Chapter 3

Public Health Implications of Intestinal Microbiota in Migratory Birds

Takehiko Kenzaka and Katsuji Tani

Additional information is available at the end of the chapter

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Abstract

Understanding the transboundary movement of microorganisms is a significant public health issue. It is possible that large amounts of various bacteria existing on the earth's surface are spreading across borders through migratory birds, but their identities and rates of migration have yet to be elucidated. Although modern bacteriology has advanced based on culture technology, many environmental bacteria may be in a "viable but nonculturable" state. To date, various novel culture-independent detection methods have been developed, including next-generation sequencing (NGS) technology that enables high-throughput sequencing and in-depth gene analysis independent of culture. By using NGS to comprehensively analyze the intestinal microbiota of migratory birds, research on bacterial and viral communities traveling over long distances has entered a new era, providing a new insight for the analysis of the livestock industry, agriculture, and human health risks. Here, we describe the current state and future outcomes of studying intestinal microbiota associated with migratory birds.

Keywords: migratory bird, avian, wild bird, gut microbiota, intestinal microbiota

1. Introduction

Understanding the transboundary movement of microorganisms is a significant issue regarding health, sanitation, and ecological conservation. Birds are susceptible to many microbial diseases that are common to humans and domestic animals [1]. The unprecedented spread of West Nile virus in North America in 1999 has raised concerns over the transport of pathogenic viruses by migratory birds [2]. Recent sporadic outbreaks of avian influenza have caused masses of avian deaths, and it is strongly suspected that the source of infection in East Asia is the feces of migratory birds. In 2014, studies revealed that the influenza virus of the Antarctic penguin had spread to migratory birds via the fecal-oral route and was highly likely to have infected horses on continents more than a few thousand kilometers away [3].

Waterfowls such as ducks, geese, coots, and cormorants can play a role in the environmental dissemination of *Giardia* cysts and *Cryptosporidium* oocysts [4]. Migratory birds are also thought to be one of the mechanisms responsible for the wide geographic distribution of various human pathogenic protozoans.

Organized and periodic surveillance methods of bacterial pathogens are not as efficient as those for the highly pathogenic avian influenza. Avian cholera, caused by *Pasteurella multocida*, occurs sporadically in various parts of the world, including North America and Europe, where migratory birds are also implicated as a vector [5]. Many human enteric pathogenic bacteria have been isolated from wild birds [6]. Additionally, studies reported that antibiotic-resistant bacteria travel long distances through migratory birds [7]. Therefore, migratory birds carry a wide range of viruses, bacteria, protozoa, fungi, and other microorganisms that may be transmitted to humans [8].

Attempts have been made to clarify the microbiota contained within migratory birds' feces using new culture-independent genetic-based methods such as next-generation sequencing (NGS). Research on the spreading of bacterial populations over the vast distances has led to the elucidation of the roles of migratory birds regarding human health risks. Further studies of the spatial and temporal distribution of pathogenic bacteria in wild birds will enhance the assessment of their roles, thereby enabling the prediction of potential outbreaks based on migratory patterns. NGS is useful for the understanding of bacterial diversity and the discovery of novel bacteria [9]. This review considers the potential role of wild birds in the transmission of intestinal microbiota and our current knowledge of microbiota associated with migratory birds using NGS technologies.

2. Intestinal microbiota in wild birds

The majority of information regarding enteropathogens in wild birds has been ascertained by applying traditional microbiological techniques [10–12]. Research on the fecal flora of wild birds in a few well-studied species has focused on the prevalence of enteropathogenic bacteria that are most likely zoonotic. Representative species that are often found in diseased wild birds include *Salmonella enterica* serotypes *Typhimurium* and *Enteritidis*, *Escherichia coli*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Clostridium botulinum*, and *Listeria monocytogenes*. The carrier birds are often diverse species, including house sparrows, brown-headed cowbirds, white-throated sparrows, tufted ducks, crows, pheasants, pigeons, hook-bills, finches, free-living flamingos, quails, pheasants, red grouse, and waterfowl [1, 6, 8].

Many classical methods such as microscopy, culture, or serology have been used to detect and isolate pathogenic protozoa or fungi from excreta of migratory birds. *Candida albicans* and *Candida tropicalis*, which can become pathogenic in immunocompromised individuals, have been isolated from excreta of migratory gulls and geese [13]. *Toxoplasma gondii* has been isolated from many migratory species including ducks and raptors. *Eimeria* spp., which cause severe intestinal coccidiosis, are distributed by geese and diving ducks. These infections are mainly caused by oral ingestion of oocysts excreted from feces. The oocysts of *Cryptosporidium baileyi*, an intracellular enteric coccidian parasite that can cause gastrointestinal and respiratory

tract disorders in birds, have been found in excreta of migratory gulls and Canada geese. Thus, waterfowls can disseminate intestinal protozoan parasites in the environment [4].

