

RESEARCH ARTICLE

Don't Deny Your Inner Environmental Physiologist: Investigating Physiology with Environmental Stimuli

An urban diet differentially alters the gut microbiome and metabolomic profiles compared with a seed diet in mourning doves

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Abstract

Urbanization influences food quality and availability for many avian species, with increased access to human refuse and food subsidies in built environments. In relation to such nutritional intakes and their presumed impact on microbes harbored in the intestinal tract and metabolic profiles of host physiological systems, our overall knowledge of the role of gut microbiome (GM) and metabolomic expression in the avian host lags far behind our understanding of mammals. Therefore, the objective of this investigation was to examine the potential differential effect of an urban modeled versus control (i.e., bird seed) diet on the GM, the metabolic profiles of plasma, liver, adipose, kidney, and muscle tissues, and circulating endotoxin and inflammatory factors in urban-caught mourning doves (*Zenaidura macroura*). We hypothesized that the urban diet would differently impact the profiles of the GM and tissue metabolomes and increase plasma lipopolysaccharide (LPS) and proinflammatory factors compared with animals fed a seed diet. After a 4-wk-diet period, contents of the large intestine were sequenced to profile the microbiome, metabolomic analyses were performed on plasma and tissue homogenates, and circulating LPS and inflammatory markers were assessed. The composition of the GM was significantly dissimilar between diets, with greater abundance of Erysipelatoclostridiaceae, Sanguibacteraceae, *Oribacterium*, and *Sanguibacter* and decreased circulating LPS in the urban-fed birds. These differences were largely not reflected in the surveyed metabolomes and plasma inflammatory markers. This research supports the notion that the microbial composition in urban doves is impacted by diet, though may only weakly associate with host physiology.

birds; gut microbiome; lipopolysaccharide; metabolomics; urban

INTRODUCTION

Research across several animal species suggest a diet high in refined carbohydrates, saturated fats, and salt promotes deleterious alterations in the composition and function of microbes harbored in the digestive tract (1, 2). This dietary pattern has also been associated with increased systemic inflammation, which is mediated, in part, through alterations in the gut microbiome (GM) (1, 3, 4). The GM plays a vital role in regulating the intestinal epithelial barrier and compositional alterations can increase the abundance of Gram-negative bacteria that release lipopolysaccharides (LPS) (5). LPS is an endotoxin with different serotypes (i.e., toxicity of the lipid A portion of LPS) harboring the ability to impair tight junction proteins and increase intestinal permeability (6). Ultimately, bacterial translocation from increased intestinal permeability can result in an inflammatory response and metabolic insulin resistance (7). Emerging evidence from animal and human studies has suggested an important role of the GM in the development of type 2 diabetes mellitus (T2D) and other

metabolic diseases (8–10). In relation, urbanization influences food quality and availability for many wild animal species, which may promote a shift in the GM that could ultimately impact host physiology. As a model organism, birds living in urban environments have access to a variety of food subsidies not available to rural birds such as bread, popcorn, restaurant waste, and other anthropogenic foods (11, 12).

Across multiple animal species, it is becoming increasingly evident that the GM plays an important role in modulating host physiology (13). In relation, the suite of small molecules present in biological samples (known as the metabolome) can complement GM data and may reveal important metabolic signatures from environmental stimuli (14, 15). Because of the unique attributes of their energetic metabolism and physiology, birds have been suggested as pathology-free models of T2D (16). In support, birds have plasma glucose concentrations 1.5–2 times that of similar sized mammals and yet can forego the regulation of glucose by insulin without any adverse effects (17). Furthermore, most avian species appear to possess mechanisms to enhance fatty acid transport and oxidation during

flight (18, 19). These traits are similar to the way energy is utilized by humans with diabetes who are unable to efficiently utilize glucose and consequently rely more on fatty acid oxidation when carbohydrates are plentiful (20). Previous research has found mourning doves (*Zenaidura macroura*) fed either a refined-carbohydrate or high-fat diet over a 4-wk period do not develop metabolic complications that mammals normally show in response to consumption of these diets (21, 22). In addition, metabolomics analyses of plasma, liver, pectoralis muscle, and kidney revealed significant differences in several individual metabolites but no changes in overall metabolic pathways (21, 22). These findings suggest that mourning doves may alter their nutritional physiology to avoid metabolic complications. In contrast to the metabolome, examination of the GM of house sparrows (*Passer domesticus*) from rural and urban environments revealed enrichment of Proteobacteria in the urban birds, which are implicated in several mammalian intestinal and extraintestinal diseases (23). Moreover, and more recently, we reported impaired vasodilation in mourning doves fed a diet modeled on food available in an urban environment compared with a bird seed diet (24). Urbanization influences food quality and availability for many wild species and has been suggested to promote a shift in the GM that could impact other nutritional physiology outcomes. Indeed, research exploring the consumption of foods available in urban environments by birds has reported significant shifts in the GM and associated physiology (25–28).

