Gut microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a wading bird

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21

22 **Abstract**

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24 Microbial communities in the gastrointestinal tract influence many aspects of host health,
 25 including metabolism and susceptibility to pathogen colonization. These relationships and the
 26 environmental and individual factors that drive them are relatively unexplored for free-living
 27 wildlife. We quantified the relationships between urban habitat use, diet, and age with
 28 microbiome composition and diversity for 82 American white ibises (*Eudocimus albus*) captured
 29 along an urban gradient in south Florida and tested whether gut microbial diversity was
 30 associated with *Salmonella enterica* prevalence. Shifts in community composition were
 31 significantly associated with urban land cover and, to a lesser extent, diets higher in provisioned
 32 food. The diversity of genera was negatively associated with community composition associated
 33 with urban land cover, positively associated with age class, and negatively associated with
 34 *Salmonella* shedding. Our results suggest that shifts in both habitat use and diet for urban birds
 35 significantly alter gut microbial composition and diversity in ways that may influence health and
 36 pathogen susceptibility as species adapt to urban habitats.

37 **Introduction**

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39 Living in urban areas can have important consequences for wildlife physiology and/or health
 40 through changes in habitat use, interactions, and diet [1]. For example, urban birds exhibit higher

stress levels and more frequent interactions with non-native species [2]. Similarly, some species experience increased exposure to pollutants and reduced immune function [3]. The provisioning of urban wildlife, either intentionally (e.g., bird feeders) or unintentionally (e.g., garbage), can also promote the use of novel foods [4] and habitat types, such as manicured lawns [5].

Such shifts in wildlife physiology and ecology may, in turn, impact the diverse microbial community inhabiting the host gastrointestinal tract, or gut microbiome. The composition and diversity of the gut microbiome regulates several aspects of host health including the production of vitamins, metabolism, and dietary efficiency (reviewed by [6]). The gut microbiome also mediates host susceptibility to infection and pathogen colonization by initiating proper development of the immune system and the production of antimicrobial metabolites (reviewed by [7]). A healthy, diverse gut microbiome can also act as a barrier to infection by competing, either directly or indirectly, with pathogens for space or resources [8]. This has direct impacts on health. For example, disrupted gut microbial communities are more likely to become colonized by enteric bacteria such as *Clostridium difficile* [9].

The importance of the gut microbiome to host health has led to many studies on humans and livestock attempting to disentangle the relative influence of host age, diet, and environment on microbial communities. These effects can occur through accumulation, whereby older individuals typically have more diverse microbiomes [10]. Host diet can also influence microbiome composition and diversity through macronutrient balance [11]. For example, diets higher in protein are typically associated with certain phyla and increased diversity [12]. Lastly,

hosts are in constant contact with microorganisms in their environments from various substrates, which can shape the acquisition and composition of intestinal microbiota (e.g., [13,14]).

The roles of environmental exposure and diet in shaping the host microbiome may cause the gut microbiome to act as an important mediator for how urban environments or changing diets may affect host health. The shifts in diet and habitat exhibited by wildlife in urban environments likely influence their gut microbial communities as they do for animals in captivity [15–18] and humans in urban vs. rural communities [19]. While a rapidly growing body of literature has characterized the avian microbiome for many species [20], little is known about how the novel foods and habitat types available to urban birds may shape microbiome diversity and composition. For example, urban house sparrows have less diverse microbiomes than their rural counterparts due to several potential mechanisms [21].

Such shifts in diet and habitat use are apparent for the American white ibis (*Eudocimus albus*), a mid-sized carnivorous wading bird native to the southeastern United States. Up until the 1960's, Florida was the primary breeding site for ibis on the mainland USA, but following dramatic wetland loss and development, breeding colonies experienced a 90% decline of nesting pairs in Florida. In the last 20 years, ibis have been observed foraging in urban environments in cities across south Florida [22,23]. Our previous work in this system has demonstrated that ibises inhabiting more developed urban areas are more likely to consume provisioned food, such as bread, but have poorer body condition [24] and are more likely to shed *Salmonella enterica* [23]. Almost half of urban ibis *Salmonella* genotypes matched those for human salmonellosis cases, indicating ibis have the ability to act as reservoirs of salmonellae for people, or share a common

environmental source [23]. In a more robust comparison of *Salmonella* shedding by ibis from developed and natural environments, site fidelity to contaminated urban parks was a significant predictor for *Salmonella* prevalence [25]. These observed changes in the diets and health of ibis with urbanization may be indicative of concurrent changes in the composition or diversity of the gut microbiome for urban birds.

In this study, we characterized the gut microbiome of white ibises with the goal of determining how gut microbiome community composition and alpha diversity changed with urbanization, the consumption of provisioned food, age, and sex. We then tested whether any differences in composition or diversity were associated with the prevalence of *Salmonella* spp. To do so, we sampled white ibises across an urban gradient in South Florida and collected feces for *Salmonella* isolation and microbiome analyses and blood for stable isotope analysis of diet.

Materials and Methods

Ibis capture and sample collection

We captured ibises in Palm Beach and adjacent counties in South Florida between October 2015 and March 2017 (Fig 1). South Florida has a tropical climate with a high biodiversity of wading birds and annual fluctuations in water levels between the dry (November - April) and rainy (May - October) seasons. Palm Beach County has dense urban developments along the coast

progressing to agricultural development and wetlands further inland. We captured ibises at 15 sites where we had observed foraging ibises and that represented an urban land cover gradient (0-93% urban land cover, see [24] for details on land cover analysis). These sites included five restored or constructed wetlands, two wildlife rehabilitation centers, a landfill, a zoo, and six urban parks. We captured ibises in wetlands using mist nets with decoys and at more urban sites we used baited manually-operated flip traps and nylon slip-knot leg lassos [24].

We assessed ibis age using plumage as adults (≥ 3 years, all white feathers) or subadults (some brown feathers) [26] and recorded mass, culmen length, wing chord length, tarsus length and width to calculate body condition. We then collected $\leq 1\%$ body weight of blood from each bird from the jugular or metatarsal vein. We used whole blood samples to confirm ibis sex using standard molecular techniques [27] and for stable isotope analysis of diet (see below). If a captured ibis defecated onto a clean surface, we collected ≥ 0.25 g of feces (0.63 ± 0.05 , mean $\pm$ S.E.) using a sterile cotton tipped applicator for microbiome analysis. We also collected approximately one gram of fresh feces into 10 ml of dulcitol selenite broth for *Salmonella* isolation.

