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## Review

### The avian gut microbiota: community, physiology and function in wild birds

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Gastrointestinal microbiota play a vital role in maintaining organismal health, through facilitating nutrient uptake, detoxification and interactions with the immune system. The gastrointestinal microbiota of birds has been poorly studied, especially in wild species under natural conditions. Studies of avian gut microbiota are outnumbered ten to one by studies of mammals, and are dominated by research on domestic poultry. Unlike domestic poultry, wild birds vary widely in environmental preferences, physiology, and life-history traits, such as migratory behavior and mating systems. Species characteristics result in a vast diversity in gut microbiota and its composition and function. Avian life-history characteristics pose selection pressures on the gut microbiota, and ultimately affect host health. Here, we review current knowledge of the gut microbiota of wild birds, including: partitioning of digestive function and microbiota among different gastrointestinal compartments, microbial diversity and function in the context of host diet, energetics and behavior, and the intrinsic and extrinsic factors impacting gut microbiota in free-living birds. The shared core microbiota of wild bird species is dominated by members of four major phyla: Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria. However, microbial communities varies inter- and intra-specifically, and among gastrointestinal tract sections. To conclude, we identify three key research areas that warrant further investigation: 1) expanding the range of avian host taxa investigated, 2) identifying the function of avian gut microbiota in physiology and immunology, and 3) transitioning from observational studies to experimental manipulations to identify key determinants of wild bird gut microbiota composition.

Keywords: 16S rRNA gene, bacteria, gastrointestinal tract

## Introduction

The microbiota of the gastrointestinal (GI) tract and its interactions with host physiology and immune function are emerging topics in microbiology, ecology and medicine. Rapid advances in molecular methods, combined with the increasing appreciation of the large impact avian gut microbiota have on host health, have resulted in an



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exponential increase in studies (Fig. 1a). Despite great diversity and ecological significance of microbes, research on gut microbiota of birds has lagged behind mammalian research. Publications on avian gut microbiota are outnumbered 10:1 by mammalian studies, and are dominated by studies of domestic poultry (Fig. 1b). Over 1300 studies on the microbiota of poultry have been published since 1948, which focus mainly on the influence of diet on microbiota, the role of microbiota in meat production, and responses of microbiota to antibiotics and probiotics. Wild birds remain understudied despite their relevance for pathogen transmission, and for understanding diet and environmental influences on gut microbial structure and function.

Gut microbiota composition could positively affect host fitness through its involvement in host health. The hologenome theory argues that organisms evolve together with their microbiomes, and that the microbial community can increase fitness parameters, such as survival, phenotypic plasticity and reproductive performance, of hosts when environmentally stressed (Zilber-Rosenberg and Rosenberg 2008). For example, changes in gut microbiota decreased fecundity in termites *Zootermopsis angusticollis* and *Reticulitermes flavipes* and the honey bee *Apis mellifera* (Hamdi et al. 2011, Rosengaus et al. 2011). In house sparrows *Passer domesticus*, altering the gut microbiome with antibiotics depressed nestling growth (Kohl et al. 2018), and the microbiome plays a role in adapting to a carrion-focused diet in birds (Blumstein et al. 2017).

Microbial gut communities of wild birds have received some attention because they can be a source of a number of human and animal diseases through direct transmission, or by acting as vectors for zoonotic pathogens (Tsiodras et al.

2008). Migratory bird species can be vectors for long-distance pathogen transmission and fecal pollution by wild birds has been well studied (Lu et al. 2011, Araújo et al. 2014, Ahmed et al. 2015), but the field has focused on one or several individual pathogens and impacts on human health. The importance of the whole gut microbiota in maintaining avian health is unknown, despite widespread declines in wild bird populations (Vickery et al. 2014).

Birds are an exciting group to investigate because the lineage (class: Aves) includes over 10 000 species. Birds are diverse and vary in life-history traits such as migratory behavior, flight capacity, diet, mating systems, longevity and physiology, all of which may impact gut microbiota. For example, migratory birds may encounter a wider variety of microorganisms that are able to colonize their gut than resident birds do. Also, different diets have different digestive requirements and thus potentially require specialized microbial communities. Kohl (2012) published an early review of the avian gut microbiota, but identified only eight studies that used 16S rRNA gene sequencing techniques to investigate gut microbial communities in wild birds. Since then, our knowledge of wild bird microbiota has dramatically increased through new applications of high-throughput sequencing and use of predictive metagenome function analysis. Waite and Taylor (2014) noted that new molecular techniques have increased the number of studies focusing on diversity of gut microbiota. Identifying the microbial diversity of the host gut is a necessary first step, but provides only limited information on functional aspects of the microbial community.

It is possible that life-history traits that differentiate birds from mammals are associated with a wider range in gut

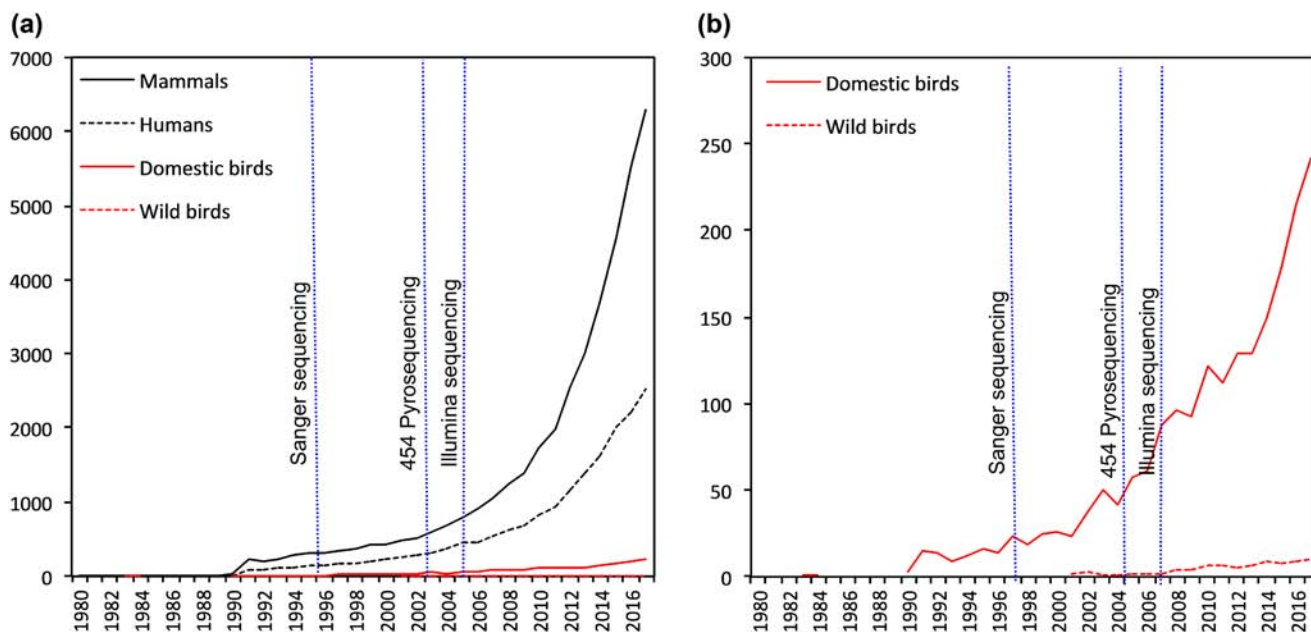


Figure 1. Studies on gut microbiota of birds and mammals (a), and wild and domestic birds (b) as indexed in Web of Science. Dashed vertical lines represent the first use of the respective sequencing technique in gut microbiota research. Data include studies using culture-dependent and culture-independent methods.

microbiota composition: there are twice as many bird species than mammals, (long-distance) flight allows exposure to many contrasting environments, and birds possibly have a greater reliance on microbiota for digestive function due to their lack of initial mechanical digestion.

