Chapter

Dissemination of Intestinal Microbiota by Migratory Birds across Geographical Borders

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Abstract

Understanding the dissemination of medically important microbiota is a significant public health necessity. Although modern bacteriology has advanced with improved culturing technology, several environmental bacteria occur in the "viable but nonculturable" state. By using next-generation sequencing (NGS) to comprehensively analyze the intestinal microbiota of migratory birds, research on microbial communities traveling over long distances has entered a new era and provides new insights that are valuable for the analysis of medical care, livestock industry, agriculture, and human health risks. The use of comprehensive analysis by NGS of not only intestinal microbiota but also diet biological communities may help elucidate the relationship between microbiological communities and the diet and succession of intestinal microbiota, including antibiotic-resistant bacteria, during migration and breeding. Here, we have described the current state and the future implications of studying intestinal microbiota associated with migratory birds.

Keywords: migratory bird, avian, gut microbiota, intestinal microbiota, antibiotic resistance, colistin

1. Introduction

The application of DNA sequence technology covers a wide range of fields. As next-generation sequencing (NGS) has progressed, it has become more widely used in an array of practical applications [1, 2]. One direction of use includes the field of precision medical care. In cases of cancer caused by genetic mutations, molecular targeted drugs can be discovered by investigating gene mutations. In addition, the application of NGS is advancing in industrial fields; for instance, genotyping of animals and plants can be performed at low cost and the genetic markers can be screened with NGS.

NGS enables profiling of complex microbial communities in nature as well as that of indigenous microbiota associated with living organisms [3, 4]. Higher forms of life coexist with huge numbers of microorganisms, including bacteria, viruses, fungi, and, in some cases, protozoa and parasites—albeit bacteria are the most important microorganisms in terms of their numbers and host interactions. There are as many viruses as numbers, but many of them are viruses (phages) that infect bacteria.

Bacteria are present on the surface bodies of living organisms, such as their skin, gastrointestinal tracts, respiratory systems, and oral cavities, and they are colonized with an inherent balance in each place. This balance constitutes a stable complex ecosystem through crosstalk between bacteria and hosts. Among these places of localization, the digestive tract has the most abundant localization in terms of both the number and the type. In humans, 90% of established bacteria inhabit the digestive tract.

The intestinal microbial community includes not only enormous numbers and types of microorganisms but also active metabolic activity. The genes of the intestinal microbial community are present in proportion of at least 100-fold more than that of the genome of the host and various metabolites are produced, which are absorbed into the host body. NGS is now an indispensable item in the study of intestinal microbial communities and is applied not only to humans but also to other living organisms, such as domestic animals, insects, poultry, and wild birds [5].

Understanding the transboundary movement of microorganisms is an important requirement from the perspective of public health and environmental science [6, 7]. Microorganisms travel geographically over distant areas on the earth via ocean currents and atmospheric movement. In addition, migratory birds carry pathogenic microorganisms when traveling over long distances to several parts of the world [8–10]. Numerous pathogenic bacteria, such as pathogenic *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Botulinum* spp., *Listeria* spp., and *Campylobacter jejuni*, have been isolated from bird feces [11–13].

Research efforts have been performed to identify the bacterial communities contained in the feces of migratory birds (Bar-headed goose, shorebirds, swallow, etc.) by a novel culture-independent method [14, 15]. We attempted to explore the stability of the intestinal bacterial communities in migratory birds, the difference in the intestinal bacterial communities among birds at the individual and species levels, and the potential of long-distance movement of antibiotic-resistant bacteria associated with migratory birds [16–19]. Research on the spread of bacterial populations over vast distances has led to the elucidation of the roles of migratory birds regarding human health risks, thereby enabling the prediction of potential outbreaks based on their migratory patterns. NGS is useful for understanding bacterial diversity and for discovering novel bacteria [20]. The present review considers the potential role of wild birds in the transmission of intestinal microbiota, including antibiotic-resistant microorganisms, and our current knowledge of microbiota associated with migratory birds using NGS technologies.

2. Methodology for analysis of bacterial community composition

Since the scientific study on bacteria began in the 19th century, pure culture methods supported the progress of microbiology in a wide range of fields such as medicine, pharmacy, biology, agriculture, and fermentation engineering. In the latter half of the 1970s, however, the method of total direct counting was developed, in which bacteria were stained with fluorescent dye and directly observed or counted under a fluorescence microscope. The use of this method revealed that several environmental bacteria cannot yet be cultivated by the conventional laboratory techniques [21, 22]. Therefore, new bacterial detection methods that were independent of culturing began to be developed in succession [23–26]. From the latter half of the 1990s, the method to directly extract DNA from the sample without culturing the bacteria and using a universal primer to target the conserved region of the 16S rRNA gene or PCR amplification with genus-specific primers to decode the DNA sequence became widespread [15, 27, 28]. Since the obtained gene information

depended on the number of bacterial clones that the researcher could handle at a time, these methods were found to be limited to about tens to thousands.