Molecular characterization of bacterial and fungal communities

Sample processing and extraction

Fecal samples for gut microbiome analyses were stored in microcentrifuge tubes at -20C in the field and subsequently at -80C until DNA extraction. Up to 0.25 mg of sample was aliquoted into 96 well extraction plates for submission to the University of Wisconsin Biotechnology Center for extraction and sequencing. Bacterial and fungal DNA were extracted using the PowerMag microbial DNA isolation kit following the manufacturer's protocol (Mo Bio Laboratories, Inc.). Extracted DNA was cleaned using a SpeedBead Clean-up protocol (GE SeraMag Beads, GE Healthcare, Chicago, IL) prior to library preparation.

Construction and sequencing of v3-v4 16s metagenomic libraries

DNA concentration was verified fluorometrically using either the Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). Samples were prepared in a similar process to the one described in Illumina's 16s Metagenomic Sequencing Library Preparation Protocol, Part # 15044223 Rev. B (Illumina Inc., San Diego, California, USA) with the following modifications: The 16S rRNA gene V3/V4 variable region was amplified with fusion primers (forward primer 341f: 5'-
ACACTCTTTCCCTACACGACGCTCTTCCGATCT(N)3/6CCTACGGGNGGCWGCAG-3',
reverse primer 805r: 5'-
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT(N)3/6GACTACHVGGGTATCTAAT
CC-3'). Region specific primers were previously described in [28] (underlined sequences above), and were modified to add 3-6 random nucleotides ((N)3/6) and Illumina adapter overhang nucleotide sequences 5' of the gene-specific sequences. Following initial amplification, reactions were cleaned using a 0.7x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences,

Union City, CA). In a subsequent PCR, Illumina dual indexes and Sequencing adapters were added using the following primers (Forward primer: 5'-AATGATACGGCGACCACCGAGATCTACAC[55555555]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', Reverse Primer: 5'-CAAGCAGAAGACGGCATACGAGAT[77777777]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT -3', where bracketed sequences are equivalent to the Illumina Dual Index adapters D501-D508 and D701-D712,N716,N718-N724,N726-N729). Following PCR, reactions were cleaned using a 0.7x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences). Quality and quantity of the finished libraries were assessed using an Agilent DNA 1000 kit (Agilent Technologies, Santa Clara, CA) and Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific), respectively. Libraries were pooled in an equimolar fashion and appropriately diluted prior to sequencing. Paired end, 300 bp sequencing was performed using the Illumina MiSeq Sequencer and a MiSeq 600 bp (v3) sequencing cartridge. Images were analyzed using the standard Illumina Pipeline, version 1.8.2. OTU assignments and diversity plots were created using QIIME analysis pipeline [29].

Bioinformatics

Output sequencing files provided by the University of Wisconsin Biotechnology Center were separated by barcode sequences by the Biotech center, resulting in 192 pairs of forward and reverse read fastq files. Paired sequences were joined, quality filtered, clustered into operational taxonomic units, assigned to taxonomic identities, and aligned to create a phylogenetic tree using the QIIME pipeline [29]. Paired end reads were joined using the multiple_join_paired.py script

and default parameters, separately for the two batches of files coming from the two Miseq runs. Unpaired sequences were discarded. Joined consensus sequences were quality filtered and combined into one fasta file using the `multiple_split_libraries_fastq.py` script with the “sampleid_by_file” option, again separately for the two Miseq runs, using default parameters. The two resulting combined files were then concatenated into one final file. Because we used the same sample identifiers on the two separate Miseq runs, by concatenating the output files from the two `split_libraries` script we effectively pool the sequences from both Miseq runs for each sample.

We clustered the resulting sequences into operational taxonomic units with 97% identity using the `parallel_pick_open_reference_otus.py` script with the UCLUST method. Open reference OTU clustering works by first comparing each of our query sequences against a reference database – we used the Greengenes 97% identity database [30]. Then, any sequences not matching to an entry in the reference database at 97% identity are separately clustered first by creating new reference databases from a random draw of the sequences for two iterations (pulling 0.1% of the unclustered sequences each time). Finally, any sequences not assigned to an OTU after these steps are clustered using de novo clustering. This process was run on 12 nodes on the University of Wisconsin Center for High Throughput Computing.

OTUs were assigned to the highest confident taxonomic resolution using the RDP Naïve Bayesian Classifier [31], using the `assign_taxonomy.py` script in QIIME. A final community matrix (samples as rows, OTUs as columns, with the number of sequences per OTU detected in each sample as cells) was created using the `make_otu_table.py` script.

Representative sequences for each OTU were aligned using the align_seqs.py script, using the PyNAST method using default parameters. Aligned sequences were used to create a phylogenetic tree, using the make_phylogeny.py script with the FastTree method [32]. For phylogenetically informed analyses, the OTU table was filtered to remove any OTU's whose representative sequences could not be aligned. The reshape2 package in R [33] was used to collapse OTUs into higher taxonomic levels for analysis (see Table S1 and Figure S1 for a summary of bioinformatics output at the genus level, which was used for analysis).

Salmonella isolation

We isolated *Salmonella* from fecal samples by modifying previously published protocols [34]. Briefly, 1 g of feces was placed in dulcitol selenite pre-enrichment broth and was maintained at ambient temperature for approximately 2-4 days until they were shipped to the University of Georgia (Athens, Georgia) for culture. We then transferred 100µl of turbid pre-enrichment broth to 10 ml of Rappaport-Vassiliadis (RV) broth and incubated for ~24 h at 43 °C (Oxoid, Hampshire, UK). We streaked for isolation from each RV enrichment onto xylose lysine tergitol 4 (XLT-4) agar plates and incubated for 24 ± 2 h. Colonies suspected to be *Salmonella* based on morphology (black colonies or yellow colonies with a black center) streaked again for isolation to obtain a pure colony. Each isolate was then confirmed by characteristic growth on CHROMagar© Salmonella-Plus after 24 h of incubation at 37 °C [34] (CHROMagar, Paris, France). We confirmed a subset of isolates as *Salmonella* via PCR [25,34]. Confirmed *Salmonella* isolates were stored at -80 °C.