Gut microbiota and microbial interactions with the animal host can strongly affect organismal health. However, a majority of past studies have been conducted on humans or animal models, such as rodents and chickens. Thus, we aim to summarize the current knowledge of gut microbiota in wild birds, identify current knowledge gaps, and suggest future research directions. We review microbial diversity and function in different parts of the avian GI tract, and discuss intrinsic and extrinsic factors affecting gut microbial communities in wild birds. Gut microbiota are predominantly bacterial but efforts to understand the relative importance of viruses, archaea and fungi are becoming more common (Morgavi et al. 2015, Huseyin et al. 2017, Mirzaei and Maurice 2017). Here, we focus on the bacterial avian gut microbiota. We focus on wild bird microbiota, but reference mammalian and poultry studies where relevant. The terms ‘microbiota’ and ‘microbiome’ are often used interchangeably, but differ in meaning. Here, we define microbiota as the collection of microbes within a given environment, and microbiome as all genomes of these microorganisms combined (Waite and Taylor 2014).

Understanding the dynamics of wild bird gut microbiota will provide us with novel insights into the interactions between free-living organisms and their environment, and will contribute to our interpretation of basic and applied aspects of microbial community structure and function.

## Literature review

To assess the current state of gut microbiota research, we compiled publications on gut microbiota for mammals, humans, domestic birds and wild birds, as recorded in the Web of Science database. We found few records between 1900–1980, and therefore present records from 1980–2016 (Fig. 1) Search terms used for mammals and humans were: gut microbiota/microbiome/gut bacteria/gastrointestinal microbiota + mammal/mouse/rat or human. Studies using experimental animals often did not include the term ‘mammal’ hence the expansion with ‘mouse’ and ‘rat’. Terms used for wild and domestic birds were: gut microbiota/microbiome/gut bacteria + wild bird/bird/chicken/broiler/poultry. Results for mammalian and human studies were too numerous to assess individually. We scanned abstracts of publications on the first 50 results. We subsampled publications that were on topic, and estimated the total number of relevant publications. The number for mammals and humans are therefore estimates. We found a total of ~600 and ~15 600 publications for mammals and humans, and 32 and ~1200 publications for wild and domestic birds from 1980–2017.

## Methods for studying gut microbiota

Identification of bacteria in microbiota studies depends on the choice of research methods and tools. Methods can be broadly divided into culture-dependent methods, that use selective culture media for growth and isolation, and culture-independent molecular methods, which often involve target gene sequencing of in situ microbial communities. We include information from culture-based studies in our review, but focus on sequence-based studies for direct comparisons of the complete gut microbiota among wild birds. Advantages and biases of both approaches have been reviewed elsewhere (Hirsch et al. 2010, Stewart 2012, Faure and Joly 2015).

Until commonly available DNA sequencing techniques, environmental microbes were mainly studied using culture-dependent techniques, in which individual strains of microorganisms were grown in specific media. Such techniques have limitations, such as the difficulty of easy and quick culturing of most microorganisms. Nevertheless, media development and fine-tuning culturing can result in overlap (56%) in detection of community membership at a genus and species level when comparing culturing and pyrosequencing microbes from human fecal samples (Goodman et al. 2011). The potential to culture > 40% of mammalian gut microbiota is high compared to other systems, such as soil or aquatic environments. The percentage of cultivable bacteria in birds is unknown, but targeted culture-based approaches are useful for detection and differentiation of known pathogens, such as *Campylobacter* spp. (Waldenström et al. 2007, Keller et al. 2011). Culture-based methods have also been used to tackle questions about the feather microbiome of birds (Leclaire et al. 2015, Vereza et al. 2017).

Molecular tools for microbial community analysis often encompass different approaches for measuring the genetic diversity of DNA sequences, representative of the genomic content of coexisting microorganisms in a sample. Early sequencing in avian research mainly consisted of constructing clone libraries directly from PCR amplicons, or indirectly by sequencing of cloned informative fragments extracted from gels used in fingerprinting (Santos et al. 2012, Grond et al. 2014, Ryu et al. 2014). Cloning-sequencing and fingerprinting techniques are time-consuming with relatively low sequence output compared to more recent high-throughput sequencing (HTS) methods, such as 454 pyrosequencing and the Illumina sequencing platform (Roesch et al. 2007, Faure and Joly 2015). The first use of HTS to measure the microbiome of a wild bird, the emu *Dromaius novaehollandiae*, was in 2013 (Bennett et al. 2013). A powerful tool yet to be employed in the study of avian microbiomes is metagenomic sequencing (Riesenfeld et al. 2004), which interrogates all gene content in the microbiome. A first use of metagenomics for identifying viruses in wild birds was recently published (Vibin et al. 2018), but the bacterial microbiome has not been investigated. Metagenomic sequencing relies on shotgun sequencing and assembly of DNA fragments from the

sample, and subsequently using database matching the relevant sequences to known microbial functional groups.

A challenge for interpreting HTS and metagenomic data is the relatively low coverage of well-annotated microbial groups in available gene reference databases (Pompanon and Samadi 2015), which makes it impossible to reliably assign all phylotypes and functional gene identities from environmental microbial sequencing libraries. Still, microbial community profiling based on 16S ribosomal rRNA gene sequencing has proven to be a reliable and reproducible representation of phylogenetic and taxonomic categorization of bacteria (Wu et al. 2009). Further, a growing literature illustrates that metagenomics approaches can address functional aspects of gut microbiota (Qin et al. 2010), which will be particularly useful for comparisons among host species with different dietary, physiological and life-history characteristics.

## The core microbiota of wild birds

Molecular microbiome surveys produce DNA sequence data which are assigned to operational taxonomic units (OTUs), defined at 97% sequence similarity of the 16S rRNA gene, with established hierarchical phylogenetic relationships among organisms. An OTU is an heuristic analog to ‘species’ defined by gene sequence similarity (Stackebrandt and Goebel 1994). An individual wild bird can house thousands of microbial OTUs within its GI tract. To identify commonly shared microbial OTUs among individuals of, for example, all wild birds, the concept of a core microbiota can be used (Turnbaugh et al. 2009, Shade and Handelsman 2012). Here, we defined core microbiota as OTUs being present in > 50% of samples.

We assessed the core microbiota of the GI tract of mammals, humans, chickens and wild birds to compare the main bacteria hosted within these groups. The wild bird core gut microbiota is more similar to domesticated chickens than to non-human mammals, and most different from the core gut microbiota of humans (Fig. 2). Relative abundance of Firmicutes is similar among humans, chickens and wild birds at around ~50% of all detections (Fig. 2), but the remaining phyla are more evenly distributed in birds than humans. The avian gut microbiota were dominated by Firmicutes and Proteobacteria, with lower abundance of Bacteroidetes and Actinobacteria (Fig. 2). Domestic and wild birds both have a high relative abundance of Proteobacteria (25%), compared to < 1% in humans. Next, we describe the general functions known for core microbial phyla in avian digestive tracts, as well as what functions gut microbiota may perform for their avian hosts. A majority of available studies on bird microbiota are descriptive have not used experimental manipulations to determine microbial function. We review available data for wild birds and use examples of known microbial functions in domestic chickens where relevant.

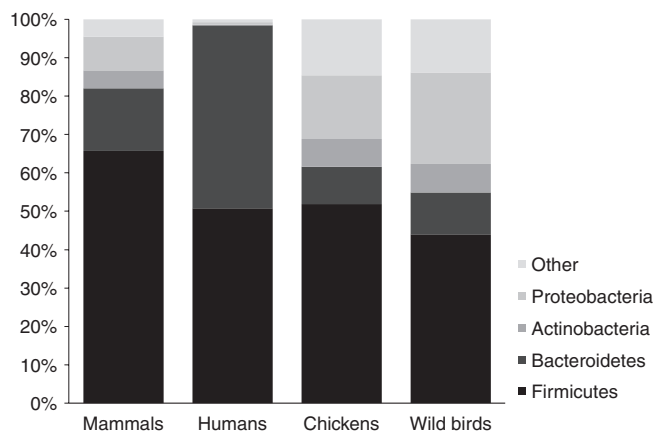


Figure 2. Core microbiota of mammals (domestic and wild; Ley et al. 2008), humans (Eckburg et al. 2005), domestic chickens (Waite and Taylor 2015) and wild birds (this study). All studies included used culture-independent sequencing methods.

## Microbial phyla in bird guts

### **Bacteroidetes**

Bacteroidetes are gram-negative bacteria that vary from strict aerobes to obligate anaerobes. Bacteroidetes occur throughout the entire GI tract as part of the normal GI community in mammals and birds (Hird et al. 2015, Waite and Taylor 2015, Colston and Jackson 2016). The four genera of the *Bacteroides*, *Prevotella*, *Porphyromonas* and *Flavobacterium* also include some potential pathogens of birds and mammals (Thomas et al. 2011).