Representative virus species that often cause fecal-oral disease transmission in wild birds include *Influenza A virus* (found in species of ducks, geese, gulls, terns, shearwaters, guillemots, shorebirds, and passerines), Newcastle disease virus (found in many species of free-living birds), anatid herpesvirus 1 (found in many species of anseriform such as ducks, geese, and swans), and aviadenovirus (found in migratory anseriform species) [6, 8].

As vectors of enteropathogens, wild birds have been associated with global outbreaks of water-borne and food-borne diseases. For example, outbreaks of infectious diseases have been attributed to fecal water supply contamination by wild birds [14]. Birds were strongly suspected as a source of enteropathogens for livestock when infected birds were found foraging on the livestock feed, and domestic cats have been known to contract enteropathogens by feeding on infected dead birds [15]. The incidence of infectious diseases in humans has also been linked to the handling of dead wild birds [16].

3. Methodology

Traditionally, studies on microbial community composition have been based on culture-dependent methods [17]. When classifying and identifying bacteria, a combination of simple methods has helped to distinguish bacteria based on morphology, dyeability, and biochemical properties. Since automated devices have been developed, it is possible to assure the quality of microbial-based data in pharmaceutical fields and diagnostics fields as standards. When in need of more specific classification, sequence decoding for a specific gene or DNA fingerprinting is carried out. When determining bacterial species, it is a standard practice to analyze the 16S rRNA gene.

However, traditional culture methods underestimate bacterial populations because many environmental bacteria cannot yet to be cultivated by conventional laboratory techniques [18, 19]. Traditional microbiological methods of detection, enumeration, and identification using culture methods are often time-consuming and labor-intensive. These practical considerations often limit the extent to which microbiological tests are routinely applied to community analysis.

Various approaches have been developed to identify microorganisms in natural samples without the requirement for laboratory cultivation [20–23]. Since the latter half of the 1990s, direct sequencing approaches for 16S ribosomal RNA gene amplicon have been popularized. In these methodologies, DNA is extracted directly from the sample without culturing the bacteria. Target DNA is amplified by polymerase chain reaction (PCR) using a universal primer set targeting a conserved region of the 16S rRNA gene or a genus-specific primer, and then sequencing follows to identify the bacterial community members. Because the amplicons are mixed molecules derived from numerous complex bacterial species, PCR products should be separated using denaturing gradient gel electrophoresis or a clone library method in combination [24, 25]. With these methods, the number of bacterial clones that can be screened at one time is limited from several tens to thousands and proves to be labor-intensive.

In this decade, a comprehensive analysis of gene sequences using next-generation sequencing (NGS) has spread rapidly [26, 27]. The NGS is a powerful technology capable of concurrently determining nucleotide sequences for tens of millions to hundreds of millions of fragmented DNA strands. Originally, NGS was used for high-throughput sequencing of a single biological genome, but now it is possible to perform high-speed processing, allowing multiple samples to be sequenced simultaneously. Therefore, a wide variety of applications have been proposed for NGS. The price of NGS contract analysis service also has declined greatly in the past few years, making it easier to use so that it is now more useful for research on genetic diseases, clinical diagnoses, relationships between human intestinal flora and diseases, analyses of environmental bacterial community composition and succession in both time and space, and searches for useful microorganisms in various environments. Metagenomic methods provided by NGS technology have facilitated a remarkable expansion of our knowledge regarding uncultured bacteria [28].

A more recent detection method, quantitative real-time PCR, is known for its excellent accuracy and sensitivity when detecting known zoonotic pathogens [29]. On the other hand, it is difficult to identify target pathogens that are not previously known with this method, and often too many samples must be handled simultaneously for it to be convenient. A comprehensive analysis by NGS enables us to comprehend a whole picture of the bacterial community contained in a sample, so it is possible to carry out further analysis with specific pathogenic bacteria based on the taxonomic information obtained by NGS.

4. Variable region of the 16S rRNA gene

The 16S rRNA gene sequence was first used in 1985 for phylogenetic analysis [30]. Because it contains both highly conserved regions for primer design and hypervariable regions to identify phylogenetic characteristics of microorganisms, the 16S rRNA gene sequence became the most widely used marker gene for profiling bacterial communities [31]. Full-length 16S rRNA gene sequences consist of nine hypervariable regions that are separated by nine highly conserved regions [32]. Limited by sequencing technology, the 16S rRNA gene sequences used in most studies are partial sequences. Therefore, the selection of proper primers is critical for studying bacterial phylogeny in various environments [32].

Recent studies utilizing high-throughput technology also have demonstrated that the use of suboptimal primer pairs results in the uneven amplification of certain species, causing either an under- or overestimation of some species in a microbial community [32, 33]. Integrated bioinformatics tools were used to evaluate the phylogenetic sensitivity of the hypervariable regions compared with the corresponding full-length sequences. Results showed that using a combination of V4–V6 regions represented the optimal subregions for bacterial phylogenetic studies of new phyla [34].