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221 **Stable isotope analysis**

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223 We quantified the proportion of provisioned food (i.e., bread and chips) in ibis diet using $\delta^{13}\text{C}$
 224 and $\delta^{15}\text{N}$ stable isotope analysis of red blood cells. These isotopes reflect the carbon sources and
 225 trophic level of consumer diets; $\delta^{13}\text{C}$ differs for marine and terrestrial communities and C4
 226 plants such as corn in processed foods [35], and $\delta^{15}\text{N}$ is biomagnified in predators [36]. Avian
 227 red blood cells have a turnover rate of approximately two months [37] and so provide an estimate
 228 of assimilated diet over that period. We estimated the importance of provisioned food, freshwater
 229 and terrestrial invertebrates, marine invertebrates, landfill refuse, and fish by creating five-source
 230 mixing models using the R package SIAR [38]. Samples were analyzed following standard
 231 methods at the Center for Applied Isotope Studies at the University of Georgia (see [24] for in-
 232 depth description of diet sources and laboratory methods).

233 **Statistical analyses**

234 Microbiome data were analyzed at the genus and phyla levels. Microbial community
 235 composition was analyzed at the site level (i.e., relative abundance of each taxon was averaged
 236 across individual samples within a site) to control for pseudoreplication when comparing
 237 microbiome characteristics to site-level characteristics. Similarity among sites in average genus-
 238 level microbiome composition was visualized using principal coordinates analysis using the
 239 capscale function in the vegan package in R [39]. Associations between the microbiome and the

geographic and demographic explanatory variables were assessed using environmental fits performed with the envfit function in the vegan package in R.

At the phylum level, association between the site-average relative abundances of each phylum and landscape urbanization were visualized using the heatmap function in the pheatmap package in R [40]. Significance of the associations between site-average relative abundance of each phylum and landscape urbanization was tested using a sample-size weighted correlation using the wtd.cor function in the weights package in R [41] with adjustment of significance thresholds using false discovery rates [42] to control for multiple comparisons. Only phyla with an average relative abundance $\geq 0.1\%$ were included in this analysis.

The relationships among microbial community characteristics at each site (e.g., Shannon diversity at the genus level and the ordination axes as univariate measures of community composition) and key environmental variables (landscape urbanization, dietary provisioning, and *Salmonella* prevalence) were assessed using linear or logistic regression as appropriate based on exploratory graphical analysis of the pair-wise relationships. Linear regression was performed using the lm function the stats package in R [43]. Linear regressions were performed on site-level measures and were weighted by samples size at each site. Logistic regression was performed on the individual sample characteristics for genus-level Shannon diversity and *Salmonella* presence using the glm function in the stats package in R with a binomial distribution. This relationship was visualized at the site level to facilitate comparison with other site-level correlations.

Explanatory variables were correlated to both microbiome measures and each other, making it difficult to determine which variables linked directly to patterns in microbial community measures and *Salmonella* prevalence. To address this issue, we performed structural equation modeling to better understand the relationships among the variables. Structural equation modeling was performed using the lavaan [44] and lavaan.survey packages [45]. We identified the best fit model starting from a complete model with all hypothesized relationships and interactions and then performed a manual backwards selection removing non-significant relationships from the model until the model fit stabilized with acceptable model fit parameters. This final model was tested further by removing one or more relationships from the model and comparing the model fit parameters for decline or improvement in model stability.

Results

We sampled 82 white ibises at 15 sites (5.3 ± 4.0 birds per site) and sampled more adult females than other age and sex classes (adult females = 36, adult males = 20, subadult females = 17, subadult males = 9). Microbiome sequence analysis of white ibis feces yielded on average 104,695 sequence reads (± 6077 SEM) and 351.6 genera (± 19 SEM) per sample (Figure S1, Table S2).

Changes in composition

The dominant shift in gut microbiome composition at the genus level correlated to the percent of urban landcover, the average percentage of diet from provisioned food, and average diversity (Shannon) at the genus level (Figure 2: Environmental Fit – Percent urban landcover $p < 0.0001$, $R^2 = 0.835$; Average percent of diet provisioned $p = 0.021$, $R^2 = 0.493$; Percent of samples from adults $p = 0.103$, $R^2 = 0.314$; Shannon diversity of genera $p = 0.014$, $R^2 = 0.519$). Urban landcover and the average percentage of diet from provisioned foods in turn were moderately correlated (sample-size adjusted $R^2 = 0.304$, $p = 0.020$).

Urban land cover and dietary provisioning were primarily associated with changes in genus-level bacterial community composition along the first ordination axis (PCoA1) (Figure 2, $R^2 = 0.82$, $F = 59.0$, $p < 0.001$). This shift in composition corresponded to notable changes in the relative abundance of several bacterial phyla (Figure 3, Table S3). The most abundant phyla in white ibis feces were Firmicutes (mean percentage of sequences across all samples: 32.5%), Proteobacteria (22.6%), and TM7 (22.6%); however, average relative abundance of dominant phyla varied considerably across the urbanization gradient (Figure 3, Table S3). Firmicutes and Cyanobacteria significantly decreased in relative abundance while Proteobacteria, TM7, Bacteroidetes, OP11, and TM6 increased in relative abundance with increasing urban land cover (Table S3).

Changes in diversity

Unlike bacterial composition, changes in bacterial genus diversity were associated with individual ibis biology rather than capture site. These changes in genus diversity, as measured by

the Shannon diversity index, were not associated with urban land cover or dietary provisioning (Urban land cover: $R^2 = 0.04$, $F_{1,13} = 1.16$, $p = 0.30$, Figure 4a; Provisioning: $R^2 = 0.03$, $F_{1,13} = 0.81$, $p = 0.38$, Figure 4b). Rather, diversity was significantly correlated with microbiome community composition (PCoA1) ($R^2 = -0.29$, $F_{1,13} = 5.2$, $p = 0.04$; Figure 4c). As predicted, ibis age, measured as the proportion of adult ibis captured at a particular site, was positively correlated with genus diversity ($R^2 = 0.16$, $p < 0.001$). Mean bacterial diversity was negatively correlated with *Salmonella* prevalence (Figure 4d), such that ibises with lower genus diversity were more likely to be shedding *Salmonella* ($\beta = -2.10 \pm 0.99$, $z = -2.12$, $p = 0.03$).