In mammals, Bacteroidetes degrade complex biopolymers, and in the GI tract they degrade polysaccharides such as carbohydrates and plant cell wall components (Thomas et al. 2011). Low relative abundance of Bacteroidetes in birds compared to mammals may be attributed to dietary differences, and the wide dietary range among different bird species warrants further investigation into this relationship. However, it is important to note that abundance may not be proportional to importance in the avian GI tract. The higher abundance of Bacteroidetes found in the ceca of Japanese quail *Coturnix coturnix*, emu and ostrich *Struthio camelus* (Fig. 3.; Matsui et al. 2010, Bennett et al. 2013, Kohl et al. 2014) may support the hypothesis that Bacteroidetes play a specific role in break-down of cellulose and other plant materials. However, to properly test this hypothesis simultaneous characterization of microbial communities in different sections of the GI tract of individual wild birds within a species would be necessary.

### **Firmicutes**

Firmicutes are predominantly gram-positive bacteria, and include the obligate or facultative anaerobe classes of Bacilli, Clostridia and Mollicutes, each of which are commonly found in GI tracts. Several pathogens from the Firmicutes phylum have been isolated from wild birds,

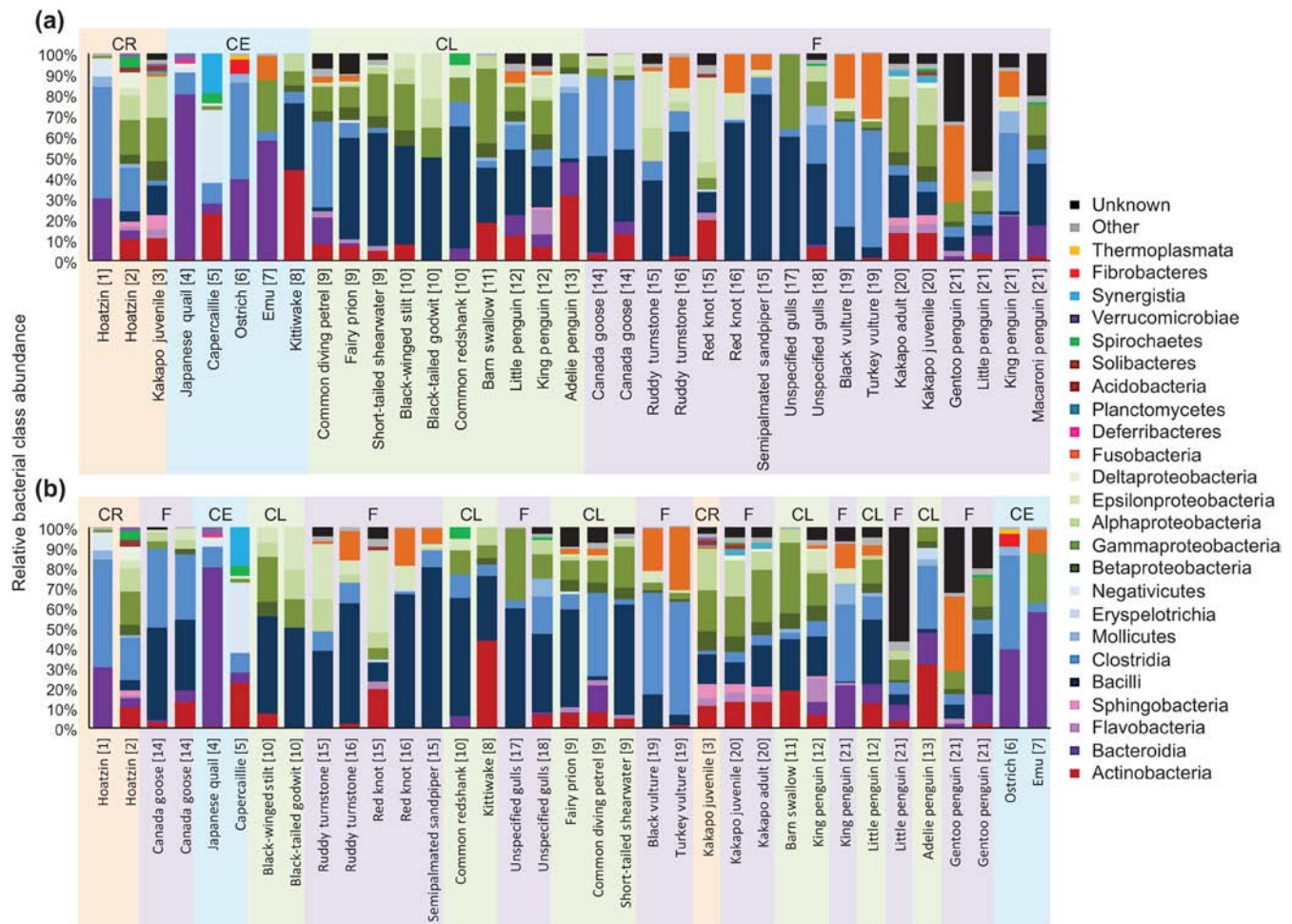


Figure 3. Bacterial diversity clustered by (a) different sections of the avian gastrointestinal tract, and by (b) bird species. We only included studies that used methods to characterize gut microbial communities, to avoid potential selective bias of culture-dependent techniques. Different sections of the GI tract are labeled above the graphs and include: crop (CR), ceca (CE), cloaca (CL) and feces (F). References: [1] Godoy-Vitorino et al. 2008, [2] [1] Godoy-Vitorino et al. 2012, [3] Waite et al. 2014, [4] Kohl et al. 2014, [5] Wienemann et al. 2011, [6] Matsui et al. 2010, [7] Bennett et al. 2013, [8] van Dongen et al. 2013, [9] Dewar et al. 2014a, b, [10] Santos et al. 2012, [11] Kreisinger et al. 2015, [12] Dewar et al. 2014a, b, [13] Banks et al. 2009, [14] Lu et al. 2009, [15] Grond et al. 2014, [16] Ryu et al. 2014, [17] Koskey et al. 2013, [18] Lu et al. 2008, [19] Roggenbuck et al. 2014, [20] Waite et al. 2012, [21] Dewar et al. 2013.

including *Mycoplasma gallisepticum*, *Clostridium botulinum* and *C. perfringens* (Benskin et al. 2009).

Firmicutes produce short-chain fatty acids as byproducts of fermentation, and these molecules can be directly absorbed by the host gut wall as an energy source (den Besten et al. 2013). An abundance of Firmicutes has been linked to obesity in humans, and to weight gain in chickens and rodents (Angelakis and Raoult 2010, Clemente et al. 2012, John and Mullin 2016). We found no studies addressing Firmicutes function in wild birds, but in domestic chickens, several studies found a positive relationship between Firmicutes abundance and mass gain and immune function, suggesting similar roles of Firmicutes between mammals and birds (Liao et al. 2012, Zhang et al. 2015). Further, supplementing chicken diets with *Bacillus subtilis*, *Enterococcus faecium*, and other Firmicutes as probiotics can increase nutrient uptake

and metabolic efficiency (Li et al. 2016, Zheng et al. 2016). Average relative abundance of Firmicutes is lower in wild birds than in domestic chickens (Fig. 2), but whether this contrast is directly associated with potential differences in capacity for weight gain is not known.

### Proteobacteria

Birds carry a higher proportion of Proteobacteria in their GI tract than mammals or domestic chickens (Fig. 2). Proteobacteria are gram-negative cells and represent 34% of all known bacteria. Phylum Proteobacteria includes five large classes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ) that vary widely in occurrence and function within and outside the GI tract. Proteobacteria also include a diversity of genera with opportunistic pathogens, such as *Campylobacter*, *Escherichia*, *Helicobacter*, *Rickettsia*, *Salmonella* and *Vibrio*, all of which have been isolated from

birds (Keller et al. 2011, Ryu et al. 2014, Wallménius et al. 2014, Diakou et al. 2016).

Within the avian digestive tract, Proteobacterial function remains undetermined. Given the high taxonomic and functional diversity within this group, extrapolating function is somewhat speculative. Some Proteobacteria may be functionally redundant with members of other better-understood phyla, whereas others may be transient through the avian GI tract. Among the Proteobacterial classes,  $\alpha$ -Proteobacteria are abundant in wild birds (45%), in contrast to only 15% relative abundance in mammalian hosts (Ley et al. 2008), suggesting this group as a target for further research on function.