5. Flyway

Bird migration is the regular seasonal journey undertaken by many species of birds. Bird movements occur as a response to changes in food availability, habitat, or weather. Approximately 1800

of the world's 10,000 bird species are long-distance migrants [35]. The bar-tailed godwit, *Limosa lapponica*, undertakes one of the avian world's most extraordinary migratory journeys. Recent research revealed that some individuals had made nonstop flights over 11,000 km, the longest continuous journey that has ever been recorded for a bird [36].

Many species migrate along broadly similar, well-established routes, known as flyways. Recent research has identified nine such pathways: the East Atlantic, the Mediterranean/Black Sea, the West Asia/East Africa, the Central Asia, the East Asia/Australasia, and four flyways in the Americas [37]. The most common pattern involves flying north in the spring to breed in the temperate or Arctic summer and then returning south in the fall to warmer wintering grounds. Migration is often annual and is linked closely with the cyclic pattern of the seasons. Migration is most evident among birds, which have a highly efficient means for traveling swiftly over long distances.

The East Asia/Australasia flyway extends from Siberia and North America to the southern limits of Australia and New Zealand. It encompasses large parts of East Asia, all of Southeast Asia, and includes eastern India and the Andaman and Nicobar Islands. The scale of avian movements along the flyway is awesome, with over 50 million migratory birds using the route annually [38]. Bacterial community compositions in migratory birds from the East Asia/Australasia flyway are described in the section below.

6. Bacterial community composition in migratory birds

A comprehensive analysis of the bacterial community structure in migratory birds using culture-independent methods is introduced below.

6.1. Confirmation of avian host

For field samples, it is important to confirm that the specimens are derived from the desired avian host. Mitochondrial DNA (mtDNA) sequences from avian hosts are ideal for this purpose because they provide phylogenetic information and a high copy number in host cells. Kenzaka et al. [39] amplified avian host DNAs by PCR with primers L5216 (5′-ACTCTTRTTTAAGGCTTTGAAGGC-3′) and H6313 (5′-GGCCCATACCCCGRAAATG-3′) targeting the NADH dehydrogenase subunit 2 (ND2) gene and determined the sequences to confirm the avian host feces [40]. The mtDNA sequences from a variety of avian species are available in DNA database (e.g., GenBank).

6.2. Eurasian wigeon

The Eurasian wigeon (*Mareca penelope* or *Anas penelope*) breeds in the northernmost areas of Europe and Asia. The size of the wigeon is approximately 50 cm in length (**Figure 1a**). The global population is estimated approximately 2.8–3.3 million individuals [41]. The species is strongly migratory, undertaking significant cold weather movements of varying magnitudes. It leaves its breeding grounds in late summer to arrive in its wintering grounds across Europe and Asia in October and November. It lives primarily in lakes, rivers, and along coastlines and

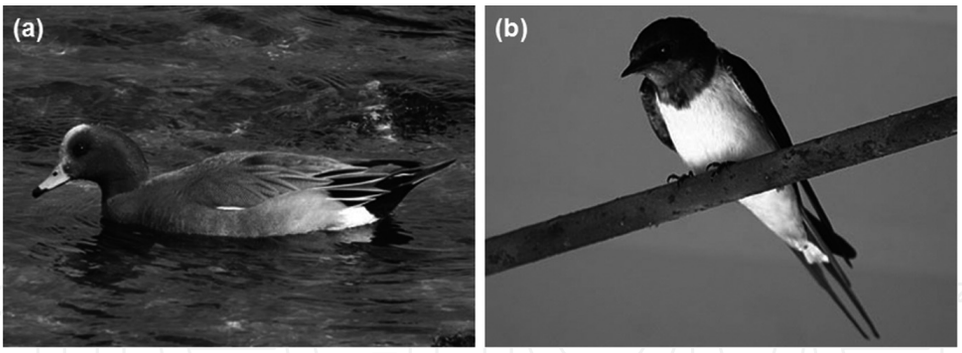

Figure 1. Photographs of (a) Eurasian wigeon and (b) barn swallow.

prefers a location near water plants and land plants that it can eat. The number of observed individuals in Japan has been about 180,000 per year.

Kenzaka et al. collected fresh feces from the Eurasian wigeon that had fallen on plant surfaces along the southern coast of Lake Biwa (Japan) [39]. From this research, most fecal sample bacterial communities were dominated by the phyla *Firmicutes* (51.7%) and *Proteobacteria* (45.1%), composing an average of about 97% (**Figure 2a**). At the family level, on average, *Enterobacteriaceae* composition was 37.6%, *Bacillaceae* was 21.5%, *Paenibacillaceae* was 16.5%, *Clostridiaceae* was 7.5%, and *Pseudomonadaceae* was 6.3% (**Figure 2b**). Although there were individual differences, these families were the dominant groups in all samples collected.