Overall, our knowledge of the role of intestinal microbes in avian hosts lags far behind our understanding of mammalian systems. However, constitutional taxa detected from birds using 16S rRNA gene amplicon sequencing have also been identified in mammalian microbial communities, suggesting that birds may harbor some shared microbial milieu (29). Moreover, in a large comparative study across 59 bird species, diet was reported to be a significant variable in explaining GM composition (30). However, more recent work has downplayed the influence of diet on the avian GM (31). Regardless, much of the work conducted in this area is cross-sectional in nature and lacks the rigor of a controlled feeding trial, which enables researchers to control for other environmental variables (e.g., pathogens, pollution, stress, etc.). Such work is critical for allowing us to better understand the role of diet in the urban environment. Therefore, as a continuation of our research we investigated the potential differential effects of an urban-type (i.e., bird seeds mixed with French fries; herein “Urban” diet) versus control (i.e., bird seeds; herein “Seed” diet) diet on the GM, metabolic profile (plasma, liver, adipose, kidney, and muscle), and circulating endotoxin and inflammatory factors in mourning doves. The common Western diet food, French fries, was used as birds have ready access to this food source in urban environments. We hypothesized that the Urban diet would differently impact the profiles of GM and tissue metabolomes, and increase plasma LPS and proinflammatory factors, compared with animals fed a Seed diet.

MATERIALS AND METHODS

Animals and Diets

Blood and tissue samples and contents from the lower intestines from a total of 13 birds were provided from a

prior study comparing Seed and Urban diets in mourning doves (24). Briefly, 16 adult male and female mourning doves (body mass: 110–130 g) were captured from the ASU Tempe campus, AZ (33°25'11.5"N–111°55'55.6"W; altitude: 365 m), using a walk-in style funnel trap baited with wild-bird seeds, as previously described (21, 22, 24). All animals were captured during the same hours and were transported to the ASU Department of Animal Care and Technology facility located on the Tempe campus in individual cloth bags with drawstring closures to minimize stress. Birds were housed individually to prevent fighting and were exposed to a 14:10 h light/dark cycle and an ambient temperature of $77 \pm 4^\circ\text{F}$ ($\sim 25^\circ\text{C}$) to mimic natural conditions at the time of capture. All study protocols were approved by the ASU Institutional Animal Care and Use Committee and were conducted under appropriate state and federal scientific collecting permits.

After a 2-wk acclimation period, birds in the original study were divided randomly and fed either an Urban diet (4 male and 4 females) or a Seed only diet (4 males and 4 females) for 4 wk, as previously described (24). Two birds died in captivity, and one was released due to poor housing acclimation resulting in 13 animals at the end of the study (Urban diet: 3 males and 4 females; Seed diet: 2 males and 4 females). Veterinarian examination (necropsy) suggested the two deaths may have been due to preexisting conditions. The Urban diet consisted of a 50:50 mixture of nutritionally balanced dove seeds mixed with mashed French fries. French fries were selected as they are an anthropogenic food item and prevalent in urban areas (32). The seed-to-French fry ratio was based on tolerability during the acclimation period and to model the expected mixed diet of birds in these environments. An overview of the study design is outlined in Fig. 1.