### **Actinobacteria**

Actinobacteria are gram-positive bacteria with members that can inhabit a wide range of environments, including soils, fresh and marine waters, as well as GI tracts (Janssen 2006, Barka et al. 2016). The Actinobacteria also include some pathogens, such as *Corynebacterium*, *Mycobacterium* and *Nocardia* species. The genus *Bifidobacterium* includes commensal bacteria (Barka et al. 2016), and has been used as a probiotic in humans and livestock (Kailasapathy and Chin 2000). Actinobacteria are the fourth most abundant phylum of microbes in the wild bird GI tract, but no studies have investigated the function of Actinobacteria in wild or domestic birds.

These four bacterial phyla are all common in a wide range of habitats. However, it is important to keep in mind that phylum is a broad category of classification. At less inclusive phylogenetic levels, microbial communities associated with different habitats are often comprised of different classes, orders, families and genera, all including OTUs with potentially different environmental preferences and functions. Comparison of microbiota on a phylum level among different environments will therefore necessitate conservative interpretation of the implications of differences. Despite the limitations of using the concept of a core microbiota, it can be a useful tool for broad investigations and comparisons among gut microbiota of different birds.

## **Function of microbes in the avian gut**

### **Nutritional uptake**

Gut microbiota are broadly involved in digestion of food products, facilitating the breakdown of dietary polymers to compounds that can be used by the host. The extent to which gut microbiota aid in nutritional uptake likely depends on host diet and physiology. For example, hoatzins *Opisthocomus hoazin* are an unusual tropical bird with a well-developed crop where foregut fermentation aids digestion of the complex polymers found in the species' folivorous diet. Foregut fermentation pre-digests food and allows hoatzins to use otherwise indigestible food sources (Godoy-Vitorino et al. 2008). At another extreme are hummingbirds, which rely largely on simple sugars for their metabolic energy. Microbiota of different species of nectarivores were not distinct at a phylum level (Hird et al. 2015) – an unsurprising

finding considering the shallow phylogenetic classification of microbiota, and the specialized diet of nectarivores. However, Preest et al. (2003) identified potential involvement of the hummingbird gut microbiota in nitrogen recycling through urate decomposition, an adaptation to the low-nitrogen content of nectar.

### **Detoxification**

The avian gut microbiota, and specifically microbiota associated with the crop and ceca, may be involved in detoxification of plant materials and other food compounds. Phenols, resins and saponins, plant defense compounds against herbivory, are usually associated with plant defenses against herbivory, and are usually indigestible or toxic to birds but common in diets of herbivorous birds. The crop of the hoatzin is currently the only known region of the GI tract to contain detoxifying bacteria (Garcia-Amado et al. 2007). The crop is the first region of the gut to process consumed food and is therefore a logical reservoir for detoxifying bacteria. High tannin concentrations in the folivorous diet of hoatzins are potentially remediated by a community of crop bacteria that also includes members of the known cellulose-degrading classes Actinobacteria and Clostridia (Garcia-Amado et al. 2007).

### **Immune function**

Interactions among gut microbiota and the avian immune system are poorly understood and remain unstudied in wild birds. In mammals, the antigen recognition spectrum of T-lymphocytes narrows over time, which may be attributed to interactions with commensal microbiota (Mazmanian et al. 2005). In birds, the development of pathogen specific antibody-producing B-lymphocytes in young birds occurs in the bursa of Fabricius (or 'bursa') – an offshoot of the intestines near the cloaca. The bursa is responsible for B-lymphocyte production until birds reach sexual maturity, after which production occurs predominantly in the bone marrow (Madej et al. 2013). At maturity the bursa atrophies and loses its function. Bacteria colonize the bursa immediately after hatch (Kimura et al. 1986), and potentially play a role in development of the bird adaptive immune response. In addition, Firmicutes could be involved in the development of T-lymphocyte immunity in birds. In chickens, antibiotic treatment early in life reduced the relative abundance of Firmicutes, but increased relative abundance of Proteobacteria (Simon et al. 2016). Also, lower T-lymphocyte dependent antibody titers were observed later in life in the antibiotic treatment group than in untreated chicks, suggesting a potential role of these bacterial phyla in adaptive immune function.

### **Competitive exclusion of pathogens**

Commensal gut microbiota can be important in maintaining host health through interactions with pathogens (Servin 2004). Commensal microbiota can interact indirectly with pathogens through stimulating or suppressing immune function, and directly through competitive exclusion and

production of antimicrobial compounds, such as bacteriocins and other toxins (Hammami et al. 2013, Kamada et al. 2013). Also, antimicrobial production generally promotes competitive exclusion through competition for space and nutrients among microbiota in the gut (Kamada et al. 2013). No studies exist on commensal-pathogen interactions in the avian GI tract, but trophic interactions in birds may be similar to those in mammals.

## Gastrointestinal physiology and microbiota

The avian gastrointestinal tract is divided into nine discrete sections: the oral cavity, esophagus, crop, proventriculus, gizzard, small intestine, ceca, large intestine, and the cloaca (Fig. 4). The esophagus and crop store food in most bird species. The proventriculus has an acidic environment that chemically digests food, whereas the gizzard is a muscular stomach that degrades food mechanically. Different

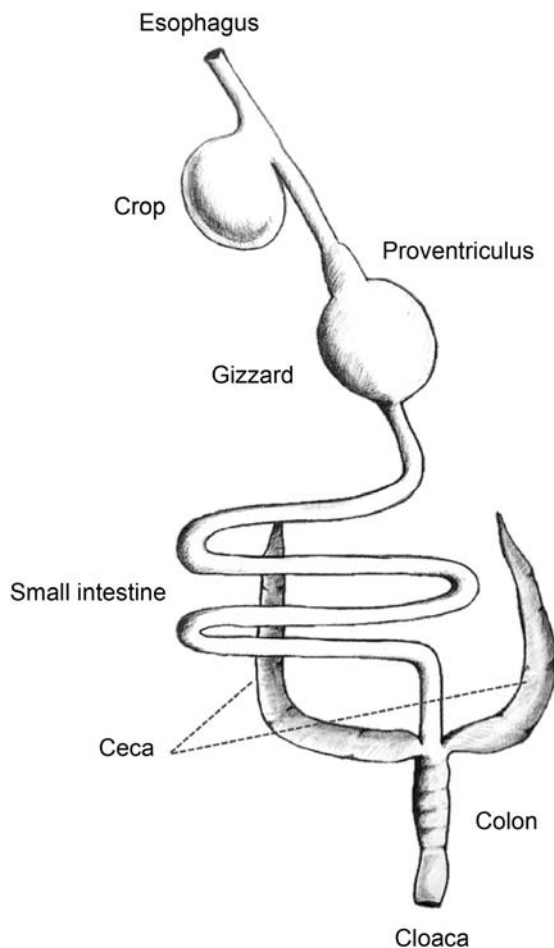


Figure 4. Schematic of the avian gastrointestinal tract. Microbiota of the different sections of the gastrointestinal tract are discussed in the corresponding chapter number in this paper. We included the crop and ceca in our figure, but want to point out that not all bird species possess these organs.

components of the GI tract vary in their biochemical properties such as oxygen content and pH, thus possibly posing a selective pressure on the microbial community. The esophagus, crop and cloaca are considered semi-oxic environments, allowing mixed communities of aerobes, microaerobes and facultative anaerobes, including members of the  $\alpha$ ,  $\beta$ , and  $\gamma$ -Proteobacteria. The internal sections of the GI tract located between the crop and cloaca are dominated by obligate or facultative anaerobes, including members of the Firmicutes and Proteobacteria (Hird et al. 2015, Waite and Taylor 2015). The cloaca does not have a digestive function, but is exposed to materials and microbial cells from the digestive system as well as the reproductive and urogenital systems, and is also the most sampled section of the GI tract among wild birds.

We focused on the microbiota of the crop, intestines, ceca and cloaca, and summarized our findings from the current literature (Fig. 3). We did not find any studies that addressed stomach or colon microbiota separately. Intestinal microbial communities have predominantly been inferred from analyzing fecal samples and cloacal swabs, two non-lethal techniques appropriate for sampling wild birds under natural conditions.

