as this is the first to reveal the alteration of canine gut microbiota depending on intake of red ginseng residue using third-generation sequencing technology that enables comprehensive analysis of the gut microbiota. While previous studies have mainly focused on the effect of red ginseng extract or products on the gut microbiota, the effect of red ginseng residue, which is rich in dietary fibers, remains unclear. Moreover, most *in vivo* studies on the effects of red ginseng compounds on the gut microbiota have been conducted using animals in strictly controlled environments, such as laboratory mice, while studies on dogs that share common environment with humans are scanty. Considering the translational value of dogs in human studies, the present study may provide a basis for the development of prebiotic products using red ginseng dietary fiber for dogs and humans.

In the present study, taxonomic composition of the canine gut microbiota was altered after the intake of ginseng dietary fiber. After intake, abundance of the phylum Bacteroidetes, which is involved in degrading polysaccharides of high molecular weights (Flint et al. 2012; Thomas et al. 2011), increased. Diets rich in plant polysaccharides increases the abundance of Bacteroidetes in the gut (Turnbaugh et al. 2009); therefore, red ginseng dietary fiber may have increased the abundance of the canine gut microbiota. The phylum Bacteroidetes consists of

various probiotic gut microbes that produce short chain fatty acids (SCFAs) that reduce inflammation and maintain host health (Marchesi et al. 2016; Xia et al. 2022). Therefore, the present study results indicate the beneficial activity of red ginseng dietary fiber that modulates the composition of the gut microbiota.

The present study showed that the intake of mid-dose red ginseng dietary fiber significantly increased the richness and evenness of gut microbiota, while low-dose was not sufficient to significantly increase the gut microbial diversity. Increased diversity of the gut microbiota positively affects host health by maintaining homeostasis of the gut (Lozupone et al. 2012) whereas decreased diversity is associated with various gastrointestinal, metabolic, and immune diseases, indicating dysbiosis of the gut microbiota (C.-Y. Lin et al. 2022; Pisani et al. 2022). Red ginseng dietary fiber may improve host health by increasing gut microbial diversity.

The present study further investigated specific genera of the gut microbiota that were significantly altered by red ginseng dietary fiber intake. Differential abundance analysis showed that several microbes that significantly increased were mainly SCFA producers, such as *Parabacteroides*, *Akkermansia*, *Leuconostoc,* and *Turicibacter*. These microbes produce SCFAs by fermenting non-digestible carbohydrates (Xia et al. 2022; Chen et al. 2021; Pan, Zhou, and Han 2021; Renu et al. 2022), which play a major role in host health. Dietary modification, particularly fiber-rich diet, is a major factor associated with the increase in SCFA producers in the gut (Tan et al. 2016). As red ginseng residue used in this study is rich in various dietary fibers, they may have promoted significant growth of SCFA producers, which is consistent with previous studies (Lange et al. 2015; Cuervo et al. 2013). Collectively, the present results

demonstrate that red ginseng dietary fiber intake may positively affect host health by enriching SCFA producers in the gut microbiota.

Notably, microbes that significantly decreased after red ginseng dietary fiber intake were mostly zoonotic pathogens, including *Helicobacter,* indicating that red ginseng dietary fiber intake confers colonization resistance to these pathogens. This may be because of 1) an increase in microbial diversity and 2) an increase in SCFA producers in the gut microbiota. Increased richness of microbiota confers resistance to pathogen colonization (Harrison et al. 2017), which is consistent with the present results. SCFAs produced by the gut microbiota suppresses the growth of pathogens *in vitro* and *in vivo* (Trachsel et al. 2022; Shealy, Yoo, and Byndloss 2021; Engevik and Versalovic 2017). Considering that *Helicobacter* is a life-threatening pathogen in both humans and dogs and is often transmitted from dogs to owners (Kubota-Aizawa et al. 2021), depletion of *Helicobacter* in the gut microbiota of dogs after intake of red ginseng dietary fiber may improve dog health and subsequently, human health.

As complicated interactions between the gut microbes regulate the stability of gut microbiota (Xiao et al. 2022; Dubin and Pamer 2017), the present study investigated the alteration of the ecological network by the intake of red ginseng residue. The present study observed that distinct alterations in network structures occurred because of the intake of red ginseng residue. The number of nodes and edges, which demonstrates the complexity of microbial interactions, increased after the intake of red ginseng residue. This may be owing to the increased richness induced by red ginseng dietary fiber intake, which is consistent with the present diversity analysis. Notably, the unique nodes observed after red ginseng dietary fiber intake were mainly SCFA producers including

Parabacteroides and *Leuconostoc*. This is coherent with the differential abundance analysis showing significant enrichment in SCFA producers after red ginseng dietary fiber intake. An increased complexity of the microbial network should increase canine health as SCFA producer-mediated microbe–microbe interactions are associated with host health factors such as immunity and metabolism.

All participating dogs enrolled in this study were clinically healthy before red ginseng dietary fiber intake and showed no abnormal signs during the study period. Therefore, low-dose and mid-dose of red ginseng dietary fiber did not affect dog health negatively. Physical examinations and blood tests may detect deterioration in health; however, directly revealing improvements in health is difficult. As the present study revealed the prebiotic potential of red ginseng dietary fiber in healthy dogs, further studies exploring the effect of red ginseng dietary fiber in dogs with abnormal states, such as diseases, may improve the knowledge of the clinical effect of red ginseng dietary fiber.

3.5 Conclusions

The present study revealed alterations in the canine gut microbiota owing to long-term intake of red ginseng dietary fiber originating from red ginseng residue. These results indicate the prebiotic potential of red ginseng dietary fiber by modulating the canine gut microbiota. The present study provides a basis for further research on the development of prebiotics using red ginseng dietary fiber.

Table 5. Ginsenosides profile and concentration of feed used in this study.

Ginsenosides Concentration (mg/kg)										
Rg1	Re	Rf	Rh1	Rg2s	Rb1	Rc	Rb2	Rd	Rg3s	Rg3r
0.35	0.11	0.39	0.60	0.31	1.36	0.37	0.36	0.14	0.76	0.92