Detected genera that have been reported in association with human pathogenicity are shown in **Table 1**. The genera *Pantoea*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Clostridium*, *Escherichia/Shigella*, *Helicobacter*, and *Serratia* were found at a rate of more than 0.1% of total sequences. On the other hand, the genus *Campylobacter*, which is present in various birds and known as causative bacteria of food poisoning [42], was detected but composed less than 0.1% of the bacterial community in 60% of the samples. Compositions for both the genus *Listeria*, a zoonotic infectious pathogen-causing listeriosis [43], and the genus *Pasteurella*, a pathogen of poultry cholera [5], were less than 0.1% in all of the samples.

6.3. Barn swallow

The barn swallow (*Hirundo rustica*) has a total length of about 17 cm, and its weight is about 18 g (**Figure 1b**). The global population is estimated more than 190 million individuals approximately. This species breeds in a wide range of climates and over a wide range of altitudes, preferring open country like farmland and near water and buildings that provide nesting sites. The barn swallow is primarily a rural species in Europe and North America, while in North Africa and Asia, it often breeds in towns and cities [44]. Many swallows migrate to Japan from Southeast Asia (i.e., Philippines, Malaysia, Indonesia, etc.) and breed near human-living environments, such as private houses and the eaves. Swallows mainly feed on insects. After breeding, they gather at river beds and reed borders, forming group roosts of 1000–10,000 of individuals, and then return to Southeast Asia in autumn. The number of observed individuals in Japan is estimated at several hundred thousand birds per year.

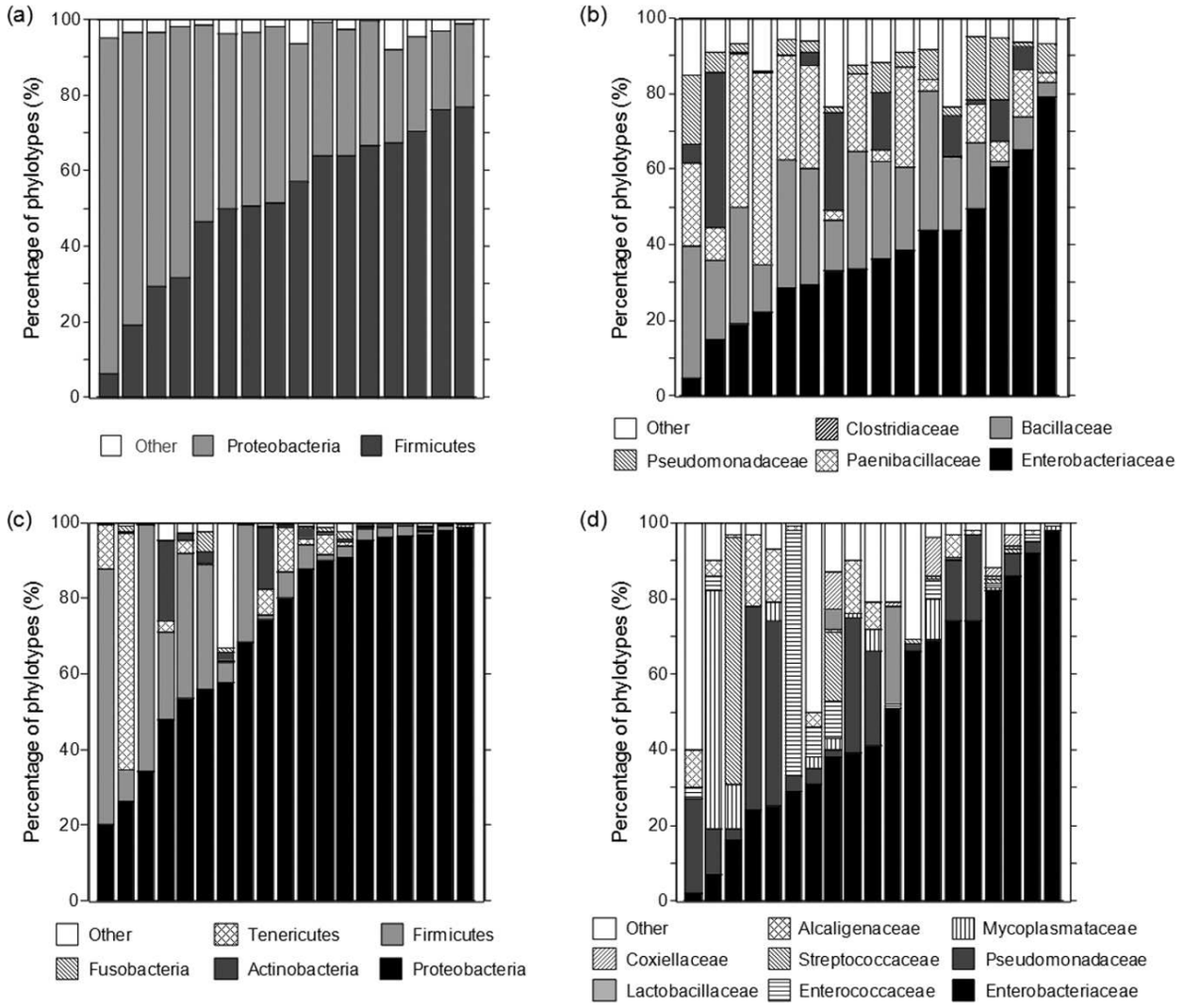

Figure 2. Relative proportions of bacterial phylotypes in individual fecal samples of barn swallow shown at the (a) phylum level and (b) family level and of Eurasian wigeon at the (c) phylum level and (d) family level.