### Crop and esophagus

The crop is a pouch below the esophagus at the beginning of the GI tract in most birds, and temporarily holds quickly ingested food. Long-term retention of food in the crop allows birds to utilize food sources that are difficult to digest, such as high cellulosic plant material. The Hoatzin is the only bird species with foregut fermenting crop system that is well-developed (Godoy-Vitorino et al. 2008). The crop of the hoatzin contains a microbial community functionally analogous to the bovine rumen, and includes methanogenic archaea, rumen bacteria and eukaryotic protozoa (Godoy-Vitorino et al. 2012). Waite et al. (2012) collected crop samples from a flightless herbivorous parrot, the kakapo *Strigops habroptila*, and observed dissimilar bacterial communities compared to the hoatzin (Fig. 3). Different subphyla of Proteobacteria, specifically  $\alpha$ , and  $\gamma$ -Proteobacteria, dominate microbiota of crops of the kakapo and hoatzin. A second study of hoatzin showed the highest proportions belonging to bacteria from Bacteroidia and Clostridia.

Hoatzin and kakapo are currently the only wild bird species for which crop microbiota have been studied, and are both unique with respect to diet, and inherent low microbial diversity (Waite et al. 2012). Among domestic birds, crop microbiota of captive bred Japanese quail were dominated by Firmicutes, Actinobacteria and Proteobacteria. Firmicutes (Lactobacillaceae and Micrococcaceae families) and then  $\gamma$ -Proteobacteria dominated in females, while the male microbiome consisted of a community of OTUs more evenly distributed among  $\gamma$ ,  $\alpha$  and  $\beta$ -Proteobacteria (Wilkinson et al. 2016).

## Proventriculus and gizzard

The avian stomach has two parts: the glandular proventriculus and the muscular gizzard. The proventriculus region of the stomach is where food and enzymes mix before the food particles enter the gizzard. The gizzard has muscular walls with a textured (koilin) lining and ingested grit where food is mechanically ground up and digested. The proventriculus is usually acidic, and likely enriching the microbiota towards acidophiles. The acidic proventriculus likely poses the first strong selection on microorganisms entering the digestive tract (Beasley et al. 2015). Stomach acidity varies among bird species, with carrion-eating birds such as vultures having highly acidic stomachs (Roggenbuck et al. 2014).

Few wild bird studies have investigated microbial communities and function in the proventriculus and gizzard, and even poultry research is limited on this topic. The gizzard microbiome of neotropical birds consisted mainly of members of the Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (García-Amado et al. 2018). Alpha diversity and bacterial community were variable by species and diet, and putative microbial function indicated a role in amino acid and vitamins metabolism pathways for the gizzard (García-Amado et al. 2018). Chicken gizzard microbiota were highly similar to crop microbiota (Sekelja et al. 2012). *Lactobacilli* comprised 43% of the gizzard microbiota in the domestic chicken GI tract (Gong et al. 2007). *Lactobacilli* are expected in the gizzard because these bacteria tolerate acidic environments, and also produce acids.

## Small intestine

The small intestine is located between the gizzard and ceca and consists of three undifferentiated sections in the avian gut (the duodenum, jejunum and ileum). The functions of the avian small intestine are similar to those in mammals, and include processing of food using enzymes and bile excreted from the pancreas and liver, as well as nutrient absorption. Microbiota analysis of the small intestine is especially challenging due to its location in the GI tract, which precludes non-lethal sampling. The limited data available on the gut microbiota of the small intestine are derived from domestic poultry and indicate that the different sections of the small intestine possess similar microbiota and are dominated by *Lactobacilli* and *Clostridia* (Amit-Romach et al. 2004).

## Ceca

Most birds possess ceca as part of the digestive tract. The ceca are long, blind pouches that attach to the gut at the boundary of the small and large intestine. The size and width of the ceca vary considerably among and within species, are generally longer in herbivorous birds, and can show facultative change to different seasonal diets. In willow ptarmigan *Lagopus lagopus* and broiler chickens, changes in dietary components, such as fiber and fermentable content of food, can also change cecal morphology (Pulliainen and Tunkkari 1983, Józefiak et al.

2006, Rehman et al. 2007). Monitoring of microbiota and volatile fatty acids after shifts in diet showed that the function of the ceca among others includes fermentation of dietary compounds (Svihus et al. 2013). In addition, ceca are important for electrolyte and water reabsorption (Thomas 1982). Ceca are emptied regularly, but at a slower rate than normal defecation occurs. Depending on the bird species, cecal material is generally retained 3–4 times longer than fecal material (Duke 1986).

Microbial communities of the ceca are distinct from the rest of the GI tract (Waite and Taylor 2014, Han et al. 2016), and may vary based on host dietary status. In Japanese quail, cecal microbiota were relatively stable during fasting (Kohl et al. 2014), with the exception of an increase in Verrucomicrobia and slight decrease in Bacteroidetes. Cecal microbiota of the northern bobwhites *Colinus virginianus* were dominated in abundance by Firmicutes, followed by Proteobacteria and Actinobacteria (Su et al. 2014), and these microbial profiles differed substantially from the microbiota of Japanese quail and capercaillie (Fig. 3). Bobwhite samples were cultured opposed to the use of direct sequencing approaches, which may partly explain differences among species. Cecal bacterial classes shared among all bird species are Clostridia and Bacteroidia, followed by Actinobacteria. In one exception to this general pattern, Actinobacteria were not detected in ostriches which belong to a flightless, primitive group of ratite birds (Matsui et al. 2010). Despite harboring several well-known microbial pathogens, most members of the class Clostridia are commensals. In mammals, this group is involved in fermentation and maintaining gut homeostasis (Lopetuso et al. 2013).

Ceca are absent in most bird species with protein or sugar rich diets, such as carnivores, piscivores, nectarivores, or frugivores. These diets cover a broad range of fiber content, indicating that fiber digestion is not restricted to the ceca (Clench and Mathias 1995). The absence of ceca in host species across a range of different diets suggests that the specific functions of cecal bacteria in birds without ceca are fulfilled in other areas of the GI tract, which should be reflected in their microbial communities.

## Colon

The large intestine of birds is short and the avian colon is located between where the ceca are attached to the GI tract and the cloaca. In birds the main function of the large intestine is water and electrolyte reabsorption. Fermentation in the avian digestive tract predominantly occurs in the ceca and, in the case of the hoatzin, the crop (Lei et al. 2012). The colon in domesticated Japanese quail was dominated by the genera *Lactobacillus*, *Bacteroides*, *Ruminococcus* and *Clostridium*, although a majority of sequences remained unclassified (Wilkinson et al. 2016). Generally, fecal bacteria are assumed to reflect the colon microbiota. However, quail fecal microbiota consisted of microorganisms of both colon and cecal origin, indicating mixing of fecal microbiota from the different sections of the GI tract.



Hird et al. (2015) described colon microbiota of 59 neotropical bird species and found a core microbiota among all host species consisting of Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. Host species was the most important factor for determining the finer-scale taxonomic composition of gut microbiota, suggesting some host specificity among resident microbes in the occurrence of unique microbial OTUs. Our literature search confirmed Firmicutes and Proteobacteria as the most abundant phyla present in fecal microbiota of a wide range of wild bird species, although abundance and occurrence of additional phyla were variable.

## Cloaca

The cloaca has no digestive function, but is the exit cavity for the digestive, reproductive, and urogenital systems, and may have a unique microbiome because of its semi-aerobic environment and the passage of waste from the multiple organ systems. The cloaca is exposed to fecal bacteria, sexually transmitted bacteria, and possibly environmental bacteria associated with the eggshell, nesting material or environmental components such as water and soil. Cloacal swabbing has been a widely used method for studying gut microbial diversity in wild birds with 15 published studies, although most have relied on culturing and fingerprinting techniques. The cloacal microbial community in wild birds was dominated by Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria (Fig. 3). Firmicutes and then Proteobacteria had the highest relative abundance in three Procellariiform seabird species (Dewar et al. 2014b), three shorebird species (Santos et al. 2012), and two penguin species (Banks et al. 2009, Dewar et al. 2014a). In contrast, the insectivorous barn swallow *Hirundo rustica* showed the opposite pattern, with Proteobacteria outnumbering Firmicutes by a 2:1 ratio (Kreisinger et al. 2015). Although phyla are shared with fecal microbiota, communities may differ on less inclusive phylogenetic levels.