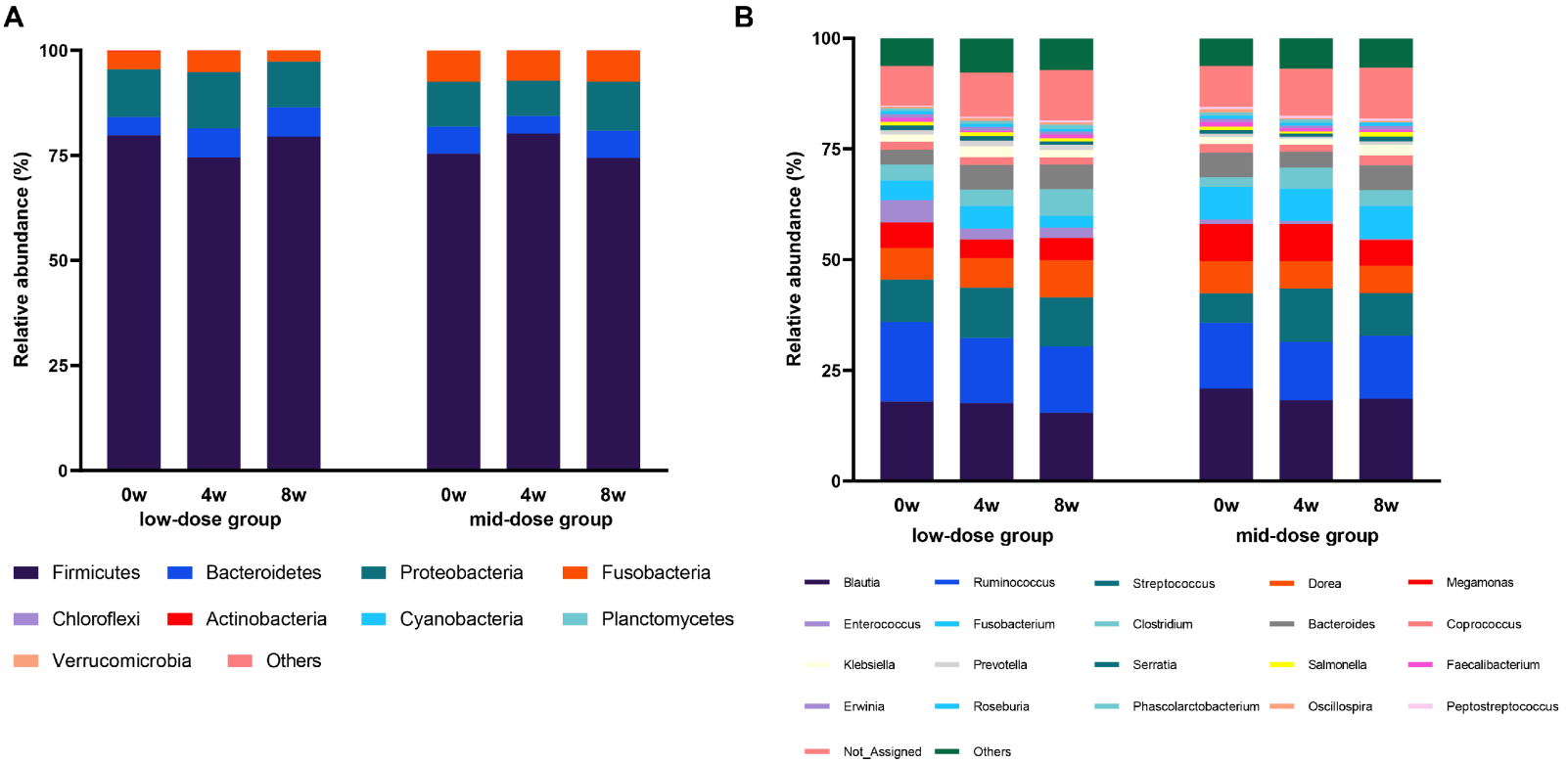

A
100
75
50
25
0
Relative abundance (%)
0w
4w
8w
low-dose group
0w
4w
8w
mid-dose group
Firmicutes
Bacteroidetes
Proteobacteria
Fusobacteria
Chloroflexi
Actinobacteria
Cyanobacteria
Planctomycetes
Verrucomicrobia
Others
B
100
75
50
25
0
Relative abundance (%)
0w
4w
8w
low-dose group
0w
4w
8w
mid-dose group
Blautia
Ruminococcus
Streptococcus
Dorea
Megamonas
Enterococcus
Fusobacterium
Clostridium
Bacteroides
Coprococcus
Klebsiella
Prevotella
Serratia
Salmonella
Faecalibacterium
Erwinia
Roseburia
Phascolarctobacterium
Oscillospira
Peptostreptococcus
Not_Assigned
Others

Figure 17. Taxonomic composition of the canine gut microbiota during the intake of red ginseng residue (A) at the phylum and (B) genus levels. Only the top 20 genera are shown

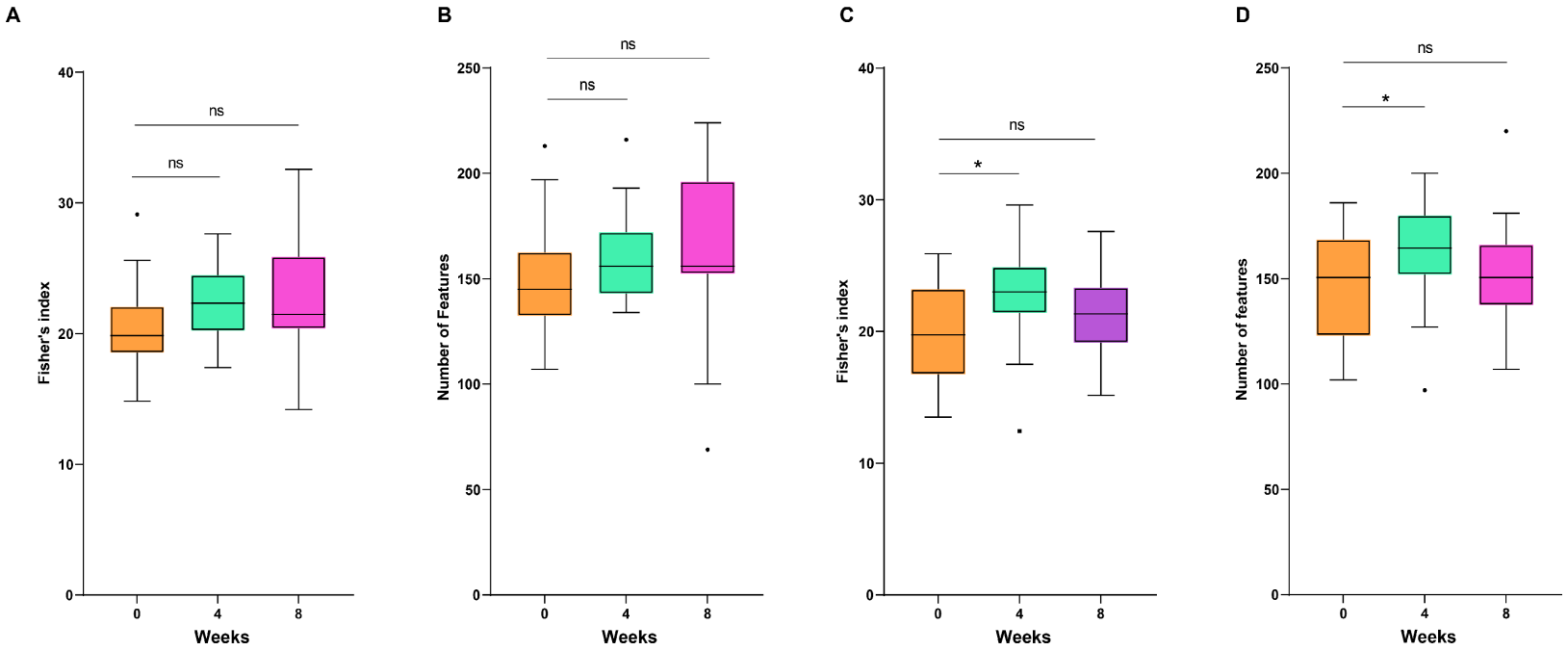

A
Fisher's index
40
30
20
10
0
ns
ns
0
4
8
Weeks
B
Number of Features
250
200
150
100
50
0
ns
ns
0
4
8
Weeks
C
Fisher's index
40
30
20
10
0
ns
*
0
4
8
Weeks
D
Number of features
250
200
150
100
50
0
ns
*
0
4
8
Weeks

Figure 18. Alteration in alpha diversity of the gut microbiota of dogs during intake of red ginseng residue. Box plots show the alteration of (A) the number of observed features and (B) Fisher's index in low-dose group. The number of observed features (C) and Fisher's index (D) in mid-dose group.