In the past few years, comprehensive analysis of DNA sequences using NGS has spread rapidly [5]. NGS is a powerful fundamental technology that is capable of concurrently determining the nucleotide sequences of tens of millions to hundreds of millions of DNA fragments. It is also capable of advanced and high-speed processing, such as multiple determinations of multiple samples. Moreover, the expenditure on equipment and operations for this method has also been reduced. The use of NGS can help acquire genetic information of tens of thousands to hundreds of thousands of bacterial species in a short time. NGS can also aid in the understanding of the entire picture, thereby enabling a greater focus on specific interesting bacteria based on the phylogenetic taxonomic information. This, in turn, would lead to further qualitative and quantitative analyses in detail.

Because 16S rRNA gene contains both highly conserved regions for primer design and hypervariable regions to identify the phylogenetic characteristics of microorganisms, 16S rRNA gene sequence has become the most widely used marker gene for profiling bacterial communities [29, 30]. Full-length 16S rRNA gene sequences consist of nine hypervariable regions that are separated by nine highly conserved regions [31, 32]. Study with bioinformatics tools attempted to evaluate the phylogenetic sensitivity of the hypervariable regions in comparison with the corresponding full-length sequences and revealed that the V4–V6 regions represented the optimal subregions for bacterial phylogenetic studies of the new phyla [33]. Since the 16S rRNA gene differs from 1 to 16 in the number of copies per cell depending on the genus [34], the relative proportion obtained by NGS does not necessarily agree with the ratio of actual community composition, although the dominant populations can be ascertained.

3. Migratory birds and flyway

The geographical route that migratory birds move annually on the earth is called "flyway," and there are nine major flyways in the world. Japan is located on the East Asia-Australia flyway, and it is estimated that more than 50 million migratory birds, such as shorebirds, birds, and seabirds, travel over the flyway every year.

Summer migrants in Japan fly from the south mainly for breeding and spend the summer in Japan, and when the breeding season ends, they return south for overwintering. Winter migrants in Japan fly from the north mainly for overwintering and spend the winter in Japan, and then in the spring, they return north for breeding. Passage migrants breed in the country north of Japan and overwinter in the country south of Japan, and so they travel through Japan during the movement and are mainly observed in spring and autumn. As a whole, some millions of migratory birds are estimated to visit Japan annually.

The direct counting method by fluorescent staining revealed that the fecal matter of migratory birds contains $\geq 10^8$ cells/g bacteria [16, 17]. Since disinfection treatments for bird feces is not performed like that for humans and livestock, there are possibilities that several live bacteria, along with 50 million migratory birds each year, travel the East Asia–Australia region flyway for a long distance. In order to clarify the dynamic of the microbiota, including pathogenic bacteria and antibiotic-resistant bacteria, as well as to verify their significance in public health and environmental microbiology, research has been performed to analyze the intestinal microbial community associated with migratory birds by NGS [16–22].

4. Intestinal microbiota of adult and young barn swallows

The barn swallow (*Hirundo rustica*) is about 17 cm in length and is widely distributed across the temperate and cold regions of Africa, the Eurasian continent, the northern end of Australia, and North America. Their global population is estimated at more than 190 million individuals [35]. Several swallows migrate to Japan from Southeast Asia (i.e., the Philippines, Malaysia, and Indonesia) and make their nests by mixing mud with plant pieces, feathers, and other such items near human living environments, such as the eaves of a house. While flying in the sky, the barn swallows catch insects to eat. After breeding, they return to Southeast Asia in the autumn season. The number of observed individuals in Japan is estimated at several hundred thousand birds per year.

For the study on the intestinal microbial communities of barn swallow, fecal specimens of the adult and young barn swallows were collected from the nest on the university campus and from various nests around the Osaka prefecture, Japan. The NGS analysis was performed on the V4 region of the 16S rRNA gene.

Diversity indices of intestinal bacterial communities in barn swallows were determined for each of the sampling locations (**Table 1**). The diversity index of young birds was found to be lower than that of adult birds. Similar results were reported from studies with swallows in Europe [36].

The stability of intestinal bacterial communities in wild birds has not yet been studied. We collected feces over a period of time from the same nest within the university campus (OOU 20) and compared changes in the intestinal bacterial communities between adult and young birds. The day when the birds were born was set as day 0, that before the birth as day –N, and that after the birth as day +N (where, N = 1, 2, 3, ...; **Figure 1**). Fecal samples in negative and positive value days represent ones of parent and young birds, respectively.

Adult samples were dominated by *Corynebacteriaceae*, *Halomonadaceae*, and *Pseudomonadaceae* at the family level before the hatch. In contrast, young samples were dominated by *Enterococcaceae*, *Mycoplasmataceae*, and *Enterobacteriaceae* (**Figure 1a**).