Cloacal sampling may fail to detect low abundance bacteria, and consequently rare taxa might be overlooked. Cloaca are sampled with sterile cotton swabs, possibly resulting in low biomass samples that can be challenging to analyze. The cloacal microbiota is likely reflects the microbiota associated with waste from the avian digestive system. However, the semi-aerobic conditions in the cloaca likely select against obligate anaerobes that occur in the intestinal microbiota.

*Salmonella* spp. were detected in 92% of fecal samples, but only in 4% of cloacal swabs collected from laying hens (García et al. 2011). Samples were not collected from the same individuals, but obtained from the same production facility hall, and indicate that cloacal swabbing captures only a fraction of the gut microbial community. Ingesa-Capaccioni et al. (2014) compared detection of bacteria from fecal and cloacal samples from chickens in production facilities, and detected *Campylobacter* spp. in 61.9% of cloacal, and 69.1% of fecal samples. In addition, Videvall et al. (2018a) showed that cloacal samples did not accurately reflect the

microbiome detected in the colon, ileum and, cecum, which invites caution with extrapolation of results from cloacal samples to the GI tract microbiome. *Campylobacter* are common in the avian GI tract (Waldenström et al. 2002, 2007, Keller et al. 2011), whereas *Salmonella* is comparatively rare. Differences in innate pathogen detection levels and sampling methods likely caused variation in observed prevalence.

## Feces

Fecal samples are advantageous as a noninvasive approach for sampling the microbial gut communities of wild birds. Fecal bacteria have been widely used as a proxy for intestinal microbiota, because many investigators and agencies for animal welfare consider the sacrifice of wild birds undesirable for microbiome studies. One challenge of studying feces instead of the actual GI microbiota is that fecal microbiota may be a mixture of bacteria from different sections of the GI tract. Also, fecal sampling can be affected by environmental contamination, depending on collection method. A study comparing microbial communities in different segments of the GI tract of domestic pigs showed that fecal microbiota was most similar to microbiota in the large intestine, with 75% similarity compared to 38% similarity with the small intestine (Zhao et al. 2015).

In birds, the only studies comparing fecal microbiota with microbiota of other GI components were conducted in ostriches and domestic chickens. The fecal microbiome of juvenile ostriches was a better representation of the colon microbiome than the cloacal microbiome, but did not represent the microbiome of ileum and ceca well (Videvall et al. 2018a). In chickens, fecal microbiota did not cluster with communities in the crop, gizzard, ileum or colon, but rather consisted of a mixture of bacteria from all components of the GI tract (Sekelja et al. 2012). Short-term fluctuations in fecal microbial communities have been attributed to differential representation of the different GI components over time. Fecal microbiota in mammals has been considered relatively stable over time (Claesson et al. 2011, Newman et al. 2012, Martinez et al. 2013, Becker et al. 2015). If fecal microbiota composition in wild birds is comparable to chickens, variable contributions of microorganisms of different parts of the GI tract could make it difficult to use fecal samples to investigate temporal relationships and internal turnover. Recapture of previously marked wild birds could provide information on turnover and microbial dynamics, but is often challenging.

## Factors affecting gut microbiota

Gut microbiota composition and dynamics are determined by a range of extrinsic and intrinsic factors (Fig. 5). Extrinsic factors include host diet, social interactions, and the pool of environmental microbial inocula, whereas intrinsic factors are inherent to the host organism, and include genetic makeup, age, sex, and health. Extrinsic and intrinsic factors are closely connected in their influence on gut microbial communities.

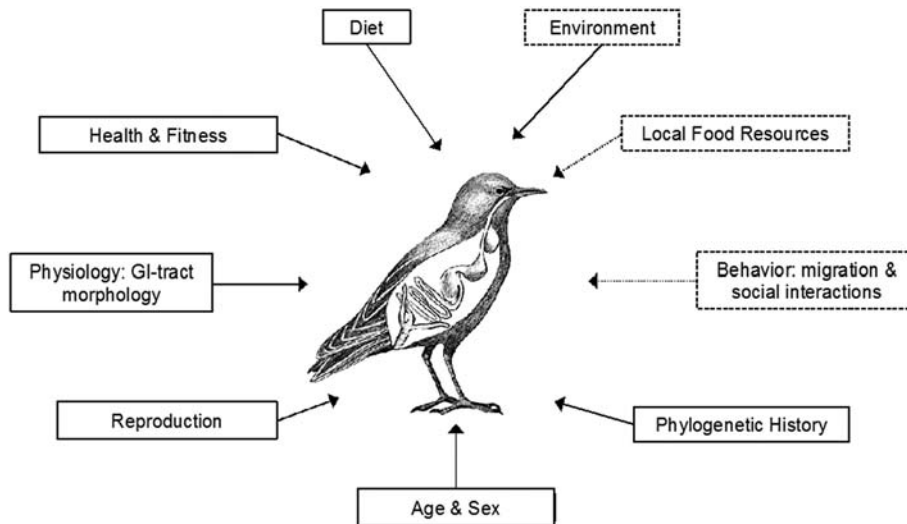


Figure 5. Extrinsic and intrinsic factors affecting the avian gut microbiota. Extrinsic factors are boxed in dashed lines, and intrinsic factors in solid lines.

For example, preferential diet of a bird may be species-specific and could be considered to be an intrinsic factor. However, food-associated microbial communities may vary by location, and food quality can pose a differential selection pressure on the gut microbiota, both extrinsic factors. Foraging location during long-distance movements has an intrinsic component related to migratory behavior, but every foraging environment includes extrinsic pressures, such as variability in food choices based on availability and competition. Disentangling the different extrinsic and intrinsic factors that affect avian gut microbial composition is challenging with descriptive study methods because of the wide range of bird life-histories. Bird species vary from strict diet and habitat specialists, e.g. some species of endemic hummingbirds, to generalists like gull species that have a broad dietary range and occupy a wide variety of environments. We consider extrinsic and intrinsic factors separately for organizational purposes, but recognize that possible effects on gut microbiota may be synergistic.

## Extrinsic factors

### Environment

Environmental microbial communities vary due to spatial and temporal heterogeneity in biotic and abiotic factors. Birds are exposed to different microbes, all potential gut inocula, through environmental conditions in preferred habitats, including diet, water, soil, nesting environments, and social interactions.

Swainson's thrushes *Catharus ustulatus* and gray catbirds *Dumetella carolinensis* shared parallel shifts in gut microbiota between spring and fall stopover, indicating a predominant environmental influence (Lewis et al. 2016). Environmental factors such as location and diet were also main factors affecting passerine gut microbiota (Hird et al. 2014). Variation in importance of site may be due to the age of sampled individuals, as young birds may have not yet developed a

stable gut microbiota. However, a study investigating the cloacal, feather, nest and skin microbiome of adult woodlarks *Lullula arborea* and skylarks *Alauda arvensis* suggested that their microbiomes as adults were shaped by the local environmental microbiome (van Veelen et al. 2017).

In contrast, sampling location was not a major determinant in gut microbial composition in adults of 59 species of Neotropical birds (Hird et al. 2015), but this could be due to low replication among species and sites. To determine environmental influence on avian gut microbiota, we need simultaneous sampling of birds and their local environments, such as sediment and water at foraging or nesting sites, and nesting materials. In addition, we need to identify successional trajectories in gut microbiota of species with varying life-histories to determine if and when birds acquire a stable microbiota.

### Local food resources

In a majority of available studies, diet has been identified as the underlying cause of differences in gut microbial communities, and the importance of gut microbiota for digestion is generally understood. The extrinsic component of diet includes the ingestion of microorganisms associated with available food sources. Different geographic locations provide different microbial environments, and birds in different locations may ingest different food-associated microorganisms. Ingestion of microorganisms with food is likely one major pathway of microbial colonization of the avian gastrointestinal tract (Grond et al. 2017). Bird eggs hatch outside of the parent, whereas neonates of mammals can be first inoculated during parturition and passage through the birth canal. To what extent ingested microorganisms contribute to or affect the mature gut microbiota in wild birds is unknown. Studies of domestic chickens identified effects of different diets on establishment of the gut microbiota in developing young (Wise and Siragusa 2007, Stanley et al. 2012, 2014), but these

studies have been unable to discern the selective effects of dietary nutrient and fiber contents versus input of microbiota from different food-associated microbial communities.