Diversity and *Salmonella* prevalence

Given the co-linearity between several of our explanatory variables and community composition, we developed a structural equation model to better understand how these variables linked through gut community composition to associations with *Salmonella* prevalence (Structural equation model weighted by sample size at each site: Robust Chi-squared goodness-of-fit $p=0.466$; Comparative Fit Index =1.000; Tucker-Lewis Index=1.032, Fig 5). The best model indicated that bacterial community composition was associated with both the degree of urbanization surrounding the sampling site and to a lesser extent the percentage of the diet attributed to provisioned food, where urbanization acted both directly on composition and indirectly through effects on diet (Urbanization: $\beta = 1.18 \pm 0.21$, $z = 5.64$, $p < 0.001$, Diet: $\beta = 1.51 \pm 0.69$, $z = 2.18$, $p = 0.029$, Figure 5). Bacterial community composition (PCo1) was negatively associated with average genus-level diversity, and diversity was positively associated with the proportion of adult ibis at each site (PCoA1: $\beta -0.39 \pm 0.15$, $z = -2.62$, $p = 0.009$,

Proportion adult: $\beta = 0.65 \pm 0.14$, $z = 4.68$, $p < 0.001$, Figure 5). Genus-level diversity was then negatively associated with *Salmonella* prevalence ($\beta = -0.41 \pm 0.17$, $z = -2.42$, $p=0.016$; Figure 5).

Discussion

In this study, we examined changes in the composition and diversity of the gut microbiome with urbanization and food provisioning for the American white ibis, a recently urbanized wading bird. We also tested if these changes were associated with an increased likelihood of shedding *Salmonella*, an opportunistic enteric zoonotic pathogen. Microbiome community composition was strongly associated with urban land cover and, to a lesser extent, the assimilation of provisioned food, such as bread. Microbial diversity was negatively associated with these changes in community composition, and ibises with less diverse microbiomes were more likely to shed *Salmonella*.

Urban land cover was the strongest driver of microbial community composition and is itself associated with many biotic and abiotic factors. Urban ibis in Australia also consume provisioned bread in parks and select for high-carbohydrate rather than high-protein foods [46]. The relative importance of macronutrients is an important predictor of microbiome composition across species [47] and so may be at least partially driving the shifts we observed in phylum

relative abundance. Ibises in urban parks may also be exposed to different environmental bacteria when foraging on lawns, managed ponds, and paved surfaces rather than shallow wetlands. Similarly, ibises and other birds in urban areas often interact with non-native or invasive species [48], further increasing their exposure to novel microbiota. Other differences in the behavior or physiology of urban birds, such as increased sedentariness [49] or altered stress levels [2] may alter microbiome composition through habitat exposure and physiological interactions. Regardless of mechanism, the taxonomic shifts we observed in microbiome composition had important consequences for community diversity.

Similarly to community composition, changes in gut microbial diversity can be caused by many different factors relating to bacterial exposure and within-host dynamics. Although urbanization was not directly associated with changes in bacterial diversity, these factors were linked indirectly through changes in composition. These findings are consistent the only other studies examining the shifts in wildlife microbiomes with urbanization, where urban house sparrows (*Passer domesticus*) and urban gulls (*Larus argentatus*) also had less diverse gut microbiomes, potentially due to less varied diets or habitat use [21,50]. Ibises that use less urban areas may be exposed to more diverse environmental bacteria because they exhibit more nomadic movements and use varied habitats such as freshwater and estuarine wetlands, coastal areas, and agricultural areas [25]. Similarly, older individuals of many species, including humans, have more diverse gut microbial communities, likely because of accumulation from environmental exposure over time [6,10].

Perhaps most importantly, lower average microbial diversity was significantly associated with higher *Salmonella* prevalence. In humans and other mammals, gut microbiome perturbations have been associated with an increase in infections and various inflammatory and chronic diseases [17,51–53]. The protective role of microbiome diversity has been demonstrated in many model species. In mice, following experimental treatment with antimicrobials to dramatically alter the composition of the gut microbiome, *Salmonella enterica* serovar Typhimurium or mice experimentally infected with *Clostridium difficile* became “supershedders”, facilitating rapid transmission [54,55]. This link between microbiome diversity and infection has also been demonstrated in other systems—Avian Influenza (AI)-positive mallards had lower OTU diversity than AI-negative birds [56]. The relationship between gastrointestinal microbiome and pathogens in free-living animals is, however, understudied. When the gut microbiome of captive cheetahs was compared to free-living cheetahs in the same habitat, captive individuals had higher abundance of pathogenic bacteria OTUs and modified disease-associated functional pathways [17]. In fact, manipulations of the gut microbiome may offer unique treatment modalities and this has been examined in production animals. For example the administration of prebiotic attenuated *Salmonella* strain or prebiotic galacto-oligosaccharides (GOS) can promote resistance to *Salmonella* colonization via increases of beneficial microorganisms in chickens [57] and similarly, *Lactobacillus* is used as a probiotic to protect against common pathogens like *Salmonella* and *Campylobacter* [58]. And specifically because antimicrobial resistance of *Salmonella* has become an urgent problem, studies on the protection against *Salmonella* colonization and infection through microbiome manipulation chickens abound [59,60]. Yet, studies where the relationships between diet, microbiome and pathogens are examined are extremely limited for free-living populations. Consistent with our results, Jungle crows (*Corvus*

macrorhyncos), which mostly consume anthropogenic food, were found to have a relatively high prevalence of pathogenic organisms in their gut microbiome [61].

Relevant to our work, the chicken gastrointestinal microbiome both interacts with the immune system within the lumen of the intestinal tract in various ways, and directly competes with pathogens. For example, greater number of intestinal lymphocytes are present in conventional versus germfree chickens and at the molecular level, the complexity of the T-cell receptor repertoire is influenced by gut microbial diversity. Additionally, in chickens, used both as human animal models and for their own economic impact, pathogen exclusion through competition appears to be the mechanism responsible for lower *Salmonella* colonization rates in older chicks with complex microbiomes [62].