In Osaka Prefecture (Japan), Kenzaka et al. collected specimens of fresh swallow feces from under a mating pair's nest, made at the edge of a private house or artificial building [45]. **Figure 2c** shows the results of the bacterial community composition analysis (at the phylum level) found in swallow feces. Most fecal samples were dominated by *Proteobacteria* (72.1%), *Firmicutes* (15.9%), and *Tenericutes* (5.7%), composing on average about 94% of the bacterial community. Moreover, the proportion of *Bacteroidetes*, which is a human gut-dominant bacterial phylum, was about 0.4%. On the family level, *Enterobacteriaceae* composition was about 53.3% on average, *Pseudomonadaceae* was 13.6%, *Mycoplasmataceae* was 5.5%, *Enterococcaceae* was 4.8%, *Streptococcaceae* was 4.6%, *Alcaligenaceae* was 4.3%, *Lactobacillaceae* was 1.7%, and *Coxiellaceae* was ~1.3% (**Figure 2d**). Although there were individual differences, any of these bacterial groups dominated more than 10% in all samples.

Table 1 shows the major genera with high abundance, namely, *Pseudomonas* spp., *Escherichia/Shigella* spp., *Enterobacter* spp., *Yersinia* spp., *Mycoplasma* spp., *Enterococcus* spp., *Achromobacter* spp., *Fusobacterium* spp., and *Serratia* spp. All of these genera include species that are reported as

Genus	Relative proportion (%)[a]	
	Eurasian wigeon	**Barn swallow**
Pseudomonas spp.	33.2	<0.1
Escherichia/Shigella spp.	21.1	<0.1
Enterobacter spp.	16.5	<0.1
Yersinia spp.	6.1	17.7
Mycoplasma spp.	5.7	<0.1
Enterococcus spp.	3.1	13.4
Achromobacter spp.	0.4	<0.1
Fusobacterium spp.	0.1	0.2
Serratia spp.	<0.1	11.2
Pantoea spp.	<0.1	9.9
Bacillus spp.	<0.1	9.2
Paenibacillus spp.	<0.1	7.2
Clostridium spp.	<0.1	4.8
Helicobacter spp.	<0.1	0.8

[a]>0.1% of total OTUs.

Table 1. Relative proportion of OTUs belonged to representative genus in feces samples determined by 16S metagenomics sequencing.

pathogenic to humans. The genus *Campylobacter* was detected in some samples but at a rate of <0.1% in only 40% of the samples. The genera *Pasteurella* and *Listeria* composed of <0.1% in all samples. Also, *Bacteroides* spp., *Bifidobacterium* spp., and *Prevotella* spp., which are all commonly dominant in the human intestine [46, 47], comprised <0.1% in more than 90% of samples.

6.4. Bar-headed goose

Wang et al. examined metagenomic profiling of gut microbial communities in both wild and artificially reared bar-headed geese in China [48]. The bar-headed goose (*Anser indicus*) breeds in the high plateaus of Central Asia in colonies of thousands near mountain lakes and winters in South Central Tibet and India. This species has been reported as migrating south from Tibet, Kazakhstan, Mongolia, and Russia, crossing the Himalayas [49].

The authors found that *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the four most abundant phyla in the gut of bar-headed geese. In wild bar-headed geese, the predominant phylum was the *Firmicutes*, with an average relative abundance of 83.2%. The second most predominant bacterial lineage, constituting 11.8%, was identified as phylum *Proteobacteria*, followed by *Actinobacteria* and *Bacteroidetes*, accounting for 2.5 and 0.9%, respectively, of the relative abundance.

At the genus level, the sequences from the wild samples represented 106 genera. Four major genera (*Streptococcus*, *Lactococcus*, *Bacillus*, and *Enterococcus*) belonged to phylum *Firmicutes*,

the genus *Pseudomona*s belonged to phylum *Proteobacteria*, and *Arthrobacter* belonged to *Actinobacteria*. Wang et al. compared the bacterial compositions between wild and artificially reared populations of bar-headed geese [48]. They found that *Bacteroidetes* was significantly more abundant in the artificially reared population compared to the wild population.

They also reported on functional profiling and found that artificially reared bar-headed geese had more genes related to carbohydrate transport and metabolism, energy metabolism and coenzyme transport, and metabolism, in general.