### **Behavior: migration and social interactions**

Resident birds may spend their whole lives in one area. Compared to migratory birds or birds with large home ranges, residents maybe less exposed to diverse inocula from which to recruit their gut microbiota. No studies have compared gut microbiota of resident and migratory individuals of the same or related species. Unexpectedly, plumage microbiota were more diverse in resident birds than in migratory birds (Bisson et al. 2009), indicating that the interaction with the local environment may be more important than the diversity of environments encountered through migration. The authors attributed differences among birds to higher microbial exposure during ground-foraging behavior, the dominant foraging strategy among resident species.

Environmental exposure may not be greater in migratory than resident birds, depending on their site fidelity and space use. Many migratory shorebird species return to the same sites during the breeding and non-breeding seasons, resulting effectively in the use of many small habitat patches over a geographical gradient (Leyrer et al. 2006, Merkel et al. 2006, Johnson et al. 2010). Retention time of local microbiota in the avian gut is currently unknown. Variation in gut microbiota turnover associated with stages of the migratory cycle therefore may not reflect sampling time and location in gut microbiota. Migratory birds often switch diets, and thus may ingest a wide variety of microorganisms associated with their different food sources. Gut microbiota of songbirds became more similar within and among species during stopover, indicating an important role of local diet and/or environment as potential drivers of change in gut microbiota (Lewis et al. 2017).

Exposure to different microbial environments during migration could be influenced by the formation of mixed-species flocks at stopover sites. Avian interactions can facilitate transfer of microorganisms through close contact and involuntary coprophagy, a potential mechanism for spread of gull fecal indicator bacteria, *Catellibacterium marimammalium*, to shorebirds (Grond et al. 2014, Ryu et al. 2014). Large aggregations of birds such as colonial breeding species could also result in the spread of gut microbiota among conspecifics, and potentially inoculation of chicks and juveniles.

## **Intrinsic factors**

### **Diet**

Dietary preferences, such as frugivory, herbivory or insectivory, are intrinsic factors of species that affect gut microbiota. Microbial communities of herbivorous bird guts are often dominated by members of the phylum Bacteroidetes. Bacteroidetes can assist in the decomposition of polysaccharides, cellulose, and other complex polymers (Thomas et al. 2011). Carnivorous bird species have broad diets, ranging

from carrion to marine invertebrates and have gut microbiota dominated by Proteobacteria and Firmicutes (Blanco 2014, Grond et al. 2014, Ryu et al. 2014). Vultures have unique adaptations and responses to their carrion-based diet, including extreme stomach acidity and resistance to toxins produced by the tissue-degrading Clostridia (Roggenbuck et al. 2014). Other species of birds seasonally shift diets during their annual cycle. Gut microbiota vary seasonally in humans and other mammals (Zhang et al. 2014, Hisada et al. 2015), including several species of primates (Sun et al. 2016), wood mice *Apodemus sylvaticus* (Maurice et al. 2015), American bison *Bison bison* (Bergmann et al. 2015) and giant pandas *Ailuropoda melanoleuca* (Xue et al. 2015). However, no studies have investigated whether seasonal variation in wild bird gut microbiota is dependent on dietary changes.

### **Phylogenetic history**

Host species and evolutionary history strongly determine the gut microbiota of mammals (Ley et al. 2008), with microbial gut communities more similar among closely related species. Coevolution in animals that have the potential for vertical transmission of complete microbial communities during birth has been hypothesized as one of the reasons for the importance of phylogeny in microbiota structuring (Ley et al. 2006). Although extrinsic factors can be driving composition of wild bird gut microbiota as discussed above, a number of studies have also shown host species to be an important determinant. Hird et al. (2015) evaluated the influence of 18 categorical variables including host species, diet and geographical location on wild bird gut microbiota, and found that variables associated with host taxonomy were the strongest determinant of gut microbial community. Waite and Taylor (2014) also observed host phylogeny as the main determinant of gut microbiota in the meta-analysis they conducted on a suite of avian species.

The gut microbiota of host young of magpies *Pica pica* and parasitic young of great spotted cuckoos *Clamator glandarius* differed among nest mates (Ruiz-Rodríguez et al. 2009), despite host and parasitic young being raised in the same nest environment. On the other hand, gut microbiota were more similar between siblings of the great tit *Parus major* within the same nest than among conspecific young in other nests (Lucas and Heeb 2005), which could be due to patterns of relatedness or a common nest environment. Manipulative experiments are needed to determine whether the genetic relatedness or the similarity in diet provided by the parents lead to these differences. For example, raising chicks from bird species with varying phylogenetic relatedness in a common garden, or reciprocal transplant experiments with control of environmental and dietary conditions, would allow us to separate genetic and environmental effects on gut microbial composition.

### **Age and sex**

Microbial colonization of bird guts is hypothesized to occur after hatching of the egg (van der Wielen et al. 2002, Kohl 2012). A study investigating microbial recruitment in

shorebird chicks showed that the chick GI tract is close to sterile at hatching, supporting this hypothesis (Grond et al. 2017). However, sampling of the microbiota during late incubation has documented the presence of several species of microbes at low abundance within the embryo gut (Kizerwetter-Świda and Binek 2008), suggesting that timing of colonization could depend on species or environmental factors. Infection of the reproductive tract in chickens can cause pathogenic bacteria to penetrate the egg shell from the outside environment, as well as to enter the egg during egg formation (Gantois et al. 2009, Cox et al. 2012, Martelli and Davies 2012). Expanding sampling of wild bird embryos before hatch could help elucidate whether the absence of an embryonic microbiota and low potential for maternal control extrapolates across species.

Microbial colonization of young bird guts may occur through various routes. Altricial birds depend on their parents for food, enabling parental influence on offspring's gut microbiota through prey selection and transfer of saliva. Precocial young leave the nest soon after hatch and often forage independently, limiting a direct parental influence on gut microbiota. Indeed, the local environment was indicated as a likely source for microbial recruitment in precocial shorebirds chicks (Grond et al. 2017). Precocial parents can potentially influence their chicks' gut microbiota through leading them to foraging areas with beneficial prey, or brooding and preening of the chicks. In addition, coprophagy by chicks could accelerate microbiome establishment.

Gut bacterial communities during early life in altricial birds vary widely in diversity and abundance (González-Braojos et al. 2011), and markedly differ from gut microbiota of conspecific adults (van Dongen et al. 2013, Waite et al. 2014). Chicks of shorebirds, ostriches and little penguins *Eudyptula minor* had dynamic gut microbial communities (Dewar et al. 2017, Grond et al. 2017, Videvall et al. 2018b), but short-tailed shearwaters *Ardenna tenuirostris* had relatively stable communities throughout development. Once mammalian hosts reach maturity, their gut microbiota are often assumed to be relatively stable (Faith et al. 2013), but long time-series with monitoring of adult bird microbiota are lacking. Waite et al. (2014) found distinct fecal microbial communities in both adult and juvenile Kakapos sampled one year apart, though adult and juvenile microbiota were not different within each year, suggesting significance of intra-annual variation. However, whether the intra-individual variation in microbiota composition is within the natural range of variation we cannot yet say, as only two samples were collected per individual. Long-term, repeated sampling of individual birds is needed to determine natural variation in their gut microbiota and to identify consistent age effects.

Males and females differ in reproductive physiology and behavior, which may manifest as different gut microbial profiles. Male and female northern bobwhites differed in three of the eight most common bacterial genera: *Enterococcus*, *Rothia* and *Streptococcus* (Su et al. 2014). Interactions between

sex hormones and the human gut microbiota are well known (summarized in Neuman et al. 2015), but little information is available for birds. Studies investigating sex hormones in birds have focused predominantly on the immunosuppressive effects of testosterone (Alonso-Alvarez et al. 2009). Escallón et al. (2017) showed experimentally that testosterone levels were positively related to cloacal diversity and *Chlamydia* spp. abundance, indicating a potential immunosuppressive effect. Alternatively, increased testosterone could increase the number of copulations with different mates and thus potentially exposure to sexual bacterial transmission.