The ability of microbiota to protect the host via resisting the colonization of pathogen parallels the increased resistance to invasion by more diverse ecological communities more generally. There are many studies demonstrating increased invasion success by pathogenic bacteria following a loss of microbial diversity parallels ecological theory that more diverse communities are more resistant to invasion [63]. Similar to other ecological systems, microbial symbionts can protect hosts inadvertently though intense competition for resources from the host's diet [64]. Likewise, just as invasive species may exploit disturbed communities [65], opportunistic pathogens such as *Salmonella enterica* exploit and more easily colonize disturbed microbial communities [63,66].

Although more diverse microbial communities can protect the host from pathogen colonization,

the negative relationship we observed between microbiome diversity and *Salmonella* prevalence may also be caused by microbiome disruption following *Salmonella* colonization. For example, *S. Typhimurium* can cause dysbiosis by inducing inflammation through the expression of virulence factors [66,67]. While we cannot determine the direction of causation between *Salmonella* prevalence and microbiome diversity with the data we collected in this study, it is a rich avenue for further research in free-living wildlife populations. For example, further characterization of the endogenous microbial communities of different wildlife species can help identify dysbiosis and any threats to conservation or public health [20].

Many studies have demonstrated changes in the prevalence of pathogen prevalence, immune function, and stress in urban wildlife [1,68,69]. Shifts in microbiome diversity with habitat use and diet may be an underappreciated mechanism by which urbanization can affect wildlife health. This shift in pathogen shedding with gut diversity for urban ibis may also have important consequences for the role of urban wildlife as carriers of zoonotic pathogens across the landscape. American white ibis, as well as many other wildlife species, can act as asymptomatic carriers of *Salmonella enterica* and ibis shed *Salmonella* serotypes shared with local human cases [23]. As such, a greater understanding of how wildlife microbiome form and function are altered in changing environments may be crucial for improving conservation and public health.

Data Availability Statement

Raw sequences will be deposited at the NCBI short-read archive

(<https://www.ncbi.nlm.nih.gov/sra>, DEPOSIT CODE TBD).

References

1. Bradley CA, Altizer S. Urbanization and the ecology of wildlife diseases. *Trends Ecol Evol.* 2007;22: 95–102. doi:10.1016/j.tree.2006.11.001
2. Ruiz G, Rosenmann M, Novoa FF, Sabat P. Hematological parameters and stress index in rufous-collared sparrows dwelling in urban environments. *Condor.* 2002;104: 162–166. doi:10.1650/0010-5422(2002)104[0162:HPASII]2.0.CO;2
3. Hawley DM, Hallinger KK, Cristol DA. Compromised immune competence in free-living tree swallows exposed to mercury. *Ecotoxicology.* 2009;18: 499–503. doi:10.1007/s10646-009-0307-4
4. Kark S, Iwaniuk A, Schalimtzek A, Banker E. Living in the city: can anyone become an “urban exploiter”? *J Biogeogr.* 2007;34: 638–651. doi:10.1111/j.1365-2699.2006.01638.x
5. Conover MR, Kania GS. Characteristics of feeding sites used by urban-suburban flocks of Canada geese in Connecticut. *Wildl Soc Bull.* 1991;19: 36–38.
6. Hollister EB, Gao C, Versalovic J. Compositional and functional features of the gastrointestinal microbiome and their effects on human health. *Gastroenterology.* 2014;146: 1449–1458. doi:10.2217/FON.09.6.Dendritic

7. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome, and immune system: envisioning the future. *Nature*. 2012;474: 327–336. doi:10.1038/nature10213.Human
8. Pickard JM, Zeng MY, Caruso R, Núñez G. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunol Rev*. 2017;279: 70–89. doi:10.1111/imr.12567
9. Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, et al. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. *Infect Immun*. 2012;80: 62–73. doi:10.1128/IAI.05496-11
10. Cui Y, Wang Q, Liu S, Sun R, Zhou Y, Li Y. Age-related variations in intestinal microflora of free-range and caged hens. *Front Microbiol*. 2017;8: 1–10. doi:10.3389/fmicb.2017.01310
11. Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*. 2013;5: 108–119. doi:10.4161/gmic.26945
12. Singh RK, Chang H-W, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. *J Transl Med. BioMed Central*; 2017;15: 73. doi:10.1186/s12967-017-1175-y
13. Hyde ER, Navas-Molina JA, Song SJ, Kueneman JG, Ackermann G, Cardona C, et al. The oral and skin microbiomes of captive komodo dragons are significantly shared with their habitat. *mSystems*. 2016;1: e00046-16. doi:10.1128/mSystems.00046-16
14. Lax S, Smith D, Hampton-marcell J. Longitudinal analysis of microbial interactions

between humans and indoor environment. *Science* (80-). 2014;345: 1048–1052.

15. McKenzie VJ, Song SJ, Delsuc F, Prest TL, Oliverio AM, Korpita TM, et al. The effects of captivity on the mammalian gut microbiome. *Integr Comp Biol*. 2017;57: 690–704.

doi:10.1093/icb/icx090

16. Xie Y, Xia P, Wang H, Yu H, Giesy JP, Zhang Y, et al. Effects of captivity and artificial breeding on microbiota in feces of the red-crowned crane (*Grus japonensis*). *Sci Rep*.

Nature Publishing Group; 2016;6: 33350. doi:10.1038/srep33350

17. Wasimuddin, Menke S, Melzheimer J, Thalwitzer S, Heinrich S, Wachter B, et al. Gut microbiomes of free-ranging and captive Namibian cheetahs: Diversity, putative functions and occurrence of potential pathogens. *Mol Ecol*. 2017;26: 5515–5527.

doi:10.1111/mec.14278

18. Delport TC, Power ML, Harcourt RG, Webster KN, Tetu SG. Colony location and captivity influence the gut microbial community composition of the Australian sea lion (*Neophoca cinerea*). *Appl Environ Microbiol*. 2016;82: AEM.00192-16.

doi:10.1128/AEM.00192-16

19. Tyakht A V., Kostyukova ES, Popenko AS, Belenikin MS, Pavlenko A V., Larin AK, et al. Human gut microbiota community structures in urban and rural populations in Russia.