Figure 1b depicts the similarity in the intestinal bacterial community at the family level by principal component analysis (PCA). The intestinal bacterial communities of the adult birds changed greatly over time, but the profiles of adult and young birds remained similar before and after hatching, and then the young birds showed a great change. The adult birds brought insects and the likes in the nest to feed the young ones; therefore, it seems that the intestinal bacterial community was temporarily similar because of the similar diets. Similar results were reported from studies with swallows in Europe [36].

To examine the extent of change in the intestinal bacterial community of the barn swallows living in the same nest, the change was compared with that in other nests. The sampling sites OOU17 and OOU20 are nests located about 50 m apart.

Site	Adult	Young
All	2.48 (1.09)[a]	1.66 (0.85)
OOU17	2.74 (0.92)	2.34 (0.88)
OOU20	3.33 (1.36)	1.82 (0.76)
Wakayama	1.86 (0.40)	1.40 (0.73)

[a]*Standard deviation.*

Table 1.
Alpha diversity of fecal microbiota in adult and young barn swallow.

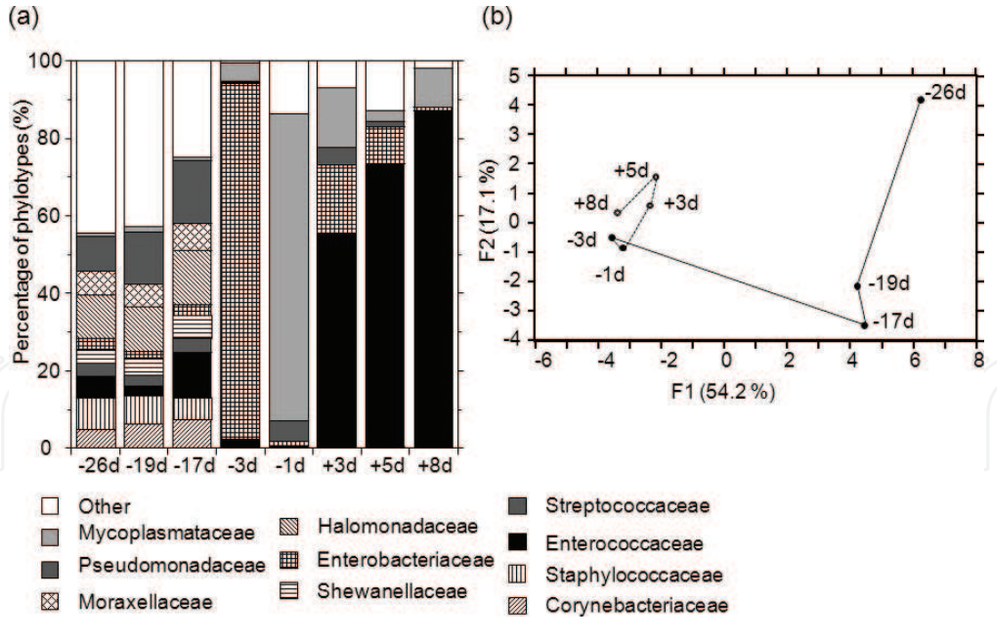

Figure 1.
Change in the bacterial community composition of fecal samples of barn swallows living in the same nest. (a) Relative portions of bacterial phylotypes in averaged fecal samples at the family level (b) Principle component analysis of family abundance data. Fecal samples in negative and positive value days represent ones of parent and young birds, respectively.

The sampling sites Wakayama were about 50 km away from the sites OOU20 and OOU17 and consisted of a plurality of nests at a distance of 100 m. As compared with these adult and young birds, no such characteristics were observed in the groups of adult or young birds or in specific nests (**Figure 2**). As seen in the figure regarding the change in the bacterial community at OOU 20, the extent of difference in the bacterial community over a period of time in the same nest was found to exceed the extent of the difference among different nests.

In order to verify whether there were similarities in the bacterial communities for each collection area, PCA was performed on those from the adult birds (**Figure 3a** and **b**). We examined four areas (northern Osaka, southern Osaka, northern Wakayama, and others); these areas were about 50 km apart and beyond the activity range of the insects that served as the swallows' diets. The distribution of the samples in the same nest at OOU20 is shown in gray in **Figure 3a**. The extent of difference in the bacterial community in the same nest surpassed that