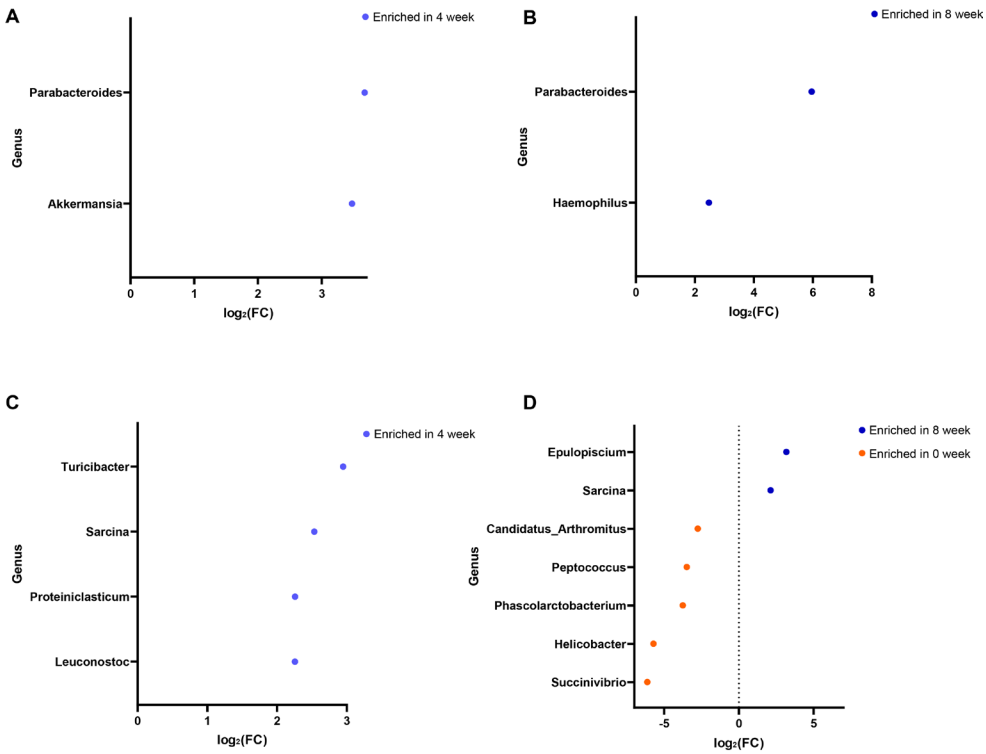


Figure 19. Differential abundance analysis of the canine gut microbiota. Results of edgeR analysis between zero and 4 weeks (A), and 0 and 8 weeks (B) in the low-dose group. Results of edgeR analysis between 0 and 4 weeks (C), and 0 and 8 weeks (D) in the mid-dose group

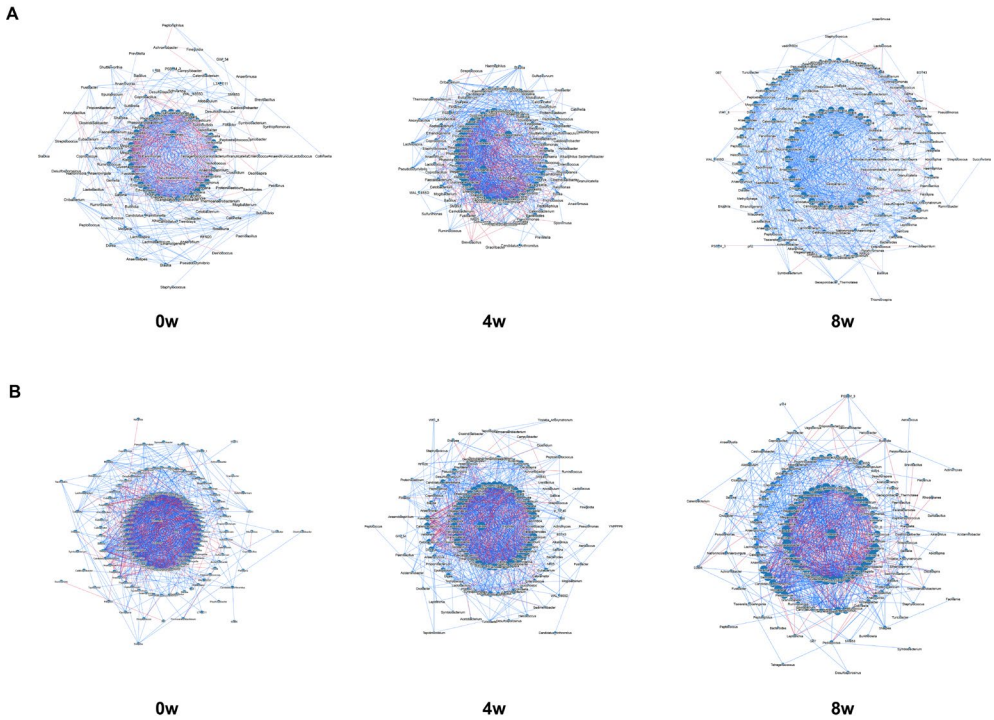


Figure 20. Alterations in ecological network of the canine gut microbiota owing to the intake of red ginseng residue. Co-occurrence network of the gut microbiome in the (A) low-dose and (B) mid-dose groups at the genus level. Co-occurrence network was generated using NAMAP with Pearson's correlation. Interactions showing $p < 0.05$ and $r > 0.7$ were considered significant. Positive and negative correlations are shown as blue and red lines, respectively.

Chapter IV

The central nervous system-demyelinating toxin cuprizone alters the gut microbiome and metabolome in mice

Abstract

Multiple sclerosis, which is characterized by the demyelination of the axons, is the most common neurodegenerative disease of the central nervous system. It is well known that the gut microbiome is associated with various neurodegenerative diseases; however, the association between multiple sclerosis and the gut microbiome and metabolome remains unclear. This multi-omics study aimed to investigate the association of the gut microbiome with multiple sclerosis using a cuprizone mouse model. Then, a 0.2% cuprizone diet was fed to C57BL/6 mice for six weeks, and the alteration of the gut microbiota and metabolome was analyzed. The results showed that six weeks of a cuprizone diet significantly decreased the gut microbiome diversity, indicating dysbiosis. Biomarker analysis showed that MS marker microbes, including *Akkermansia*, were enriched in the cuprizone group. Moreover, gut metabolome was significantly altered by cuprizone intake. Branched-chain amino acids, such as isoleucine, valine, and leucine, were depleted in the cuprizone group. These alterations of the gut microbiota and metabolome showed similar characteristics to human MS patients. This study may provide a basis further research on the role of gut microbiome and potential biomarkers in neurodegenerative diseases.

Keywords

Gut microbiome, metabolome, multiple sclerosis, gut-brain axis, multi-omics, cuprizone

4.1 Introduction

Multiple sclerosis (MS), characterized by damage of oligodendrocytes and demyelination of axons, is the most common neurodegenerative disorder of the central nervous system (Baecher-Allan, Kaskow, and Weiner 2018). In MS, the the myelin sheath of neurons is destroyed by immune cells, resulting in disorder of interactions between the brain and the body. Therefore, MS causes irreversible degeneration of nerve system. Previous epidemiological studies have revealed that various host and environmental factors, such as gender, birth date, geographic location, smoking, and vitamin D insufficiency, are associated with MS, but the exact etiology of the disease remains unknown (Olsson, Barcellos, and Alfredsson 2017; Ramagopalan et al. 2010).