Nat Commun. Nature Publishing Group; 2013;4: 1–9. doi:10.1038/ncomms3469

20. Waite DW, Taylor MW. Exploring the avian gut microbiota: Current trends and future directions. *Front Microbiol*. 2015;6: 1–12. doi:10.3389/fmicb.2015.00673

21. Teyssier A, Rouffaer LO, Saleh Hudin N, Strubbe D, Matthysen E, Lens L, et al. Inside the guts of the city: Urban-induced alterations of the gut microbiota in a wild passerine.

Sci Total Environ. Elsevier B.V.; 2018;612: 1276–1286.

doi:10.1016/j.scitotenv.2017.09.035

22. Frederick P, Ogden J. Philopatry and nomadism: contrasting long-term movement behavior and population dynamics of White Ibises and Wood Storks. Colonial Waterbirds. 1997;20: 316–323. doi:10.2307/1521699
23. Hernandez SM, Welch CN, Peters VE, Lipp EK, Curry S, Yabsley MJ, et al. Urbanized White Ibises (*Eudocimus albus*) as carriers of Salmonella enterica of significance to public health and wildlife. PLoS One. 2016;11: 1–22. doi:10.1371/journal.pone.0164402
24. Murray MH, Kidd AD, Curry SE, Hepinstall-Cymerman J, Yabsley MJ, Adams HC, et al. From wetland specialist to hand-fed generalist: Shifts in diet and condition with provisioning for a recently urbanized wading bird. Philos Trans R Soc B Biol Sci. 2018;373. doi:10.1098/rstb.2017.0100
25. Murray MH, Hernandez SM, Rozier RS, Kidd AD, Hepinstall-Cymerman J, Curry SE, et al. Site fidelity is associated with food provisioning and Salmonella in an urban wading bird. Ecohealth. In Press.
26. Bildstein KL. White ibis: wetland wanderer. Washington, D.C.: Smithsonian Institution Press; 1993.
27. Fridolfsson AA, Ellegren H. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol. 1999;30: 116–121.
28. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41: 1–11.

doi:10.1093/nar/gks808

29. Caporaso J, Kuczynski J, Stombaugh J, Bittinger K, Bushman F, Costello E, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2011;7: 335–336. doi:10.1038/nmeth.f.303.QIIME
30. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol*. 2006;72: 5069–5072. doi:10.1128/AEM.03006-05
31. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*. 2007;73: 5261–5267. doi:10.1128/AEM.00062-07
32. Price MN, Dehal PS, Arkin AP. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol*. 2009;26: 1641–1650. doi:10.1093/molbev/msp077
33. Wickham H. Reshaping data with the reshape Package. *J Stat Softw*. 2007;21: 1–6. doi:10.5325/soundings.101.1.0006
34. Luo Z, Gu G, Giurcanu MC, Adams P, Vellidis G, van Bruggen AHC, et al. Development of a novel cross-streaking method for isolation, confirmation, and enumeration of *Salmonella* from irrigation ponds. *J Microbiol Methods*. Elsevier B.V.; 2014;101: 86–92. doi:10.1016/j.mimet.2014.03.012
35. Jähren AH, Kraft RA. Carbon and nitrogen stable isotopes in fast food: signatures of corn and confinement. *Proc Natl Acad Sci*. 2008;105: 17855–60. doi:10.1073/pnas.0809870105

36. DeNiro MJ, Epstein S. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica Cosmochim Acta*. 1981;45: 341–351.
37. Hobson KA, Clark RG. Turnover of ^{13}C in cellular and plasma fractions of blood: Implications for nondestructive sampling in avian dietary studies. *Auk*. 1993;110: 638–641.
38. Parnell AC, Inger R, Bearhop S, Jackson AL. Source partitioning using stable isotopes: coping with too much variation. *PLoS One*. 2010;5: e9672. doi:10.1371/journal.pone.0009672
39. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. Vegan: Community ecology package, R package version 2.5-5. 2019; url: <https://cran.r-project.org/package=vegan>.
40. Kolde R. pheatmap: Pretty Heatmaps. R package version 1.0.12. 2019; url: <https://CRAN.R-project.org/package=pheatmap>.
41. Pasek J, Tahk A, Culter G, Schwemmler M. weights: Weighting and Weighted Statistics. R package version 1.0. 2018; url: <https://CRAN.R-project.org/package=weights>.
42. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Stat Soc Ser B*. 1995;57: 289–300.
43. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2019; url: <https://www.R-project.org/>.
44. Rosseel Y. lavaan: An R package for structural equation modeling. *J Stat Softw*. 2012;48: 1–21. doi:10.18637/jss.v048.i02
45. Oberski D. lavaan.survey: An R package for complex survey analysis of structural

equation models. J Stat Softw. 2014;57: 1–27. doi:10.18637/jss.v057.i01

46. Coogan SCP, Machovsky-Capuska GE, Senior AM, Martin JM, Major RE, Raubenheimer D. Macronutrient selection of free-ranging urban Australian white ibis (*Threskiornis moluccus*). Behav Ecol. 2017;28: 1021–1029. doi:10.1093/beheco/axx060
47. Muegge BD, Kuczynski J, Knights D, Clemente JC, Gonzalez A, Fontana L, et al. Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science (80-). 2011;332: 970–974. doi:10.1126/science.1198719
48. Galbraith JA, Beggs JR, Jones DN, Stanley MC. Supplementary feeding restructures urban bird communities. Proc Natl Acad Sci. 2015;112: E2648–E2657. doi:10.1073/pnas.1501489112
49. Partecke J, Gwinner E. Increased sedentariness in European Blackbirds following urbanization: a consequence of local adaptation? Ecology. 2007;88: 882–90. Available: <http://www.ncbi.nlm.nih.gov/pubmed/17536705>
50. Furst M, Veit RR, Hahn M, Dheilly N, Thorne LH. Effects of urbanization on the foraging ecology and microbiota of the generalist seabird *Larus argentatus*. PLoS One. 2018;13: e0209200. doi:10.1371/journal.pone.0209200
51. Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. Microb Ecol Heal Dis. 2015;26. doi:10.3402/mehd.v26.26191
52. McKenna P, Hoffmann C, Minkah N, Aye PP, Lackner A, Liu Z, et al. The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. PLoS Pathog. 2008;4. doi:10.1371/journal.ppat.0040020
53. Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, et al. Obesity-induced

gut microbial metabolite promotes liver cancer through senescence secretome. *Nature*.
Nature Publishing Group; 2013;499: 97–101. doi:10.1038/nature12347