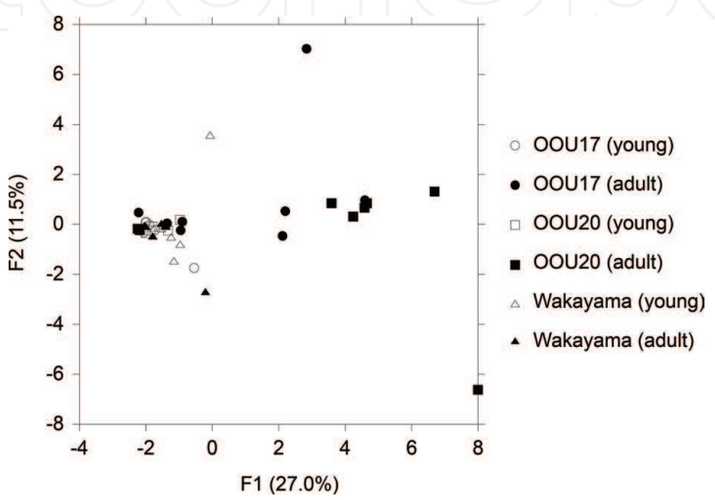

Figure 2.
Principal component analysis of class abundance data from adult and young barn swallows.

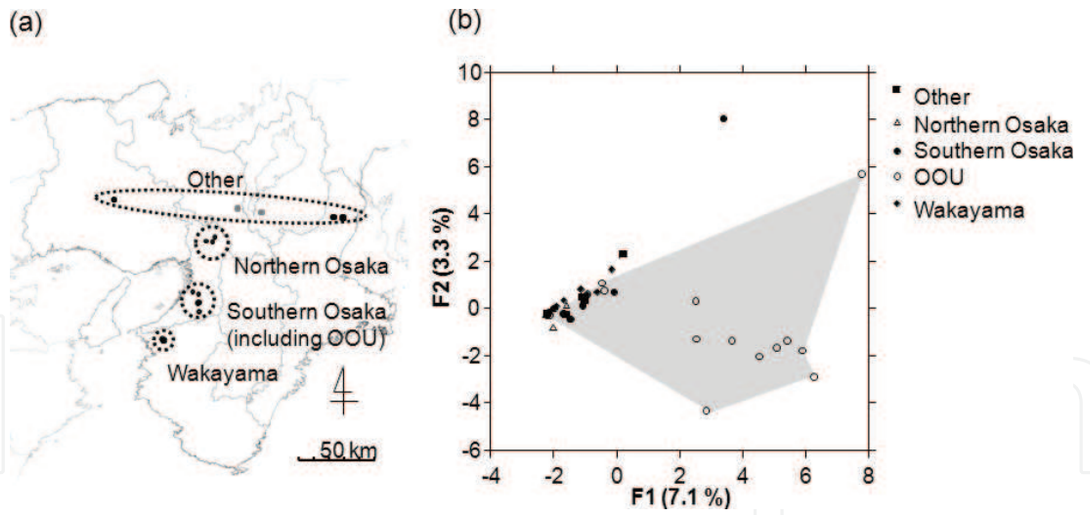

Figure 3.
Similarity analysis of 16S rRNA gene data from adult barn swallows. (a) Sampling sites around Osaka, Japan. (b) Principal component analysis of class abundance data.

among different nests. No relationship was found between the bacterial community composition and the geographical area in the fecal samples. Since the intestinal tract of swallows is short, it was speculated that the intestinal microbial community might be influenced by the daily diet and may accordingly change greatly in only a couple of days.

5. Succession of intestinal microbiota of a Eurasian wigeon while spending a winter

In order to examine the stability of the intestinal bacterial communities of other birds, we examined the succession of intestinal bacterial communities in the feces of a Eurasian wigeon, which was flying to the northern part of Osaka at the beginning of the winter season, staying in the same area when spending a winter. The Eurasian wigeon (*Mareca penelope* or *Anas penelope*) is about 50 cm in length. It breeds in the northern part of the Eurasian Continent, and in winter, it crosses southern Europe, North Africa, and East/South Asia. The global population of Eurasian wigeons is estimated at 2.8–3.3 million individuals [37]. This bird lives primarily in quiet sea, estuaries, lakes, and rivers. In addition to eating plants such as grass leaves and algae, it eats aquatic insects and mollusks. The number of wigeons observed in Japan is reportedly 180,000 per year.

In December 2017, when the researchers flew to Japan, we examined the succession over time with monthly intestinal bacteria of Eurasian wigeon around the Ai River in north Osaka (**Figure 4**). Community analysis at the class level revealed that Clostridia constituted 64.7% in December, but the proportion decreased to 18.4% in April 2018 ($P < 0.01$). The proportion of Bacilli, Fusobacteria, Alphaproteobacteria, and Gammaproteobacteria significantly increased from 0.3 to 7.4%, 1.2 to 10.4%, 1.2 to 6.3%, and 4.1–29%, respectively ($P < 0.01$), while spending a winter in Japan. The intestinal bacterial community composition of Eurasian wigeon, which flew to Japan at the beginning of the winter season, significantly changed while staying in Japan for about 4 months.