### **Reproduction**

In addition to the innate physiological differences associated with sex, there are reproductive behaviors specific to each sex that could affect gut microbiota composition, such as mating system. Sexually monogamous bird species are exposed to fewer microorganisms during mating than species with multiple sexual partners. Studies that have investigated sexual transfer of microbiota in wild birds and other vertebrates have predominantly used cloacal swabbing to investigate the affected microbial community. The effects of mating on the cloacal microbiota in black-legged kittiwakes *Rissa tridactyla* were transient (White et al. 2010), and unlikely to have a lasting effect on their gut microbiota. Different mating systems could result in differential pathogen transfer, which can affect the partners' health.

Breeding can affect gut microbiota directly through transfer of microorganisms during the mating process and indirectly through increased close contact between mates during incubation and chick provisioning. No differences were found in cloacal microbiota between male and female barn swallows *Hirundo rustica* when birds were sampled during the breeding season (Kreisinger et al. 2015). In addition, many bird species segregate geographically sex outside of the breeding season (Cristol et al. 1999, Alves et al. 2013), exposing males and females to different diets and habitats. How differential migration affects microbial exposure for males and females is not known, but could be investigated by sampling during the non-breeding season.

### **Physiology: GI-tract morphology**

Animal behavior can affect their microbiota and vice versa (Ezenwa et al. 2012). In birds, migration is one behavior that can influence gut microbial composition. In addition to the extrinsic effects of migration on gut microbiota, there may be intrinsic physiological effects due to plasticity in bird gut morphology during migration. Migratory shorebirds down-regulate the mass of their GI tract before long-distance movements, resulting in an average 30% reduction in GI tract length (Battley et al. 2000). Large reductions in GI tract length affect physiological function and uptake rates, and could reduce physical habitat for microorganisms. In addition, the lack of nutrient input during flight, could lead to an impoverished microbiota. Many migratory birds frequent one or more staging sites during migration, which are usually

food-rich locations that birds use as mid-migration fueling sites. Intensive foraging at staging sites results in ingestion of large quantities of local food-associated microorganisms, potentially inoculating impoverished or altered communities with local microbes.

During spring migration at Delaware Bay (DE, USA), shorebirds use the same habitat and food source, consisting of eggs of horseshoe crabs *Limulus polyphemus* (Clark et al. 1993). Three shorebird species using this site, the red knot *Calidris canutus*, ruddy turnstone *Arenaria interpres* and semipalmated sandpiper *C. pusilla*, differed in their fecal microbial communities, in a common foraging environment (Grond et al. 2014, Ryu et al. 2014). Interspecific differences support the potential importance of phylogeny as a strong determinant of gut microbial communities, which differs from studies where environmental drivers of gut microbiota are important (Hird et al. 2014, Lewis et al. 2016, 2017). Similarly, shorebirds staging in the Tagus estuary in Portugal use the same habitat and resources, but each species possesses distinct microbial communities (Santos et al. 2012). It is plausible that the microbiota already present in the GI tract upon arrival can outcompete incoming food-associated microorganisms. Alternatively, retention time of food in shorebirds during stopover may be too short to allow settlement of food-associated bacteria.

To investigate how migratory movements affect gut microbiota, it would be necessary to sample birds prior to migration, upon arrival at staging sites, and during the staging period. Timing of migration and physiological adaptations to migration are endogenous traits, which are still expressed by captive birds. Pre-migratory fattening and GI tract shrinkage can be observed in captive wild birds, which, in combination with wind tunnel experimental flights, could be valuable tools for assessing the effect of migration on gut microbiota.

### **Health and fitness**

The influence of the gut microbiota on health has been studied intensively in humans and, to a lesser extent, in domestic poultry. How gut microbiota affect health in wild birds is unknown, due to the difficulties of determining health status for free-living birds and rarity of recovering dead individuals.

Gut microbiota can improve health by aiding nutrient uptake and through positive interactions with the immune system. A study investigating the effect of immune supplementation and challenge on the cloacal microbiota in homing pigeons *Columba livia domestica* revealed effects on evenness of the microbial community, but not on richness or diversity (Matson et al. 2015). The authors did not identify the microbial communities, and it is unclear which bacteria were affected by the experimental manipulations. A better understanding of how gut communities interact with the immune system under normal and immunologically challenging conditions will require extensive surveys combining health measurements, immunological assays and assessment of the

gut microbiota in wild bird populations. In addition, insight into how gut microbiota contribute to host fitness in birds warrants manipulating of gut microbial communities of birds under field conditions, followed by monitoring components of fitness such as fecundity, recruitment and survival.

## **Discussion and future directions**

In our review, we have highlighted the many opportunities provided by microbiome research of GI tracts of wild birds to learn how the environment and host interact to determine the composition and function of the gut microbiota. Three topics warrant future investigation: 1) descriptive studies of a wider range of bird taxa to increase understanding of baseline, or 'core', gut microbial diversity found within and among avian taxa, 2) use controlled experiments to identify the functional importance of gut microbiota to host performance, and 3) identify function of avian gut microbiota using meta-omics tools.

At present, broad inferences about gut microbial diversity are limited, as studies on wild birds are relatively new, and to date, have mainly focused on one or a few species. It is possible that life-history traits that differentiate birds from mammals are associated with a wider range in gut microbiota composition: there are twice as many bird species than mammals, (long-distance) flight allows exposure to many contrasting environments, and birds possibly have a greater reliance on microbiota for digestive function due to their lack of initial mechanical digestion. Expanding the range of avian species studied will improve our understanding of variation in inter-specific gut microbial diversity, a necessary first step in addressing the knowledge gaps in the areas we have identified.

Sampling a large variety of bird species is challenging, but collaborations among different fields and research groups or organizations could offset some of the challenges. Hird et al. (2015) investigated gut microbial communities in 59 neotropical bird species from 14 different orders by collaborating with the Museum of Natural Science at Louisiana State Univ. during specimen collection. Alternatively, international collaborations have the potential for sample collection over large geographical ranges. One such collaboration, the Arctic Shorebird Demographics Network (ASDN) consisted of a collaborative network of 17 field sites in the Russian and North American arctic with standardized field protocols for collecting demographic data on shorebirds (Weiser et al. 2018). The infrastructure already in place for the ASDN facilitated multi-year collection of shorebird fecal samples throughout the arctic (Grond et al. 2017).

In addition to increasing the variety of species studied, it is important to investigate the within-individual variation in the microbiota in different parts of the GI tract. Collecting fecal and cloacal samples as proxies for gut microbiota is a common approach. It is unclear what fraction of the microbiota in upstream sections of the GI tract are represented in

these samples, which affects our ability to use fecal samples to understanding links with digestive function. However, investigating upstream sections of the avian GI tract requires sacrifice of the host, which is undesirable for most populations of wild birds. Study of abundant populations of introduced house sparrows *Passer domesticus* or Eurasian starlings *Sturnus vulgaris*, could be an option for lethal or invasive experiments.

One challenge of studying wild birds under natural conditions is disentangling the large number of intrinsic and extrinsic factors that can influence host microbial communities. Statistical methods exist to separate the importance of different factors on gut microbial communities, but require broad sampling across a wide range of intrinsic and extrinsic factors. Bringing wild birds into captivity changes their environment, diet and social interactions from natural conditions, thus complicating the extrapolation of experimental results to natural systems. In the future, controlled experimental studies between paired free-living and captive populations should be used to address questions regarding microbial function, and responses to extrinsic and intrinsic factors. At a phylum level, we observed substantial similarity between domestic chickens and wild birds (Fig. 2), indicating that captive organisms can serve as an important tool and starting point to form hypotheses on the microbiota responses to changes, such as changes in diet and habitat.

In summary, the function of gut microbiota in birds remains poorly understood. Birds are fundamentally distinct from mammals with a different gut morphology due to their evolutionary history. As a result, our assessments of avian gut bacterial roles based on their equivalent function in the mammalian gut microbiota requires caution, and we stress that these are predictions based on available information that should be corroborated with future research. To assess broad functional patterns in gut microbial diversity, identifying a ‘core’ microbiota of different hosts can be a good starting point. Identifying core taxa could aid in a better, targeted selection of focal taxa for physiological or genome studies. Host-specificity of gut microbiota is likely, and we encourage reporting and assessment of microbial communities at finer taxonomic levels than phylum, and greater use of metagenomics approaches in the appropriate contexts to help link microbiome structure and function.

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