Intestinal Content Sample Collection and Extraction

After birds were euthanized, contents of the large intestine (proximal to the cecum) were harvested. Although we understand the disadvantages of lacking a baseline measurement, it was not feasible to collect intestinal samples in birds without highly invasive techniques thus preventing pre- and postmeasures within the same individual. Although cloacal swab or fecal samples have been used in the past, they are often unreliable and less accurate compared with the methods used presently and outlined previously (33). Intestinal contents were stored at -80°C until processing for microbial analyses. Microbial genomic DNA was extracted from intestinal samples using a commercially available kit following the manufacturer's protocol (PowerSoil DNA Isolation Kit, MoBio Laboratories, Inc., Carlsbad, CA). DNA concentration was quantified using a μDrop plate adaptor (Cat. No. N12391, Thermo Fisher Scientific, Waltham, MA) and Multiskan GO microplate spectrophotometer (Cat. No. 5119300, Thermo Fisher Scientific). The $\text{OD}_{260}/\text{OD}_{280}$ ratio of all samples were ≥ 1.80 , demonstrating purity.

Microbiome Analysis

Microbial DNA samples were sent to the Genomics Core Facility in the Biodesign Institute at Arizona State University (Tempe, AZ) for bacterial community analysis via next-generation paired-end sequencing on the MiSeq Illumina platform.