54. Lawley TD, Bouley DM, Hoy YE, Gerke C, Relman DA, Monack DM. Host transmission
of *Salmonella enterica* serovar Typhimurium is controlled by virulence factors and
indigenous intestinal microbiota. *Infect Immun*. 2008;76: 403–416.
doi:10.1128/IAI.01189-07

55. Lawley TD, Clare S, Walker AW, Goulding D, Stabler RA, Croucher N, et al. Antibiotic
treatment of *Clostridium difficile* carrier mice triggers a supershedder state, spore-
mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun*.
2009;77: 3661–3669. doi:10.1128/IAI.00558-09

56. Ganz HH, Doroud L, Firl AJ, Hird SM, Eisen JA, Boyce WM. Community-level
differences in the microbiome of healthy wild mallards and those infected by influenza A
viruses. *mSystems*. 2017;2: 1–15.

57. Azcarate-peril MA, Butz N, Cadenas B, Koci M, Ballou A, Mendoza M, et al. An
attenuated *Salmonella enterica* serovar Typhimurium strain and galacto-oligosaccharides
accelerate clearance of *Salmonella* infections in poultry through modifications to the gut
microbiome. *Appl Environ Microbiol*. 2018;84: e02526-17.

58. Patterson JA, Burkholder KM. Application of prebiotics and probiotics in poultry
production. *Poult Sci*. 2003;82: 627–631. doi:10.1093/ps/82.4.627

59. Kumar S, Chen C, Indugu N, Werlang GO, Singh M, Kim WK, et al. Effect of antibiotic
withdrawal in feed on chicken gut microbial dynamics, immunity, growth performance
and prevalence of foodborne pathogens. *PLoS One*. 2018;13: 1–23.

doi:10.1371/journal.pone.0192450

60. Hughes R-A, Ali RA, Mendoza MA, Hassan HM, Koci MD. Impact of dietary galacto-oligosaccharide (GOS) on chicken's gut microbiota, mucosal gene expression, and *Salmonella* colonization. *Front Vet Sci.* 2017;4: 1–14. doi:10.3389/fvets.2017.00192
61. Maeda I, Siddiki MSR, Nozawa-Takeda T, Tsukahara N, Tani Y, Naito T, et al. Population abundance of potentially pathogenic organisms in intestinal microbiome of jungle crow (*Corvus macrorhynchos*) shown with 16S rRNA gene-based microbial community analysis. *Biomed Res Int.* 2013; 1–5. doi:10.1155/2013/438956
62. Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, et al. The chicken gastrointestinal microbiome. *FEMS Microbiol Lett.* 2014;360: 100–112. doi:10.1111/1574-6968.12608
63. Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA. The application of ecological theory towards an understanding of the human microbiome. *Science* (80-). 2012;336: 1255–1262. doi:10.1126/science.1224203.The
64. Yurdusev N, Ladire M, Ducluzeau R, Raibaud P. Antagonism exerted by an association of a *Bacteroides thetaiotaomicron* strain and a *Fusobacterium necrogenes* strain against *Clostridium perfringens* in gnotobiotic mice and in fecal suspensions incubated in vitro. *Infect Immun.* 1989;57: 724–731.
65. Hobbs RJ, Huenneke LF. Disturbance, diversity, and invasion: Implications for conservation. *Conserv Biol.* 1992;6: 324–337. doi:10.1046/j.1523-1739.1992.06030324.x
66. Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M, et al. *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the

intestinal microbiota. PLoS Biol. 2007;5: 2177–2189. doi:10.1371/journal.pbio.0050244

67. Thiennimitr P, Winter SE, Winter MG, Xavier MN, Tolstikov V, Huseby DL, et al.

Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the

microbiota. Proc Natl Acad Sci. 2011;108: 17480–17485. doi:10.1073/pnas.1107857108

68. Brearley G, Rhodes J, Bradley A, Baxter G, Seabrook L, Lunney D, et al. Wildlife disease

prevalence in human-modified landscapes. Biol Rev. 2013;88: 427–442.

doi:10.1111/brv.12009

69. Murray M, Sánchez C, Becker D, Byers K, Worsley-Tonks K, Craft M. City sicker? A

meta-analysis of wildlife health and urbanization. Front Ecol Environ. 2019;In Press.

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Author Contributions

Competing Interests Statement

The authors declare no competing interests.

Figure legends

Figure 1. Map of 15 sampling sites on the eastern coast of South Florida where American white ibises were captured to collect fecal specimens to determine their gastrointestinal microbiome. Sampling sites represented a gradient of urban land cover from 0% (blue) to 91% (red; see also Table S1). Inset circles demonstrate the range of difference in urban habitat across the urban land cover gradient, ranging from open wetland (blue inset) to densely inhabited residential areas (red inset).

Figure 2. Relationship between bacterial community composition to urbanization, dietary provisioning, age, and genus diversity. Principal coordinates analysis demonstrated site-level patterning in bacterial community composition at the genus level that corresponded to urban land cover (red to blue gradient) and use of provisioned food by ibises (circle size) at capture sites, but not age composition of sampled birds (See text for statistics). Genus-level Shannon diversity associated with the community composition, increasing in the opposite direction of these independent variables.

Figure 3. Correlation between urban landcover and average relative abundance of dominant bacterial phyla. The heat map shows several bacterial phyla (bolded) significantly increased (green) or decreased (yellow) in relative abundance across the urban landcover gradient (top bar, on the same color scale as Figure 2).

Figure 4. Correlation of bacterial diversity to urbanization (a), dietary provisioning (b), and bacterial community composition (c), and *Salmonella* prevalence (d). Circles represent average values for 15 capture sites and colored along an urban gradient from red (most urban) to blue (least urban). Bacterial diversity was not significantly correlated to landscape and dietary factors but was significantly associated with overall community composition and *Salmonella* prevalence (see text for statistics).