6.5. Shorebirds

Ryu et al. examined intestinal microbiota of migrating shorebirds in Delaware Bay (Delaware, United States) on Atlantic flyway using a 16S rRNA clone library [50]. The authors collected the pellets from ruddy turnstones, red knots, and semipalmated sandpipers, which use the Atlantic flyway. The flyway route generally follows the Atlantic Coast of North America and the Appalachian Mountains.

The ruddy turnstone (*Arenaria interpres*) is a small wading bird. The global population is estimated approximately 460,000–730,000 individuals [51]. The ruddy turnstone breeds in northern latitudes around the sea. A subspecies occurs in Northern Alaska and in Arctic Canada, Greenland, Northern Europe, and Northern Russia. In the America, the species winters on coastlines from Washington and Massachusetts southward to the southern tip of South America. The red knot (*Calidris canutus*) is a medium-sized shorebird. The global population is estimated approximately 891,000–979,000 individuals [52]. The species has an extremely large range, breeding from Alaska across the Arctic to Greenland and Northern Russia. It winters on the Atlantic and Pacific coasts of North and South America, as well as Northwestern Europe. The semipalmated sandpiper (*Calidris pusilla*) is a very small shorebird. The global population was estimated at about 2 million individuals in 2006 [52]. It is a common breeder in the Arctic and subarctic, from Far Eastern Siberia east across Alaska and Northern Canada to Baffin Island and Labrador. In the non-breeding season, the species uses coastal estuarine habitats, wintering on the Pacific coast from Mexico to Peru and on the Atlantic coast from the Yucatan and the West Indies south to central Argentina. At one particular staging site in Delaware Bay, thousands of these shorebirds aggregate every spring to refuel for their migration to the Canadian Arctic.

Of about 4000 16S rRNA clone sequences analyzed from these shorebirds, the bacterial community was mostly composed of *Bacilli* (63.5%), *Fusobacterium* (12.7%), *Epsilonproteobacteria* (6.5%), and *Clostridia* (5.8%). The high abundance of *Firmicutes* in shorebird excreta was consistent with other avian studies. At the genus level, three main genera, *Bacillus* spp., *Catellicoccus* spp., and *Lysinibacillus* spp., constituted about 60% of the total sequences. The relatively low abundance of phylum *Bacteroidetes* and genus *Bacteroides* in shorebird excreta also was consistent with other avian studies. Analysis of epsilonproteobacterium-specific 23S rRNA gene clone libraries showed that sequences were dominated by *Campylobacter* (82.3%) or *Helicobacter* (17.7%) spp. In particular, *Campylobacter jejuni*, *C. coli*, and *C. lari* are known to be pathogenic species causing human gastroenteritis worldwide. *C. lari* constituted about 30% of the total *Epsilonproteobacteria* clones, but the pathogenic species of *C. jejuni* and *C. coli* were not detected in the feces of the three shorebird species.

6.6. Bacterial community composition in migratory and nonmigratory birds

Application of NGS for analyzing the intestinal flora of various animals, including humans, is rapidly increasing. In studies on nonmigratory birds, such as chickens, turkeys, ducks, and penguins, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* are reported to be high at the phylum level in all birds [53–55]. In particular, *Firmicutes* was present in almost all bird samples, while *Proteobacteria* and *Bacteroidetes* were present in about 90% of samples. It has been reported that *Tenericutes* was present in about 60% of samples. In the swallow samples, it was characteristic that *Proteobacteria* occupied a high percentage of the community, 50% or more, but the proportions of phylum *Bacteroidetes*, represented by genera *Bacteroides*, *Bifidobacterium*, and *Prevotella*, which are widely present in human intestines, were low.

In the case of the Eurasian wigeon, it was characteristic that the proportions of *Bacteroidetes*, *Actinobacteria*, and *Tenericutes* were lower, which is different from other birds. Also, genera *Bacteroides* and *Bifidobacterium*, which were widely present in human intestine, were low although the genera which belonged to *Firmicutes* and *Proteobacteria* were high.

Figure 3 shows the relative proportions of bacterial phylotypes in intestinal microbial communities of the Eurasian wigeon, the barn swallow, other birds, and mammals registered in DNA database GenBank. **Figure 4** shows the results of principal component analysis comparing the similarities between the intestinal microbial communities of the migratory birds with other birds and