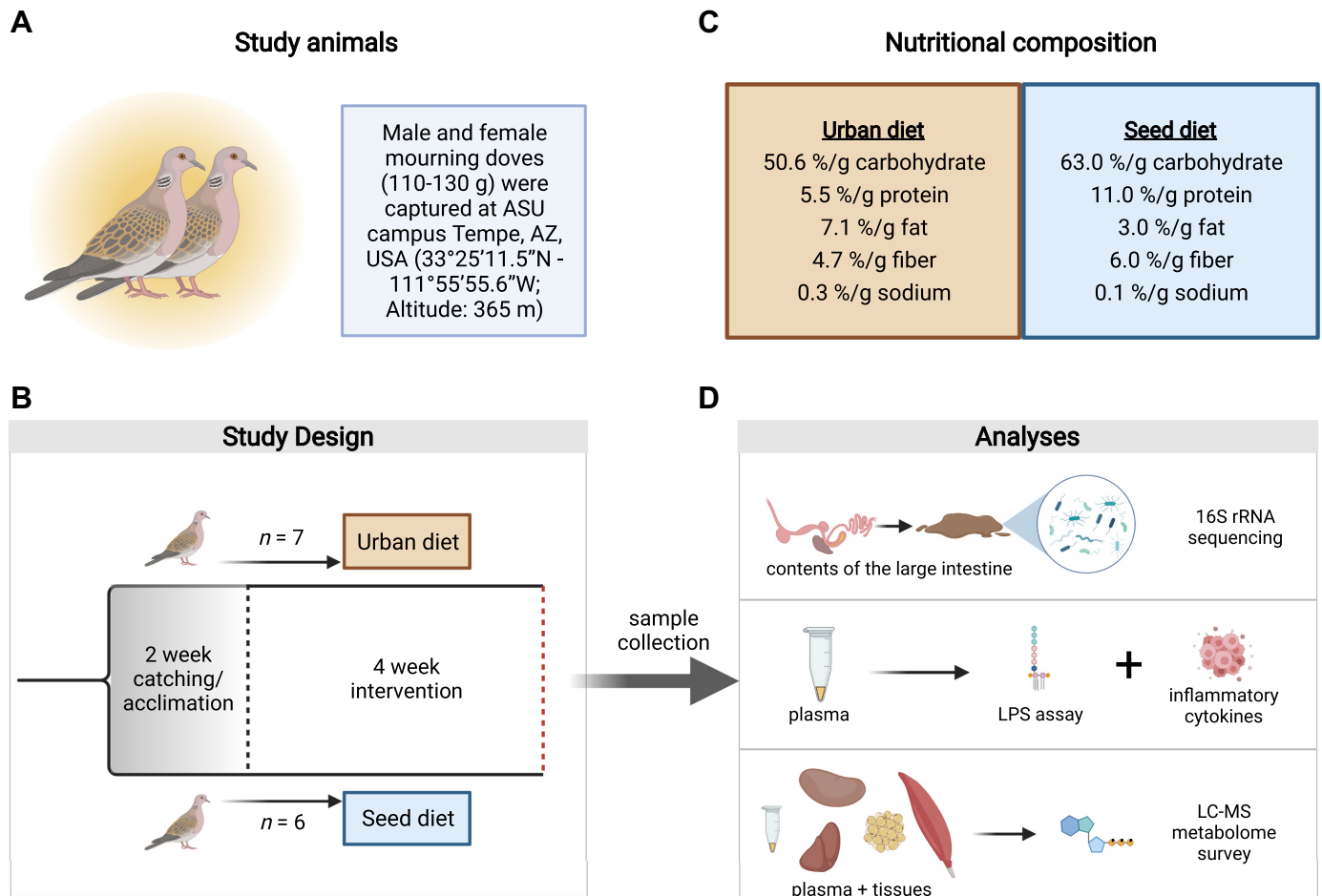


Figure 1. Schematic of study design. **A:** a total of 16 male and female mourning doves (*Zenaida macroura*) were captured in Tempe, AZ. **B:** after capture, birds were acclimated to their conditions for a 2-wk period. Next, birds were randomly assigned to the Urban diet (4 male and 4 females) or a Seed only diet (4 males and 4 females). Two birds died and one was released, leaving 7 birds in the Urban diet group and 6 in the Seed diet group. **C:** the Urban diet consisted of a 50:50 ratio of French fry to seed based on tolerability. The Seed diet consisted of bird seed. **D:** after the intervention, contents of the large intestine were removed, and microbial DNA was extracted for 16S rRNA gene sequencing. Plasma samples were assessed for lipopolysaccharide (LPS) and inflammatory cytokine concentrations. Plasma, liver, kidney, adipose, and pectoralis and gastrocnemius muscle samples were taken, and metabolomics analyses were performed. LC-MS, liquid chromatography–mass spectrometry.

Specifically, amplicon sequencing of the V4 region of the 16S rRNA gene was performed with the barcoded universal forward (515 F: GTGYCAGCMGCCGCGGTAA) and reverse (806 R: GGACTACNVGGGTWTCTAAT) primers containing Illumina adapter sequences and following the protocol by the Earth Microbiome Project (EMP; <https://earthmicrobiome.org/protocols-and-standards/16s/>) for the library preparation (34). PCR amplifications for each sample were done in triplicate, then pooled and quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). A no-template control sample was included during the library preparation as a control for extraneous nucleic acid contamination. DNA (200 ng) per sample was pooled and then cleaned using QIA quick PCR purification kit (QIAGEN, Valencia, CA). The pool was quantified by Illumina library Quantification Kit ABI Prism (Kapa Biosystems, Wilmington, MA). The DNA pool was diluted to a final concentration of 4 nM then denatured and diluted to a final concentration of 4 pM with a 25% of PhiX. Finally, the DNA library was loaded in the MiSeq Illumina and run using the version 2 module, 2 × 250 paired end, as per the directions of the manufacturer.

The resultant paired end, demultiplexed data were imported and analyzed using QIIME 2 software v. 2021.2 (35). Briefly, the sequencing generated 1,014,547 reads with a median of 77,450 reads per sample. After sequence quality plots were viewed based on 10,000 randomly selected reads, the first 25 bases of the forward and reverse reads were trimmed. Next, quality control was performed via the DADA2 denoise-paired method to remove low quality regions and construct a feature table using Amplicon Sequence Variants (ASVs) (36). The feature-classifier plugin was used to classify ASVs taxonomically. A pretrained I Bayes machine-learning classifier was trained to differentiate taxa present in the 99% SILVA (version 138.1) reference set trimmed to 250 bp of the V4 hypervariable region (corresponding to the 515 F-806R primers). This classifier works by identifying k-mers that are diagnostic for particular taxonomic groups and using that information to predict the taxonomic affiliation of each ASV (37). A phylogenetic tree was then constructed using the fragment-insertion plugin where the sequences were inserted into the SILVA identity reference tree backbone. Based on the inherent issues with rarefaction (38) and lack of a large difference in sequencing

depth between samples, data was not rarefied (Supplemental Fig. S1; all Supplemental material is available at <https://doi.org/10.6084/m9.figshare.20084090>). A *phyloseq* (v1.38.0) object was created, and downstream analyses and visualizations were performed in R (v4.1.2). Sequences were removed including mitochondrial and plant DNA. In addition, ASVs that had a mean read count of less than 2 across all samples were removed.