The gut microbiome consists of diverse community of microbes residing in the gut of host. It is common knowledge that complex host-microbe interactions regulate the broad spectrum of host fitness, including gastrointestinal health, metabolic function, and immunological function (Ley et al. 2006; Bäckhed et al. 2007; Lamont, Koo, and Hajishengallis 2018). The significance of the association between the gut, the gut microbiome and the brain is now being explored and elucidated (Sgritta et al. 2019; Sampson et al. 2016). The gut microbiome drives host synthesis of metabolites and neurotransmitters that regulate gut-brain communication, as well as produce neuroactive substances. Through neural pathways of the vagus nerve, molecules produced by the gut microbiota transmit signals to the brain or affect the immune system (Dinan and Cryan 2017; Grenham et al. 2011a). Therefore, maintaining homeostasis of the gut microbiota is critical for neural health.

Various animal models such as cuprizone, lysolecithin, and the experimental autoimmune encephalomyelitis (EAE) mouse model have been established to investigate the pathophysiology of MS (Torkildsen et al. 2008; Dehghan et al. 2021; Baxter 2007). Among these models, the cuprizone model is widely used for its reproducibility. Dietary intake of the copper-chelator cuprizone induces the apoptosis of oligodendrocytes and further activation of innate immune cells in the brain, including astrocytes and microglia, resulting in demyelination in the CNS. Consequently, the cuprizone model incorporates several significant aspects of progressive MS, including 1) the processes underpinning innate immune cell-driven myelin and axonal degradation and 2) the remyelination of demyelinated axons. The majority of previous studies on the cuprizone mouse model have focused on the immunological response (Almuslehi et al. 2020; Ghaiad et al. 2017; L. Liu et al. 2010), but studies on the alteration of the gut microbiome are scarce.

This study investigated the effects of cuprizone-induced demyelination of central nerve system on the gut microbiome in mice using multi-omics analysis, including metagenome and metabolome analysis. The mice were fed a 0.2% cuprizone diet for six weeks, with a standard diet for the final two weeks; control mice were fed a standard diet for the entire eight weeks. The present study observed alterations in the taxonomic composition and diversity of the gut microbiota, as well as changes in amino acids and SCFAs in the metabolome, which are associated with host neural health. Thus, this study elucidates the association of gut microbiota in MS and potential biomarkers for neurodegenerative diseases.

4.2 Material and Methods

Mice experiments

This study is reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University (approval number: SNU-200427-4-5). A graphical scheme of the study design is shown in Figure 17. Briefly, eight-week-old mice were divided into control (n = 6) and cuprizone (n = 6) groups. The control group was fed a standard chow diet for eight weeks. The cuprizone group was fed a 0.2% cuprizone diet for six weeks and a standard chow diet for two weeks (Figure 21). Fecal samples from the mice were collected at baseline, six weeks after baseline, and eight weeks after baseline. The collected fecal samples were transferred to the laboratory and processed for metagenome sequencing and metabolome analysis.

Metagenomic DNA extraction and 16S rRNA Nanopore sequencing

Mice feces were processed using a Qiagen Power Fecal DNA Pro kit to extract metagenomic DNA. Full-length bacterial 16S rRNA amplicons were produced from 10 ng of extracted DNA by PCR using barcode primers supplied in the 16S Barcoding Kit. Using AMPure XP beads, the amplicons from each sample were purified and cleaned. Equivalent concentrations of purified amplicons were pooled, the pooled barcoded libraries were fed into flow cells (version R9.4), and full-length 16S rRNA sequencing was performed using a MinION sequencer.

Bioinformatics

For quality control on the raw sequencing data, barcode sequences were trimmed, and reads with lengths shorter than 1400 bp were discarded. The base-calling of preprocessed sequence data was performed using MinKNOW with the super-accuracy base-calling option. Using the Greengenes database (version 13.5) and Kraken2, the taxonomy profile and feature table of the sequencing data were produced. R packages were used for the downstream analysis. The alpha diversity of the microbiota was analyzed using Shannon's index, which evaluates the richness and evenness. The differences in alpha diversity was determined using the paired t-test for paired samples. The edgeR R package was used for differential abundance analysis of the gut microbiota.

Metabolome analysis

For metabolome analysis of amino acids in the fecal samples, 0.5 g of each fecal sample was homogenized in 2 mL of distilled water for 5 min. The homogenized samples were centrifuged at 16,000 × *g* for 20 min, and the supernatant was used for high-performance liquid chromatography (HPLC) analysis with a Dionex Ultimate 3000 (Thermo Dionex, USA) and Agilent 1260 infinity FL detector (Agilent, USA). Downstream analysis was performed with R packages.

4.3 Results

Taxonomy composition of the gut microbiota in mice

To assess the effects of the cuprizone diet, the present study first analyzed the taxonomic composition of the gut microbiota at the phylum and genus levels (Figure 22). Before the intake of cuprizone (0 weeks, baseline), Bacteroidetes and Firmicutes were the most dominant phyla in both the control and cuprizone groups (98.64% and 94.54%, respectively). Firmicutes was the most abundant phyla in both the control and cuprizone groups (average 88.01% and 76.75%, respectively), and Bacteriodetes was the second most abundant phyla in both groups (average of 10.63% and 17.80%, respectively). At six weeks after baseline, the cuprizone group showed a higher abundance of Firmicutes (average 98.08%) than the control group (average 92.32%) and a lower abundance of Bacteroidetes (average 1.67%) than the control group (average 6.75%). At eight weeks after baseline, the cuprizone group showed a higher abundance of Firmicutes (average 95.30%) than the control group (average 90.24%) and a lower abundance of Bacteroidetes (average 3.80%) than the control group (average 7.95%). At the genus level, *Ruminococcus* and *Oscillospira* were the most dominant genera at baseline in both the control and cuprizone groups (average 33.67% and 39.34%, respectively). At six weeks after baseline, *Lactobacillus* was the most abundant genus in the control and cuprizone groups (average 21.64% and 62.52%, respectively). At eight weeks after baseline, the abundance of *Lactobacillus* decreased in both the control and cuprizone groups (average 14.93% and 18.30%, respectively). Collectively, the present results showed that the overall taxonomy composition of the gut microbiota was altered by the intake of cuprizone for six weeks.

The shift in diversity of the gut microbiota in mice

The present study analyzed the effect of cuprizone on the alpha and beta diversities of the gut microbiota in mice. In the control group, the alpha diversity (based on Shannon's index) showed no significant differences ($p > 0.05$) between any time points (Figure 23A). In contrast, the alpha diversity in the cuprizone group significantly decreased after six weeks ($p < 0.05$) and recovered to a level comparable to baseline at eight weeks. The shift in beta diversity was analyzed using PCoA based on the Bray-Curtis dissimilarity (Figure 23B). At baseline, the control and cuprizone groups formed homogenous clusters. After six weeks, the cuprizone group formed distinct clusters from the control group (PERMANOVA, $p < 0.05$). After eight weeks, the clusters of the cuprizone group showed no distinct difference from those of the control group.

Differential abundance analysis of the gut microbiota in mice

To investigate the specific components of the gut microbiome contributing to the diversity shift after cuprizone intake, the present study conducted differential abundance analysis between the control and cuprizone groups at six weeks after baseline. At the phylum level, four phyla including Firmicutes, Verrucomicrobia, Actinobacteria, and Tenericutes were significantly enriched (adjusted $p < 0.05$) in the control group (Figure 24A). At the genus level, *Desulfovibrio* was significantly increased in the control group (adjusted $p < 0.05$), and 18 genera were significantly increased (adjusted $p < 0.05$) in the cuprizone group, including *Turicibacter*, *Allobaculum*, *Lactobacillus*, and *Akkermansia* (Figure 24B).