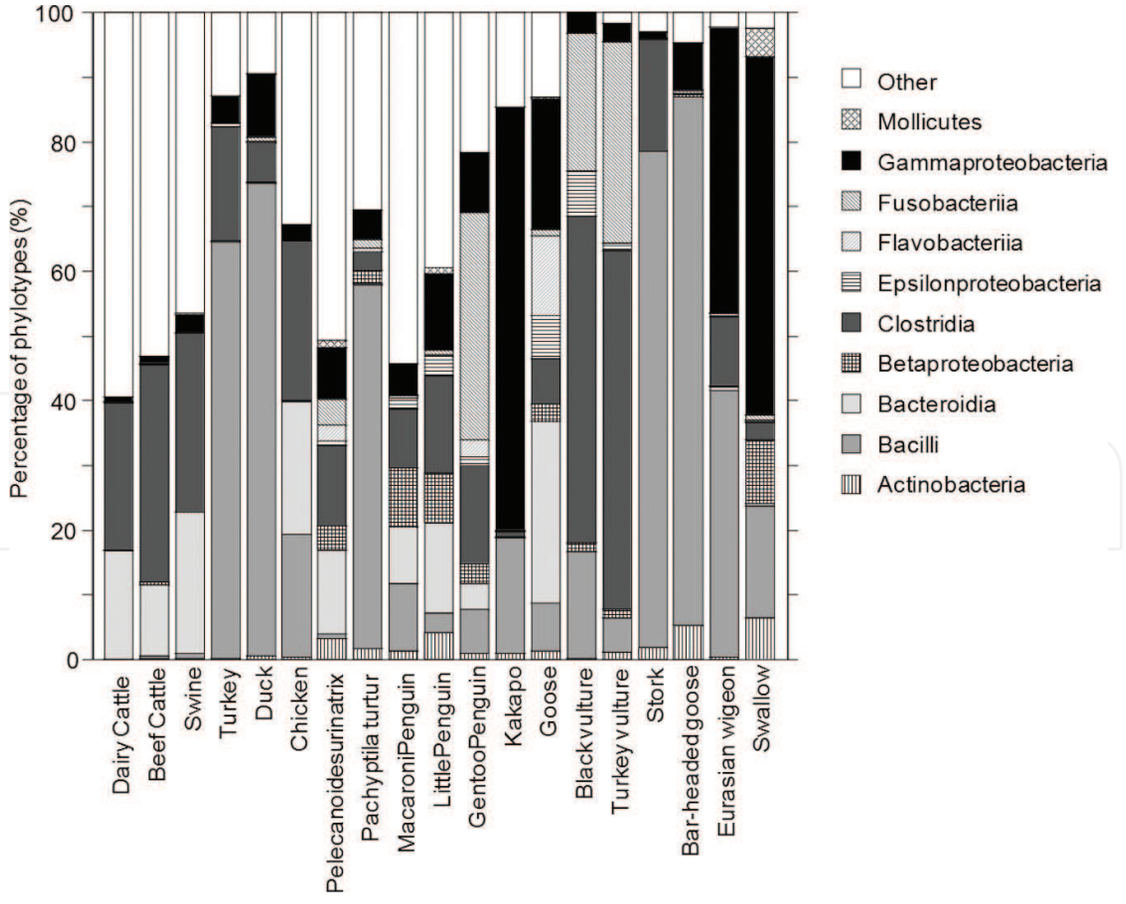

Figure 3. Relative proportions of bacterial phylotypes shown at the class level in gut samples of migratory birds and others.