α Diversity (intracommunity diversity) was measured using the Shannon index, which accounts for richness and evenness, and phylogenetic diversity (PD), which is a phylogenetically weighted measure of richness, using the *phyloseq* and *picante* (v1.8.2) packages, respectively. β Diversity (between-sample diversity) was calculated using the Aitchison distance rooted in a centered log ratio (CLR) transformation as it is robust to high levels of sparsity (39). Before transformation, a pseudocount of 1 was assigned, and CLR was performed using the package *microbiome* (v1.18.0). Differences in GM composition by diet group were visualized via a Principal Component Analysis (PCA) of the Aitchison distance. To predict the function of GM, data analysis was performed through the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUST2) pipeline (40). PICRUST2 output was based on structured pathway mappings of Enzyme Commission gene families to the MetaCyc database (41).

Metabolomics Analysis

Plasma, liver, kidney, adipose, as well as pectoralis and gastrocnemius muscle samples were analyzed by the Arizona Metabolomics Laboratory core facility at Arizona State University using targeted liquid chromatography-mass spectrometry techniques as previously described (21, 22). Contents of the large intestine were not used in this analysis due to very low sample amount. All samples were thawed overnight at 4°C before extraction and analyses. Internal quality control samples were created by pooling aliquots of plasma from all animals. These quality controls were analyzed at the beginning, middle, and end of the analyses. Data for tissue samples were normalized to tissue mass before analyses. Sample analyses were carried out using an Agilent 1290 UPLC-6490 QQQ-MS (Santa Clara, CA) system equipped with an electrospray ionization (ESI) source. The resulting data were statistically analyzed as described subsequently.

LPS Assay and Inflammatory Cytokine Analyses

After the 4-wk diet, plasma and tissue samples were collected, as previously described (24). LPS concentrations were quantified in duplicate using undiluted cardiac plasma samples that were collected at euthanasia for both diet treatments. A commercially available kit was used per the manufacturer's protocol [Cat No. KTE30162, Abbkine, Inc., China; intra-assay coefficient variation (CV) <10%]. Undiluted plasma samples were sent to Eve Technologies (Calgary, Alberta, Canada) for assessment of inflammatory cytokines [interferon- α (IFN α), interferon- γ (IFN γ), interleukin (IL)-2, IL-6, IL-10, IL-16, IL-21, macrophage inflammatory protein-1 β (MIP)-1 β , MIP-3 α , macrophage colony-stimulating factor (M-CSF), regulated on activation, normal T cell expressed and secreted (RANTES), vascular endothelial growth factor (VEGF)] using a 12-plex chicken cytokine array (Millipore, Burlington, MA).

Statistical Methods

For the GM, α diversity metrics were compared with Mann-Whitney *U* tests and β diversity was compared with permutational multivariate analysis of variance (PERMANOVA; permutation $n = 999$) using the “adonis” function in the *vegan* package (v2.6.2). To compare dispersion, a permutation test for homogeneity in multivariate dispersion (PERMDISP) was conducted using the “betadisper” function in the *vegan* package. Next, to evaluate whether taxa frequencies in the two groups were equal, a Dirichlet-multinomial distribution was assumed and difference in the mean distribution of taxa was tested (accounting for the overdispersion). This was conducted at the phyla level after pooling rare taxa using the *HMP* package (v2.0.1) (42). For differential abundance testing, analysis of compositions of microbiomes with bias correction was employed on taxa present in at least 30% of all the samples using the R package *ANCOMBC* (v1.4.0) (43). Adjustment for multiple testing was conducted with a Bonferroni correction. Features with a q value <0.05 were considered significant. Data generated from the PICRUST2 pipeline was first filtered, retaining features present in at least 30% of all samples, and then assigned a pseudocount of 1 and transformed via CLR using the package *microbiome*. Analyses with Mann-Whitney *U* tests were then performed with *P*-value corrections performed using the Bonferroni approach (set at $q < 0.05$).

To determine significantly affected metabolites, data were log-transformed and subjected to independent sample *t* tests (Seed versus Urban diets) for all plasma and tissue samples using IBM SPSS Statistics for Windows, version 27.0 (SPSS, Inc., Chicago, IL). If data were not normally distributed, a Mann-Whitney *U* test was used. To adjust for multiple hypothesis testing, a false discovery rate (FDR) correction was used with a significance level of $P < 0.05$. The online software package MetaboAnalyst (metaboanalyst.ca) was used for the pathway analyses, integrating enrichment analyses, and pathway topology analyses of metabolomic data. The data were log₁₀-transformed before model construction and the reported Holm adjusted *P* value was used to determine significance (44). The pathway analysis was calculated from the chicken metabolic pathway library. PubChem Open Chemistry Database (<https://pubchem.ncbi.nlm.nih.gov/>) was used for interpretation of individual metabolites and metabolic pathways.