The shift in the gut metabolome of mice

To investigate the alterations in the gut metabolome induced by the cuprizone diet, the present study analyzed the shift in amino acid concentrations in fecal samples of the control and cuprizone groups at six weeks after baseline. Principle component analysis showed that the metabolome of the control and cuprizone groups formed distinct clusters (Figure 25A). Differential abundance analysis showed that five amino acids (isoleucine, serine, valine, leucine, and alanine) were significantly enriched ($p < 0.05$) in the control group, while two amino acids (aspartic acid and histidine) were significantly enriched ($p < 0.05$) in the cuprizone group (Figure 25B).

4.4 Discussion

There is growing evidence that the gut microbiota may play a role in the development of wide range of neurodegenerative diseases (Haikal, Chen, and Li 2019; Cox et al. 2022; Sochocka et al. 2019). Therefore, the gut-brain axis which represents the complex interactions between the gut microbiome and the CNS is drawing more concern from researchers. The gut microbiome influences the wide range of circulating metabolites in the host, such as SCFAs or branched-chain amino acids (BCAAs), which are closely associated with regulating homeostasis of the gut and brain (Agus, Clément, and Sokol 2021; Biswas, Duffley, and Pulinilkunnil 2019). Conventional gut microbiome analysis focusing on the characterization of diverse microbial communities by 16S rRNA sequencing cannot precisely identify the metabolic pathways inside each bacterial genome. Gut metabolomics provides valuable insights into the metabolic interaction between the host, diet, and gut microbiota, complementing sequencing-based methods by giving a functional readout of the microbiome. Therefore, this study aimed to investigate the dynamics of the gut microbiome and metabolome in a cuprizone mouse model, which is a well-established model for studying the pathogenesis of MS.

Results showed that the gut microbiome composition in mice was altered after the cuprizone diet. This alteration showed a consistent pattern with previous studies in human MS patients (Cox et al. 2021; Jangi et al. 2016). In the case of humans, increased Firmicutes and decreased Bacteroidetes phyla have been consistently reported in MS patients. Consistently, the present study showed that Bacteroidetes, which are involved in degrading polysaccharides of high molecular weights (Thomas et al. 2011), dramatically decreased after six weeks of cuprizone

intake. The phylum Bacteroidetes consists of various probiotic gut microbes that produce SCFAs that reduce neuro-inflammation and maintain homeostasis of the gut-brain axis. Therefore, decreased Bacteroidetes in the gut may have promoted neuro-inflammation during cuprizone-induced demyelination.

The present study revealed that the cuprizone diet significantly decreased the alpha diversity of the gut microbiome. As various research has suggested that dysbiosis is characterized by a decrease in alpha diversity (Hooks and O'Malley 2017), the present results suggested that six weeks of cuprizone diet induced dysbiosis of the gut microbiome in mice. Dysbiosis is associated with several neurodegenerative diseases (Grenham et al. 2011a). Moreover, considering that the alpha diversity recovered to near baseline levels after cuprizone diet cessation and two weeks of standard chow, it can be inferred that remyelination of the CNS is associated with the increase in alpha diversity. Notably, several previous studies on human MS patients showed no significant differences in alpha diversity compared to healthy controls (Cantarel et al. 2015; Jangi et al. 2016), which is in contrast to the present study. This may be due to the difference in factors associated with shaping the gut microbiota between humans and mice. Laboratory mice have a controlled genetic background and environment. However, factors that shape the gut microbiota such as genetic background, diet, environment, and lifestyle vary among human populations and thus cannot be perfectly controlled. Moreover, unlike the murine model, the MS patients in human studies were under treatment, which may have affected the alpha diversity of the gut microbiota.

The present study further analyzed the specific genera of the gut microbiome that were significantly altered by cuprizone diet to identify the components involved in dysbiosis. The results showed that microbes significantly

enriched in the cuprizone group included *Akkermansia* and *Lactobacillus*. This is consistent with previous studies on the gut microbiome of human MS patients and EAE mouse models (Cantarel et al. 2015; Jangi et al. 2016; Moles et al. 2021). Interestingly, these microbes are known as beneficial microbes. For instance, *Akkermansia* is known to be negatively correlated with a wide range of metabolic diseases, including obesity, diabetes, and metabolic syndrome (X. Zhang et al. 2013; Dubourg et al. 2013; Sidiropoulos et al. 2020; Derrien, Belzer, and de Vos 2017). There may be two possibilities for these results: 1) the response of the gut microbiome to ameliorate the demyelination and 2) the fact that *Akkermansia* may induce CNS-related diseases. Considering that a previous study showed that administration of *Akkermansia* reduced the MS-like symptoms in an EAE mouse model (S. Liu et al. 2019), enrichment of *Akkermansia* may be a protective response of the gut microbiome associated with recovery rather than a pathogenic mechanism. Further studies on the administration of *Akkermansia* in cuprizone models may produce interesting results.

The present study showed that a cuprizone diet significantly altered the gut metabolome of mice. Interestingly, BCAAs, including isoleucine, valine, and leucine, were depleted in the cuprizone group. This is consistent with studies on human MS patients, which reported lower levels of BCAAs in the blood and cerebrospinal fluid (Kasakin et al. 2019; Podlecka-Piętowska et al. 2019). BCAAs are essential amino acids and are known to play essential roles in the homeostasis of host fitness including immune and metabolic function (Zeng et al. 2020). Moreover, the depletion of these amino acids can trigger a cellular stress response in oligodendrocytes. Indeed, increased immune cells during the demyelination process in MS express enzymes including branched-chain aminotransferase and

branched-chain α-ketoacid dehydrogenase, which are involved in metabolism of BCAAs (Brosnan and Brosnan 2006). Therefore, it can be inferred that the depletion of BCAAs after cuprizone intake is due to the inflammatory response during demyelination and may be utilized as a putative biomarker for demyelination diseases.

4.5 Conclusions

The present study revealed that the intake of cuprizone alters the gut microbiome and metabolome in a mouse model. Evidence of dysbiosis of the gut microbiota was shown, including decreased diversity and enrichment of biomarker microbes that are consistently observed in human MS patients. Moreover, gut metabolites known as biomarkers of MS (including aspartic acid) were enriched in the cuprizone group. These signs of dysbiosis shared characteristics of the gut microbiota of human MS patients. This study suggests the association of gut microbiome with MS, and may improve the comprehension of the role of gut microbiome and potential biomarkers in neurodegenerative diseases.