Figure 5. Path analysis of factors associated with *Salmonella* prevalence in American white ibises. Path analysis showing significant positive (solid arrows) and negative relationships (dashed arrows) between urban land cover, ibis use of provisioned food, and ibis age with microbial composition (ordination axis values), diversity (Shannon index of genus diversity), and *Salmonella* prevalence. Thicker arrows indicate larger effect sizes.

694 Figures

695 Figure 1 (low resolution for review)

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718 Supplemental Information

719 Table S1: Landcover surrounding capture sites

	Number of Samples	Percent Surrounding Landcover		
		Wetland	Urban	Other*
JW Corbett	1	91.8%	0.0%	8.2%
TetraTech	3	91.8%	0.0%	8.2%
Loxahatchee Slough	3	74.4%	15.4%	10.2%
Loxahatchee Wildlife Refuge	3	73.8%	4.3%	21.9%
Green Cay	5	26.8%	65.5%	7.7%
Kitching Creek	2	15.2%	25.5%	59.3%
Solid Waste Authority	4	11.1%	49.4%	39.4%
Busch Wildlife Sanctuary	1	9.3%	62.5%	28.2%
Lion Country Safari	14	8.9%	46.8%	44.3%
Juno Beach	10	3.8%	61.4%	34.8%
Dreher Park	9	0.0%	89.7%	10.3%
Dubois Park	5	0.0%	53.0%	47.0%
Gaines Park	6	0.0%	84.0%	16.0%
Indian Creek Park	12	0.0%	91.1%	8.9%
Lake Worth	3	0.0%	76.4%	23.6%
CROW Sanibel Island	1	-	-	-

720 *Other land classifications included agricultural, coastal, other water, and other terrestrial

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Table S2. Summary of sample sizes and sequence outputs

Site	Number of Samples	Sequences		Genera	
		Mean Number	Standard Error	Mean Number	Standard Error
Busch Wildlife Sanctuary	1	67605	-	633.0	-
CROW Sanibel Island	1	63960	-	581.0	-
Dreher Park	9	108408	15334	317.3	68.4
Dubois Park	5	77400	18890	315.2	80.0
Gaines Park	6	121111	21644	329.3	59.6
Green Cay	5	113303	32279	289.8	66.2
Indian Creek Park	12	115061	18700	343.4	52.6
Juno Beach	10	120359	11575	387.6	54.0
JW Corbett	1	64762	-	370.0	-
Kitching Creek	2	175378	62761	457.5	5.5
Lake Worth	3	74650	22398	351.3	137.4
Lion Country Safari	14	102983	18523	302.3	42.0
Loxahatchee Slough	3	89203	14894	410.0	119.1
Loxahatchee Wildlife Refuge	3	131894	31528	420.0	61.2
Solid Waste Authority	4	56060	17078	327.8	117.6
Tetra Tech	3	81505	194	465.0	27.5
All Samples	82	104695	6077	351.6	18.8

Table S3. Individual and site average relative abundance of dominant phyla and correlations to landscape urbanization

Phyla	Percentage of Sequences				Correlation coefficient	p-value
	Mean (SEM) (82 samples)	Mean (SEM) (15 sites)	Median (15 sites)	Range (15 sites)		
Firmicutes	32.5 (3.1)	39.9 (5.3)	40.9	10.9-71.4	-0.773	<0.0001*
Proteobacteria	22.6 (2.6)	20.2 (1.7)	19.0	12.6-36.1	0.772	<0.0001*
TM7	22.6 (3.2)	15.2 (3.8)	16.7	0.4-37.7	0.763	<0.0001*
Actinobacteria	6.4 (1.0)	6.9 (1.0)	7.5	2.5-14.2	0.131	0.671
Chlamydiae	6.2 (1.6)	6.0 (2.4)	0.8	0.1-34.0	0.297	0.086
Fusobacteria	3.0 (1.2)	3.0 (0.8)	2.0	0.0-8.7	-0.261	0.181
Cyanobacteria	1.6 (0.4)	2.9 (1.0)	1.4	0.1-11.2	-0.578	0.004*
Chloroflexi	1.1 (0.2)	1.5 (0.4)	1.1	0.1-4.8	-0.514	0.112
Tenericutes	0.8 (0.4)	1.4 (1.1)	0.2	0.1-16.9	-0.466	0.104
Plantomycetes	0.8 (0.2)	0.9 (0.2)	0.7	0.2-2.6	-0.225	0.507
Bacteroidetes	1.1 (0.2)	0.9 (0.2)	0.9	0.1-2.6	0.528	0.010*
OP11	0.4 (0.1)	0.3 (0.1)	0.2	0.0-1.1	0.516	0.007*
TM6	0.3 (0.2)	0.2 (0.1)	0.1	0.0-1.5	0.457	0.020*
Verrucomicrobia	0.1 (0.0)	0.2 (0.0)	0.1	0.0-0.5	-0.067	0.811
Acidobacteria	0.12 (0.03)	0.1 (0.0)	0.1	0.0-0.5	0.037	0.935

* Significant correlation after adjustment for false-discovery rate (Benjamini, Yoav; Hochberg, Yosef (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society, Series B. 57 (1): 289–300.)

Table S4. Path analysis statistical results

Regression	Estimate	SEM	p-value
Salmonella Prevalence~			
Mean Bacterial Diversity	-0.413	0.171	0.016
Mean Bacterial Diversity~			
Bacterial Composition (PCoA1)	-0.392	0.149	0.009
Percent Adult	0.647	0.138	<0.001
Bacterial Composition (PCoA1)~			
Percent Urban Habitat	1.180	0.209	<0.001
Mean Percent of Diet Provisioned	1.514	0.694	0.029
Mean Percent of Diet Provisioned~			
Percent Urban Habitat	0.210	0.053	<0.001

Model fit: Overall model fit p=0.223, Comparative fit index =0.950, Tucker-Lewis index=0.900

Figure S1. Rarefaction curves of bacterial genera detected in American white ibis feces. Rarefaction was performed using the rarecurve function in the vegan package [39].

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure S1