We also compared the intestinal microbial communities of Eurasian wigeon in December and April at different sites and in different years. We used samples collected from different sampling sites, Lake Biwa in 2016 and Ai River in 2017, as samples in the early winter. As samples for the spring season, fecal samples were collected from Biwa Lake and Ai River between 2016 and 2018.

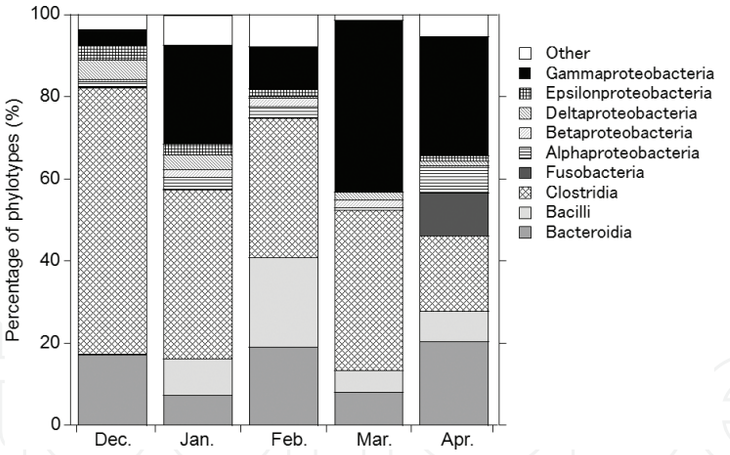

Figure 4.
Changes in the bacterial community composition of fecal samples from the Eurasian wigeon while staying in western Japan. Fecal samples were collected from around Ai River, north Osaka, Japan.

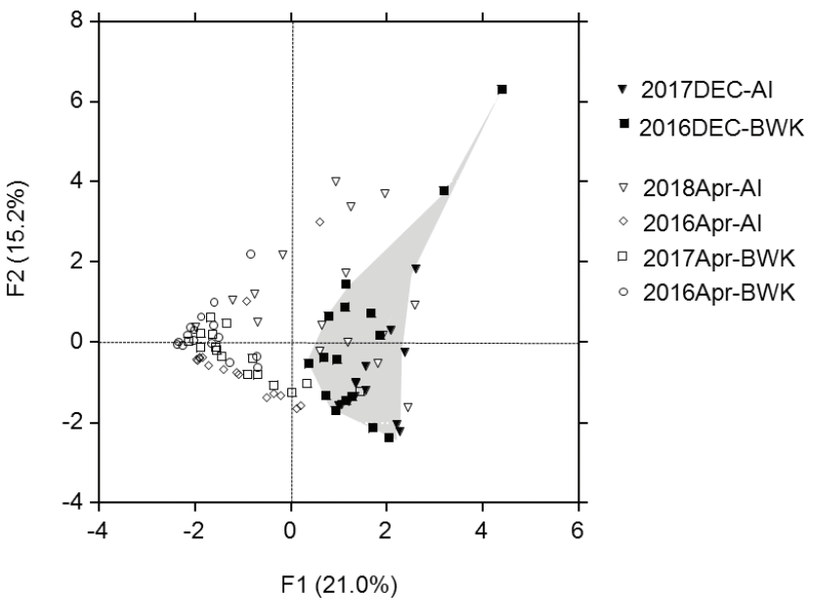

Figure 5.
Comparison of the bacterial community composition of fecal samples from the Eurasian wigeon in the early winter (December) and spring (April) seasons. Fecal samples were collected from around Ai River and lake Biwa, Japan in different years.

All samples in December were dominated by Clostridia, and samples in April were dominated by Gammaproteobacteria. Regardless of the differences in the location and year of sampling, the major bacterial communities were found to be similar. PCA revealed obvious difference in the bacterial community—namely, the samples in December were distributed in gray (right panel) and the samples in April shifted to the left panel in **Figure 5**. These results revealed that the intestinal bacterial community composition of Eurasian wigeon, which flew to Japan at the beginning of winter, changed while they spend a winter in Japan.

6. Colistin-resistant bacteria associated with Eurasian wigeon

The dissemination of antibiotic-resistant bacteria across borders has become an important issue in public health, and the involvement of migratory birds has been indicated as a mechanism by which resistant bacteria spread at the global level [13]. In addition, colistin is regarded as an important antimicrobial agent; it is even an