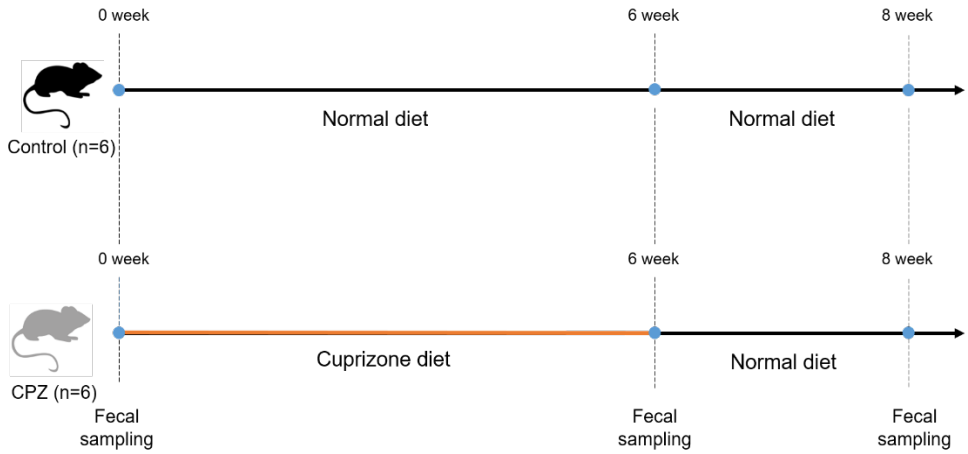


Figure 21. Graphical scheme of the present study

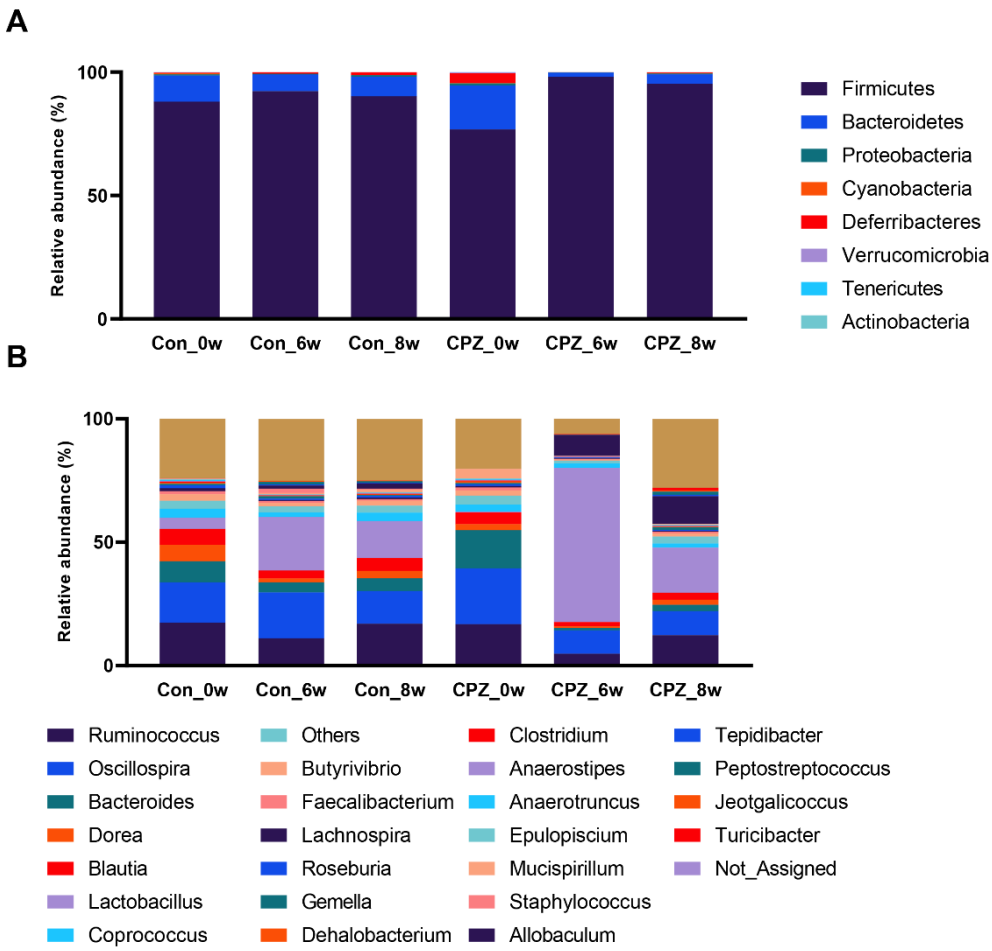


Figure 22. Taxonomy composition of the gut microbiota in mice at (A) the phylum and (B) genus levels.

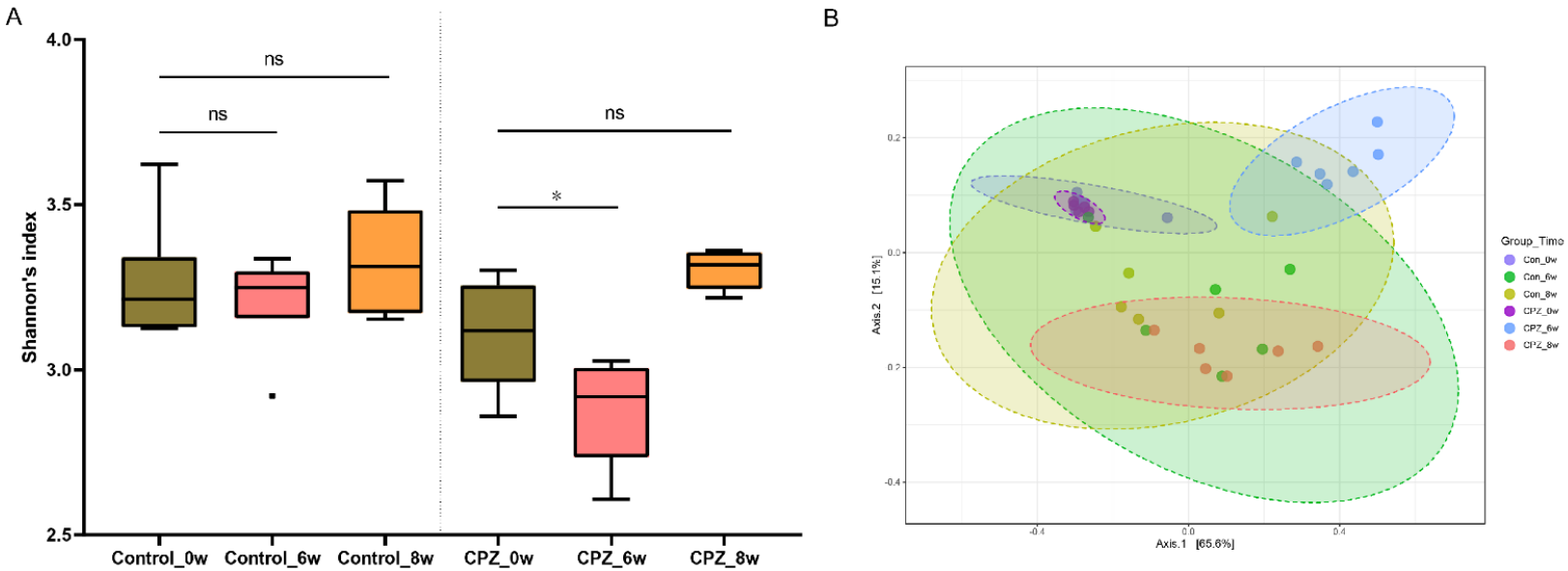
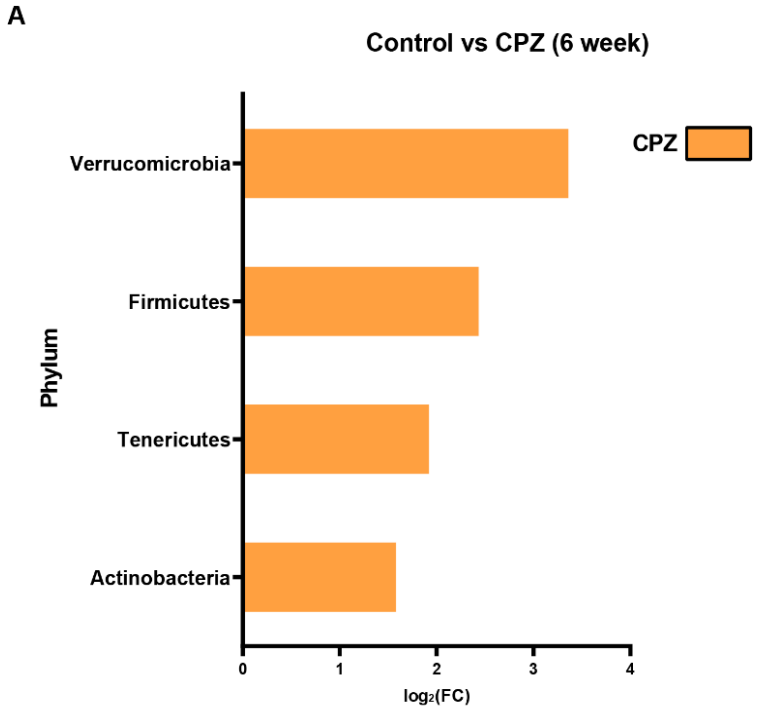
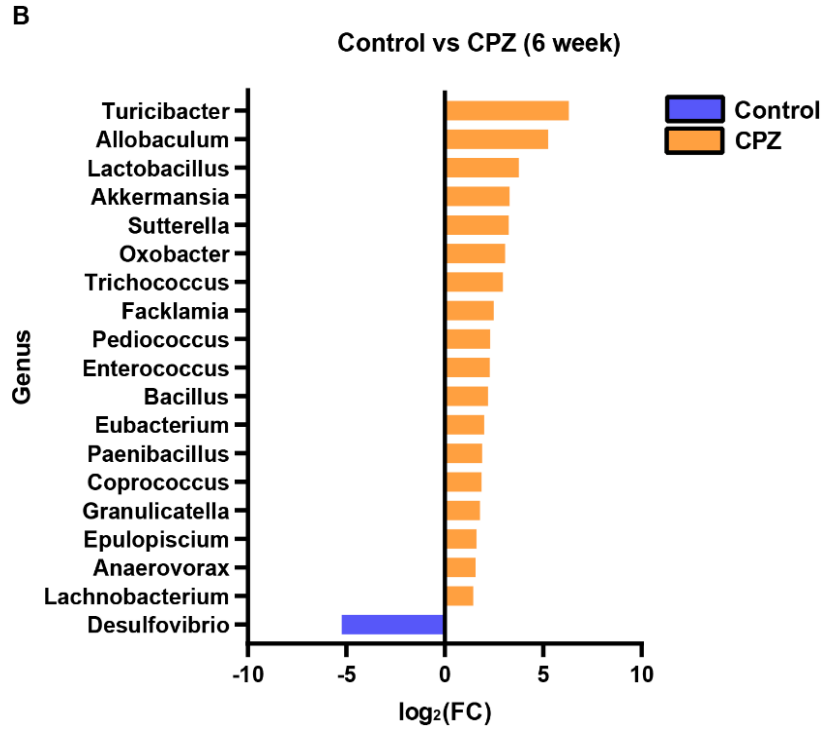