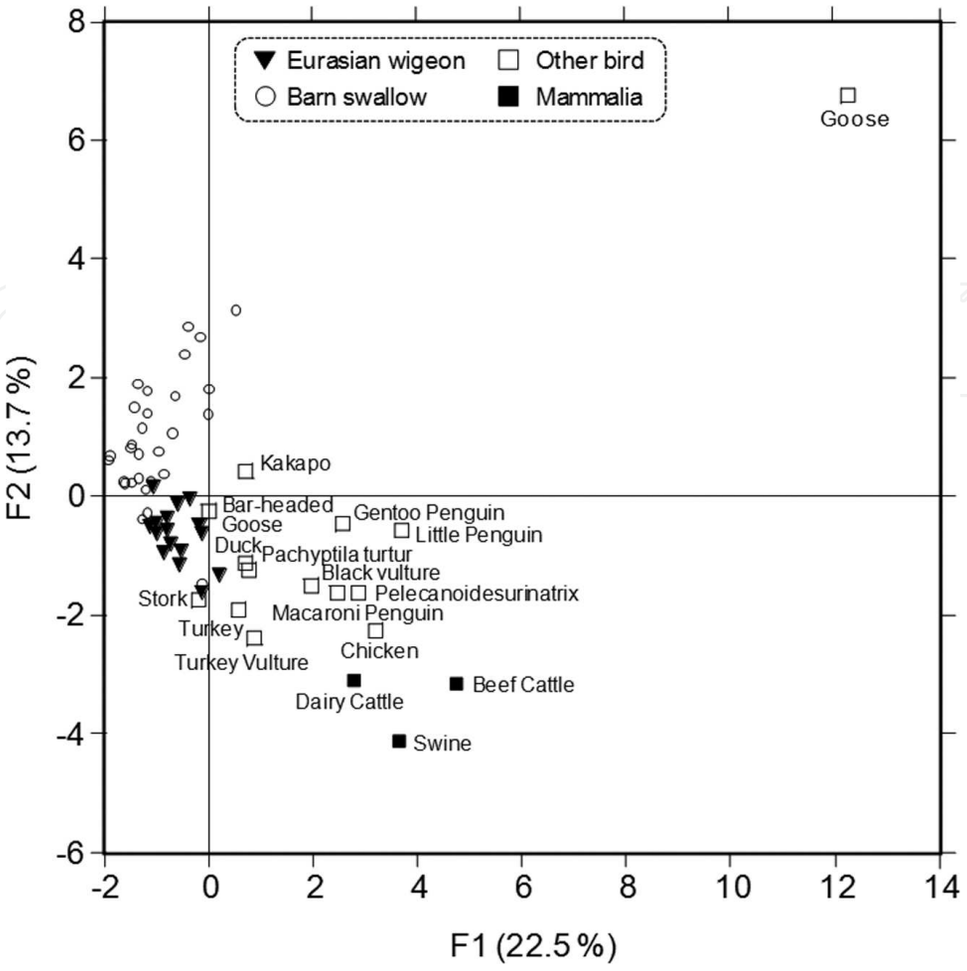

Figure 4. Principal component analysis of class abundance data from migratory birds and others.

mammals registered. It is highly likely that migratory birds may eat different foods from individual to individual, so differences across individuals are large compared to poultry; however, compared with other organisms (□, ■ in **Figure 4**), individual intestinal microbiota from the Eurasian wigeon (▼) and the swallow (○) were relatively similar. In particular, intestinal bacterial composition was found to be greatly different from mammals, such as swine, beef cattle, and dairy cattle (■). It seems that each intestinal bacterial community is formed by the food consumed, whether it is an insect meal, an herbivorous meal, an omnivorous meal, a carnivorous meal, and so on.

7. Protozoa and fungi in migratory birds

For comprehensive analysis by NGS of eukaryotic parasite, 18S rRNA gene, 28S rRNA gene, or cytochrome c oxidase I (COX1) gene on mitochondrial DNA has been used. The universal primers can amplify species across a broad variety of taxa, making them a time- and cost-effective alternative to group-specific primers. Using multiple markers may provide a broader taxonomic resolution of biological communities including diet. The diversity of sequences that can be detected by universal primers is often compromised by high concentrations of DNA templates of some groups. Moreover, up to 90% of the sequences obtained from NGS can be

less-degraded host DNA [56]. If the DNA within the sample contains a small number of interesting sequences in relatively high concentrations of non-interesting sequences, less sequences are often not amplified. In this case, the use of annealing inhibiting primers which overlap with the 3′ end of one of the universal primers is effective [57]. The inclusion of primers to block host DNA amplification can increase the number of nonhost sequences significantly.

As fungi contained in the intestinal tract of seabirds, *Blastocladiomycota*, *Chytridiomycota*, *Entomophthoromycotina*, *Ascomycota*, *Mucoromycotina*, and *Basidiomycota* have been detected [58, 59]. *Nebela* spp., Alveolata, Stramenopila, Rhizaria, Amoebozoa, Excavata, Choanoflagellatea, Glaucophyta, Cryptophyceae, Chlorophyceae, Trebouxiophyceae, Ulvophyceae, Prasinophyceae, and Mamiellophyceae have been detected as protozoa contained in the intestinal tract of seabirds.

8. Conclusion and future perspectives

The use of culture-independent methods for studying bird-associated microbial communities could prove invaluable in the expansion of our current knowledge. NGS targeting the 16S rRNA gene allows comprehensive clarification of the sampled bacterial communities and their associated movement with migratory birds. This methodology also is clarifying the details of bacterial communities, which are moving long distances with migratory birds. Since the 16S rRNA gene differs from 1 to 16 in the number of copies per cell depending on genus [60], the relative proportion obtained by NGS does not necessarily agree with the ratio of actual community composition, but the dominant populations can be ascertained. Applications of NGS will lead to a better understanding of the whole picture of the bacterial communities in migratory birds. Narrowing down the target bacteria using NGS will enable us to identify unknown pathogens or reveal the potential migration status of known pathogens that have not been noticed thus far due to methodological constraints.

The dynamics of individual pathogenic bacteria and drug-resistant bacteria need to be examined in detail in the future. By conducting community composition investigations in parallel with functional investigations (e.g., drug resistance), these methods will lead to an understanding of the mechanisms by which multidrug-resistant bacteria spread around the world.

Addressing the current implications of birds as potential vectors of pathogenic 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 microbial transfer associated with natural avian migratory patterns. When wild birds are vectors of disease, it is important to identify the true source of the infectious organisms. NGS, being a culture-independent method, will facilitate further understanding of the complexities and interactions of the genera inherently present in the avian gut and of those acquired from the environment.

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Author details

Takehiko Kenzaka* and Katsuji Tani

*Address all correspondence to: kenzat@osaka-ohtani.ac.jp

Faculty of Pharmacy, Osaka Ohtani University, Osaka, Japan

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