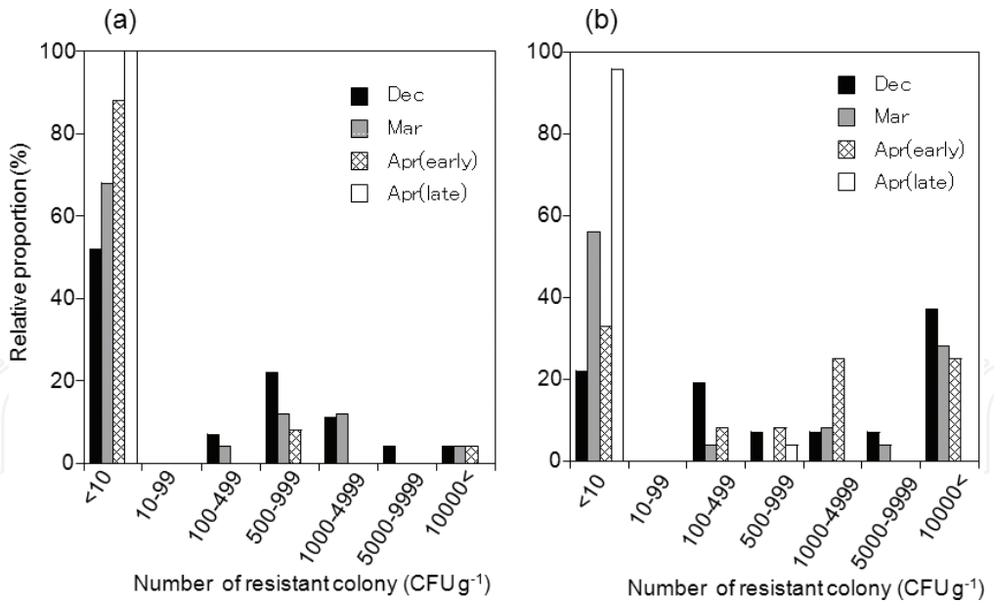

Figure 6.
Frequency distribution of the number of colistin-resistant E. coli (a) and coliform (b) in fecal samples of Eurasian wigeon. Fecal samples were taken around Ai River.

antibiotic used as the last resort for carbapenem-resistant Enterobacteriaceae bacteria at the World Health Organization. Thus, the succession of number of colistin-resistant *E. coli* and coliform in Eurasian wigeon was investigated in this study.

The frequency distribution of the number of colistin-resistant *E. coli* per gram of the sample is shown for each count range in **Figure 6**. In December 2017, the proportion of fecal samples below the detection limit was approximately 50%, and >100 CFU/g was about 50%. In March, the proportion gradually increased to approximately 70%, about 90% at the beginning of April, and 100% at the end of April 2018. For the colistin-resistant coliform, it was also found that the proportion of samples below the detection limit increased gradually while spending a winter in Japan. These results suggest that colistin-resistant *E. coli* and coliform may have been carried over to Japan after ingestion by the Eurasian wigeon in the northern area.

7. DNA barcoding and diet

DNA barcoding is a technique that allows identification of species by using a short nucleotide sequence of a specific gene region. By using a gene region that reflects the difference in species as a standard DNA barcode, it has become possible to specify species. This method can be used to identify species of plants, animals, and fungi. It also helps to discover new varieties by combining with other information.

For animals, about 650 bases in length at the 5′ end of the cytochrome C oxidase subunit I (*COI*) gene on the mitochondrial genome is regarded as a standard barcode region, but the primer used differs, depending on the research project [38, 39]. The reason that the *COI* of mitochondria was selected as a standard barcode region of DNA barcoding of animals is that the universal primers are available to cover most taxa of the animal kingdom and the region contains several mutations at the species level. With a few exceptions, the cells of all eukaryotic species contain the mitochondria. The mitochondrial genome comprises a double-stranded DNA molecule of approximately 16-kb length, accounting for 1–2% of the total DNA in mammalian cells.

Target gene	Name	Nucleotide sequence (5′-3′)
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA
matK	3F_KIM f	CGTACAGTACTTTTGTGTTTACGAG
	1R_KIM r	ACCCAGTCCATCTGGAAATCTTGGTTC
rbcL	rbcLa_F	ATGTCACCACAAACAGAGACTAAAGC
	rbcLa_R	GTAAAATCAAGTCCACCRCG
12S	M13U12S-F	TGTAAAACGACGGCCAGTCAAACTGGGATTAGATACCC
	M13U12S-R	CAGGAAACAGCTATGACCGAGGGTGACGGGCGGTGTGT
16S	M13U16S-F	TGTAAAACGACGGCCAGTACCGTGCAAAGGTAGCATAAT
	M13U16S-R	CAGGAAACAGCTATGACCTCCGGTCTGAACTCAGATCAC

**Underlined portions are M13 tag sequences [39].*

Table 2.
Representative primers used for DNA barcoding.

On the other hand, for plants, a few mitochondrial interspecific mutations and *COI* cannot be used for species-level identification. Therefore, researchers propose the use of the chloroplast DNA region in plants, with *rbcL* and *matK* as standard barcode regions of terrestrial plants [40].

For analyzing the intestinal contents of various types of wildlife, the DNA barcoding method described above can be used in combination with NGS [41, 42]. Representative primers used for DNA barcoding were shown in **Table 2**. Various primer sets are available from the following site (http://www.boldsystems.org/index.php/Public_Primer_PrimerSearch).