A
4.0
3.5
3.0
2.5
Shannon's index
ns
ns
ns
*
Control_0w
Control_6w
Control_8w
CPZ_0w
CPZ_6w
CPZ_8w
B
Axis.2 [15.1%]
Axis.1 [65.6%]
Group_Time
Con_0w
Con_6w
Con_8w
CPZ_0w
CPZ_6w
CPZ_8w

Figure 23. The shift in diversity of the gut microbiota in mice. (a) Box plot demonstrating the Shannon's index of the gut microbiota. (b) PCoA plot of the gut microbiota in mice.


A
Control vs CPZ (6 week)
CPZ
Verrucomicrobia
Firmicutes
Tenericutes
Actinobacteria
Phylum
0
1
2
3
4
log2(FC)


B
Control vs CPZ (6 week)
Control
CPZ
Turicibacter
Allobaculum
Lactobacillus
Akkermansia
Sutterella
Oxobacter
Trichococcus
Facklamia
Pediococcus
Enterococcus
Bacillus
Eubacterium
Paenibacillus
Coprococcus
Granulicatella
Epulopiscium
Anaerovorax
Lachnobacterium
Desulfovibrio
Genus
-10
-5
0
5
10
log2(FC)

1 2 3

Figure 24. Differential abundance analysis of the gut microbiota in mice at (A) the phylum and (B) genus levels.

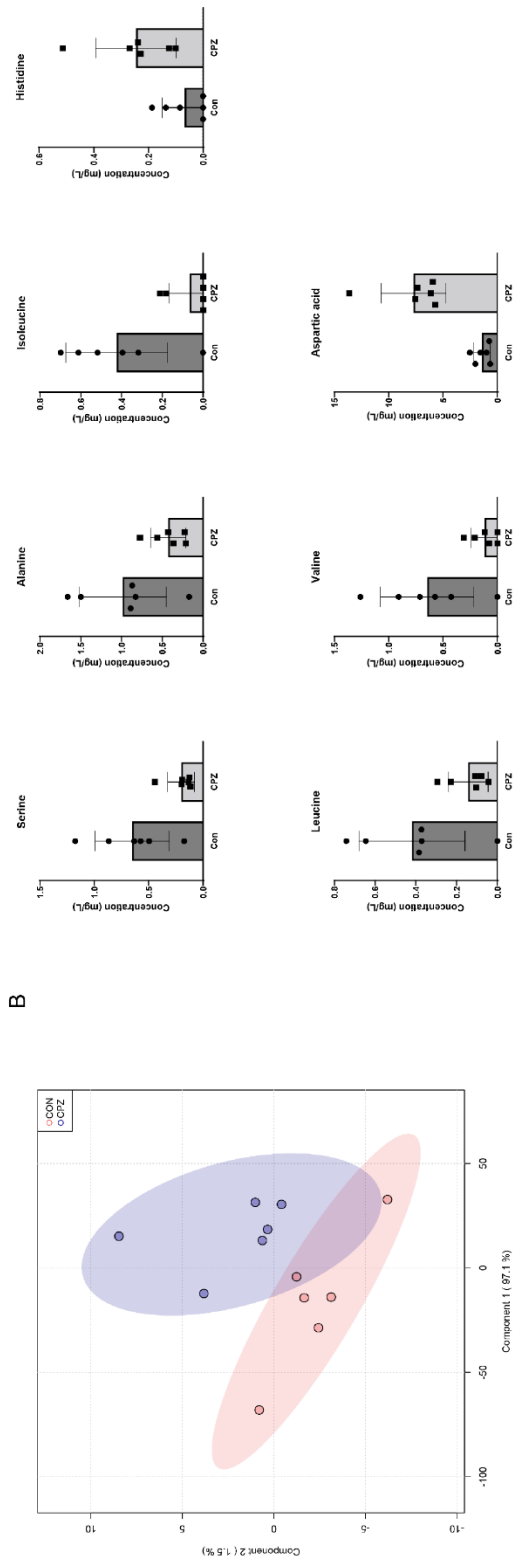

A
CON
CPZ
Component 2 (1.5 %)
Component 1 (97.1 %)
B
Histidine
Isoleucine
Aspartic acid
Alanine
Valine
Serine
Leucine
Concentration (mg/L)
Con
CPZ

Figure 25. The shift in the gut metabolome of mice. (A) PLS-DA plot demonstrating the beta diversity of the gut metabolome of mice. (B) Bar plot demonstrating the concentration of fecal metabolites showing significant differences between the control and cuprizone groups.

General conclusions

The present study was conducted to investigate the impact of host and environmental factors, including host genetics, environment, and diet, on the gut microbiota of animals. First, the present study revealed the distinct differences in the gut microbiome of Korean wild mice, including *Micromys minutus* and *Mus musculus* sharing the same habitat. Metagenomic analysis showed that *Micromys minutus* is a reservoir for *Campylobacter*, whereas *Mus musculus* did not harbor *Campylobacter* in the gut. The distinct proportion of *Campylobacter* and *Lactobacillus* in the wild mice gut microbiome may explain the discrepancies in *Campylobacter* presence. Second, the present study revealed that environmental perturbations alters the gut microbiome and antibiotic resistance acquisition in wild migratory birds during wildlife rehabilitation. Dysbiosis of the gut microbiota was observed, including decreased diversity, depletion of SCFA producers, decreased microbial network complexity, and enrichment of zoonotic pathogens. Moreover, antibiotic resistance including tetracycline and ciprofloxacin resistance of gut microbiome significantly increased after rehabilitation, and the majority of the wild birds acquired multidrug resistance. Third, the present study revealed the impact of long-term dietary intake of red ginseng dietary fiber on the canine gut microbiota. Long-term intake of red ginseng dietary fiber increased the diversity and SCFA producers and decreased zoonotic pathogens, including *Helicobacter*, suggesting the prebiotic potential of red ginseng dietary fiber. Lastly, the present study revealed the effect of a cuprizone diet, which induces demyelination in the CNS, on the gut microbiota and metabolome of mice. Dysbiosis of the gut microbiota was shown, including decreased diversity. Moreover, the enrichment of biomarker

microbes, including *Akkermansia*, and metabolites, including BCAAs, was consistent with human MS patients.

Thus, the present study suggests that the animal gut microbiome is significantly shaped by internal and external factors, including host species, environment, and diet. Moreover, the findings of this study suggest that alteration of the gut microbiome may alter the host-microbe interactions, thereby affecting the host's health. The alteration of the gut microbiome by the various interventions in this study may provide a basis for further studies on animals' gut microbiomes, in particular, further studies exploring beneficial microbes and prebiotic diets to modulate the gut microbiota. Moreover, the present study suggests novel perspectives for the utilization of microbiome analysis for development of sustainable rehabilitation strategies for wild animals. In addition, the findings elucidate the interactions between the brain and the gut microbiome, which may help to overcome neurodegenerative diseases.

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국문 초록

장내 마이크로바이옴에 대한 숙주와 환경 요인의 영향 및 멀티오믹스를 활용한 숙주-미생물 상호작용 연구

송효근

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수의학과

수의병인생물학 및 예방수의학 전공

장내 마이크로바이옴은 장내에 공생하는 박테리아, 바이러스, 곰팡이를 모든 포함한 미생물과 그 유전체의 군집이다. 최근 연구들을 통해 장내 마이크로바이옴이 정교한 숙주-미생물 상호작용을 통해 소화

기능, 면역 기능 및 대사 기능 등의 숙주 건강에 밀접한 영향을 준다는 것이 밝혀졌다. 장내 마이크로바이옴은 숙주 유전체, 식이, 행동 습관 및 환경 등의 다양한 요인에 의해 형성된다. 장내 마이크로바이옴을 형성하는 요인에 대한 대부분의 연구는 인간과 실험실 마우스에 대해 수행되었으며 반려 동물 및 야생 동물의 장내 마이크로바이옴에 대한 연구는 아직 부족한 상황이다. 따라서 본 연구는 동물의 숙주 유전체, 환경 및 식이 등의 요인들이 장내 마이크로바이옴에 대한 영향을 밝히기 위해 진행되었다.

그 결과 첫째, 본 연구는 동일한 서식지를 공유하는 두 종의 한국 야생 마우스, *Micromys minutus*와 *Mus musculus*의 장내 마이크로바이옴이 뚜렷한 차이를 보인다는 것을 밝혔다. 메타유전체 및 배양 분석 결과, *Micromys minutus*는 *Campylobacter* 를 풍부하게 보유하고 있었지만 *Mus musculus*는 장내에 *Campylobacter*가 존재하지 않았다. 두 종류의 야생 마우스 사이에 *Campylobacter*의 존재 차이는 각각의 장내 마이크로바이옴에서 *Campylobacter*를 억제하는 *Lactobacillus*의 상호작용으로 인한 것일 가능성을 제시하였다.

둘째, 본 연구는 재활 기간 동안의 환경 변화가 야생 조류에서 장내 마이크로바이옴의 변화 및 항생제 내성 획득을 유발한다는 것을 밝혔다. 재활 기간동안 야생조류의 장내 마이크로바이옴은 다양성의 감소, 단쇄 지방산 생산미생물의 고갈, 생태학적 미생물 네트워크 복잡성의 감소 및 인수공통병원체의 증가 등의 장내 마이크로바이옴의 불균형을 나타내는 변화들이 일어났다. 또한 재활 기간 동안 ciprofloxacin과

tetracycline 등의 항생제 내성이 유의하게 증가하였으며, 대부분의 야생조류는 항생제 다제내성을 획득하였다.

셋째, 본 연구는 홍삼 식이섬유의 장기간 식이 섭취가 반려견의 장내 마이크로바이옴에 미치는 영향을 밝혔다. 홍삼 식이섬유의 섭취는 장내 마이크로바이옴의 다양성, 단쇄지방산 생성 미생물 및 생태학적 미생물 네트워크의 복잡성을 증가시켰고 헬리코박터를 포함한 인수공통병원체를 감소시켰으며 이는 홍삼 식이섬유의 프리바이오틱스로 활용될 가능성을 시사한다.

마지막으로 본 연구는 중추신경계의 탈수초화를 유도하여 다발성 경화증 모델에 사용되는 cuprizone 식단이 마우스의 장내 마이크로바이옴과 장내 대사체에 미치는 영향을 밝혔다. Cuprizone 식이 급여 결과 마우스 장내 마이크로바이옴 다양성의 감소, 베타다양성의 변화 등 불균형의 지표들이 확인되었다. 또한, cuprizone 급여는 마우스에서 *Akkermansia*를 포함한 미생물 증가 및 분지쇄아미노산 등의 포함한 대사산물을 증가시켰으며 이는 사람 다발성 경화증 환자의 장내 마이크로바이옴 분석 결과와 유사하였다.

결론적으로 본 연구는 동물의 장내 마이크로바이옴이 숙주 종, 환경, 식이 등 숙주 요인과 환경 요인에 의해 영향을 받는 다는 것을 밝혔으며, 이러한 요인들에 의해 변화한 장내 마이크로바이옴이 숙주–미생물 상호작용을 통해 동물의 건강에 영향을 줄 수 있다는 것을 밝혔다. 본 연구 결과는 추후 동물에서의 장내 마이크로바이옴 특히, 장내 마이크로바이옴 조절을 위한 프리바이오틱스와 유용 미생물 발굴 연구에 기

초 자료를 제공할 수 있다. 또한 지속 가능한 야생동물 재활을 위해 장내 마이크로바이옴 분석을 적용할 수 있는 새로운 관점을 제시하고 있다. 마지막으로 뇌와 장내 마이크로바이옴의 상호작용을 밝힘으로써 장내 마이크로바이옴 조절을 통한 퇴행성 신경질환 치료 연구의 기초 자료를 제공할 수 있다.

키워드: 장내 마이크로바이옴, 메타지노믹스, 멀티오믹스, 숙주-미생물 상호작용, 장-뇌 축

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