8. Migratory bird diet

We focused on the DNA barcoding method to examine the relationship between diet and intestinal microbial communities in migratory birds. It can be hypothesized that diet—based on foods available in the area—has an influence, leading to change in the intestinal bacterial community of Eurasian wigeon and the number of colistin-resistant *E. coli* while spending a winter in Japan. Therefore, we performed NGS analysis on the intestinal contents of the Eurasian wigeon using eukaryotic *COI* gene as the target (**Figure 7**).

The *Orthocladiinae* sp. belonging to Arthropoda were found to be abundant at any time period, although there were differences in their proportion depending on the season. In December, *Demodex folliculorum* and *Orthocladiinae* sp., which belonged to Arthropoda, and *Adineta vaga*, which belonged to Rotifera, were abundant. Rhodophyta and Ochrophyta were observed only in the February samples. In the April samples, *Cricotopus annulator,* which belonged to Arthropoda, and *Adineta vaga*, which belonged to Rotifera, were abundant.

Pearson's correlation coefficients did not show any strong relationships between the number of both colistin-resistant *E. coli* and coliform and the diet. The abundance of Actinobacteria and Bacilli showed a significant but week correlation with abundance of *Scaptomyza montana* ($r = 0.340$, $P < 0.05$ and $r = 0.417$, $P < 0.01$, respectively). The abundance of Clostridia was negatively related with *Orthocladiinae* sp. ($r = -0.372$, $P < 0.05$) and *Scaptomyza montana* ($r = -0.327$, $P < 0.05$) and positively related with abundance of *Talaromyces pinophilus* ($r = 0.409$, $P < 0.01$).

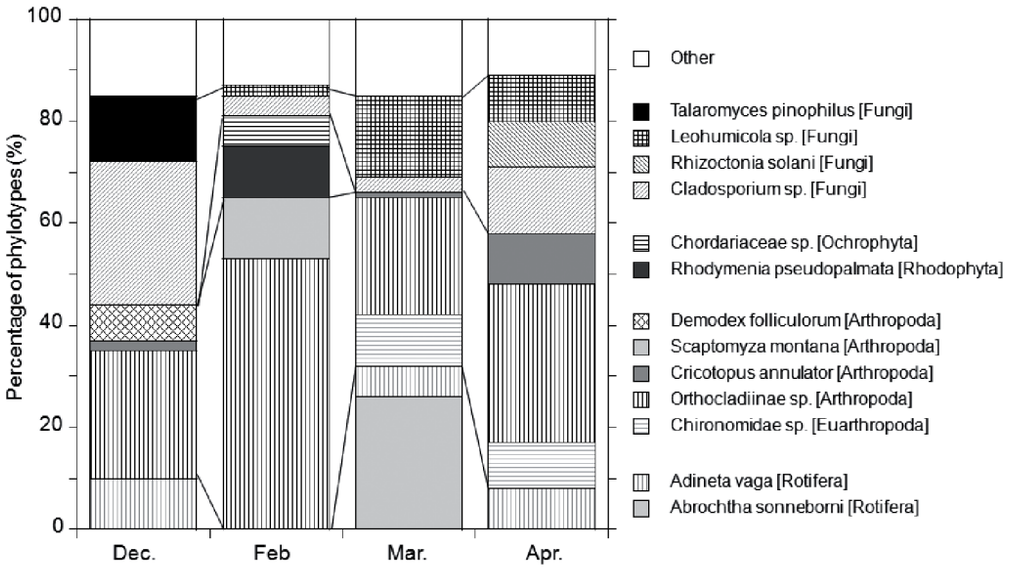

Figure 7.
Relative proportions of phylotypes of diet in the Eurasian wigeon.

The abundance of Gammaproteobacteria was positively related with abundance of *Abrochtha sonneborni* (r = 0.354 P < 0.05) and *Orthocladiinae* sp. (r = 0.393, P < 0.05, respectively). All correlation coefficients were not strong, and thus other parameters or multiple parameters might affect the intestinal microbial communities.

Although the Eurasian wigeon mainly ingests terrestrial plants, *COI* sequences of terrestrial plants were less detected in this study. For organisms that feed on plants, it seems better to use the sequences of *rbcL* and *matK* on chloroplast DNA. In order to investigate the relationship between the intestinal microbial community and diet in wild birds, it is necessary to select appropriate primers suitable for particular types of living organisms that are consumed as part of the diet.

9. Bacterial community composition in migratory and nonmigratory birds

In order to clarify the intestinal bacterial communities among different avian species, those of seagulls, Eurasian wigeon, and barn swallow were compared at their class levels (**Figure 8**). For this research, feces of the European herring gull and Slaty-backed gull were collected in Hokkaido (northern Japan). Slaty-backed gull (*Larus schistisagus*) is a large gull, measuring about 60 cm in length, breeding around northern Japan in the summer season and traveling to the south of mainland Japan and South Korea in the winter season. The global population was estimated to be 25,000–1,000,000 individuals, while the national population was estimated to include >1000 wintering individuals in Japan [43]. The European herring gull (*Larus argentatus*) is a large seagull with a total body length of approximately 60 cm, breeding in eastern Siberia in the summer season and traveling to Japan or to more southern areas in the winter season. These seagulls mainly eat fish, but they also eat crustaceans, insects, and other bird eggs. The global population was estimated to be 2,060,000–2,430,000 individuals, but the population was estimated to be decreasing at a rate of approximately 30% in 39 years [44].

Although the intestinal bacterial community of the Eurasian wigeon was dominated by Clostridia and Bacteroidia, those of the seagulls were dominated by Gammaproteobacteria. **Figure 8** shows the results of PCA, comparing the similarities between the intestinal bacterial communities of the migratory birds with other birds

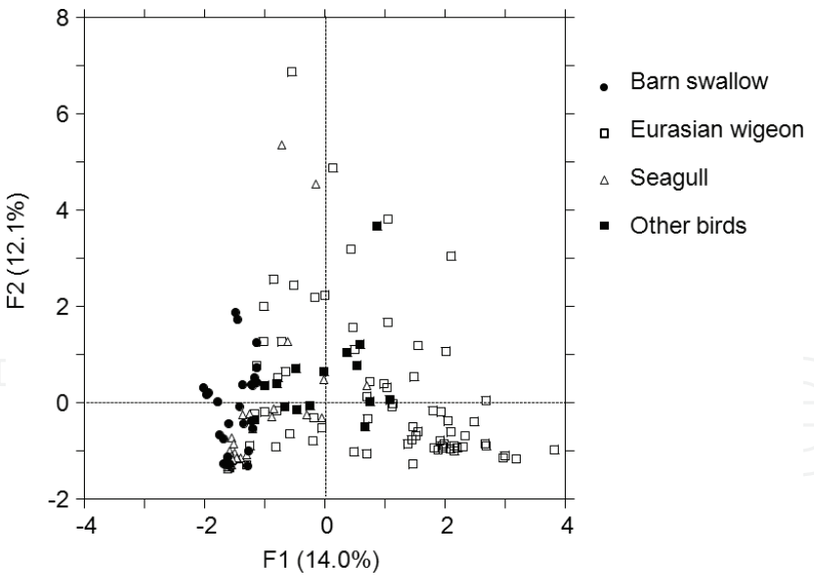

Figure 8.
Principal component analysis of class abundance data from migratory birds and nonmigratory birds.

registered on the public database. It is highly likely that migratory birds may eat different foods; therefore, differences across individuals were large as compared to those in poultry. However, as compared with other birds, individual intestinal microbiota from the barn swallow was relatively similar. In particular, intestinal bacterial composition from the Eurasian wigeon (□) collected from different seasons (December and April) was found to be highly diversified. The extent of the difference in them surpassed the extent of the difference among other birds. It may be reasonable that each of the intestinal bacterial communities was formed by the food consumed, be it an insect meal, an herbivorous meal, an omnivorous meal, or a carnivorous meal.

10. Conclusion

The use of culture-independent methods for studying wild bird-associated microbial communities could have been shown to be beneficial in the expansion of our current knowledge. The NGS targeting the 16S rRNA gene allows a comprehensive clarification of the bacterial communities, their succession while spending a winter or breeding, and their associated movement with migratory birds. The application of NGS is expected to improve our understanding of the overview of not only bacterial communities but also organisms ingested as part of the diet in wild birds. Narrowing down the target organisms using NGS will enable us to identify unknown pathogens or reveal the potential migration status of known pathogens that have escaped noticed so far due to methodological constraints. In addition, the relationship between intestinal microbial communities and diet of living organisms needs to be studied in greater detail.

Investigation of community composition in parallel with functional investigations (e.g., drug resistance) is expected to improve our understanding of the mechanisms by which multidrug-resistant bacteria spread around the world. Addressing the current implications of birds as potential vectors of antibiotic-resistant bacteria is of great interest. Analysis of the indigenous bacterial flora of migratory birds may highlight the importance of human hygiene and the environmental significance of the transfer of antibiotic-resistant bacteria associated with natural bird migratory patterns. When wild birds act as vectors of diseases, it is important to identify the true source of infectious organisms. NGS, as a culture-independent method,

facilitates further understanding of the complexities and interactions of the genera inherently associated with birds, such as sputum, feces, and feather, as well as of those acquired from the wintering or breeding environment.

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Conflict of interest

The author has no conflicts of interest directly relevant to the content of this article.

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