For LPS and inflammatory markers, normality statistics and probability plots were generated to test normality assumptions, and log transformations were performed as appropriate. Plasma data were analyzed by *t* tests (Urban vs. Seed diet). Statistical analyses were performed using IBM SPSS Statistics for Windows, version 27.0 (SPSS, Inc., Chicago, IL). A *P* value of < 0.05 was considered statistically significant.

RESULTS

Gut Microbiome Analysis Revealed Compositional Differences between Seed and Urban Diet Groups

Aitchison distance matrices of CLR transformed ASV counts were used to compare overall GM composition between the Seed and Urban diet groups. Significant clustering by diet was observed accounting for 11.37% of the

explained proportion of variance [PERMANOVA: $F_{(1,12)} = 1.41$, $R^2 = 0.11$, $P = 0.04$; Fig. 2A]. Importantly, we did not detect a significant difference in group dispersion for Seed versus Urban diet groups [PERMDISP: $F_{(1,12)} = 0.80$, $P = 0.78$], increasing our confidence that the significant compositional differences were not an artifact of variance in group dispersion. In comparison, there were no significant differences between Seed and Urban diet groups for the α diversity metrics, Shannon index, or PD (Mann–Whitney U test: $P \geq 0.37$; Fig. 2, B and C; Supplemental Table S1). These findings suggest that diet was not associated with GM richness or evenness in these animals.

Overall, we identified 178 unique ASVs after filtering providing taxonomy assignment for 75 features at the genus level (phylum: 9; class: 14; order: 28; family: 49). The predominant phyla across the two groups, irrespective of diet, were Firmicutes, Actinobacteriota, Proteobacteria, Bacteroidota, and Desulfobacteria (Fig. 3A). On evaluation of the distribution of phyla, we did not detect a significant difference between the Seed and Urban diets (χ^2 statistic = -29.51 , $P = 1.00$). However, there was more visually apparent variation at the genus level. Of the top 10 most abundant genera, we noted several biologically notable taxa including, *Lactobacillus*, *Enterococcus*, and *Veillonella* (Fig. 3B).

In relation to differential abundance testing, significance was detected at the family and genus levels, with several differentially abundant taxa between the two diet groups (ANCOMBC: $q < 0.05$). Specifically, families more abundant in the GM of birds fed the Urban diet included, Erysipelatoclostridiaceae [log-fold-change (log-FC) = 2.18] and Sanguibacteraceae (log-FC = 1.28; Fig. 4A; Supplemental

Table S2). In the GM of Seed birds, Aeromonadaceae (log-FC = 1.23) was more abundant. At the genus level, two taxa were more abundant in the Urban diet group, including *Oribacterium* (log-FC = 2.06) and *Sanguibacter* (log-FC = 1.04), whereas three were more abundant in the Seed diet group, including *Aeromonas* (log-FC = 1.58), *Galibacterium* (log-FC = 2.51), and *Solobacterium* (log-FC = 2.04; Fig. 4B; Supplemental Table S2).

The PICRUST 2 pipeline was implemented to predict the function of GM based on the 16S rRNA gene amplicon sequencing data. Overall, there were 342 predicted pathways, though after filtering and statistical testing only 4 pathways displayed significant differences in enrichment between the Seed and Urban Diet groups (Fig. 5, A–D). The Urban diet had greater abundance in “methanol oxidation to carbon dioxide,” whereas the Seed diet had greater abundance in “glucose and glucose-1-phosphate degradation,” “superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis,” and “2-methylcitrate cycle I” (Mann–Whitney U test: $q < 0.05$).

Metabolome Analysis of Host Tissues Displayed Subtle Differences between Seed and Urban Diet Groups

The metabolomics analyses yielded numerous metabolites per plasma/tissue sample (plasma: $n = 154$; pectoralis muscle: $n = 123$; liver: $n = 125$; kidney: $n = 188$; gastrocnemius muscle: $n = 105$; adipose: $n = 92$). Although 32 metabolites were initially significantly different between diet groups, after adjusting for multiple hypothesis testing, only one pectoralis muscle metabolite remained significantly different between diet groups (Table 1; Supplemental Fig. S2). The

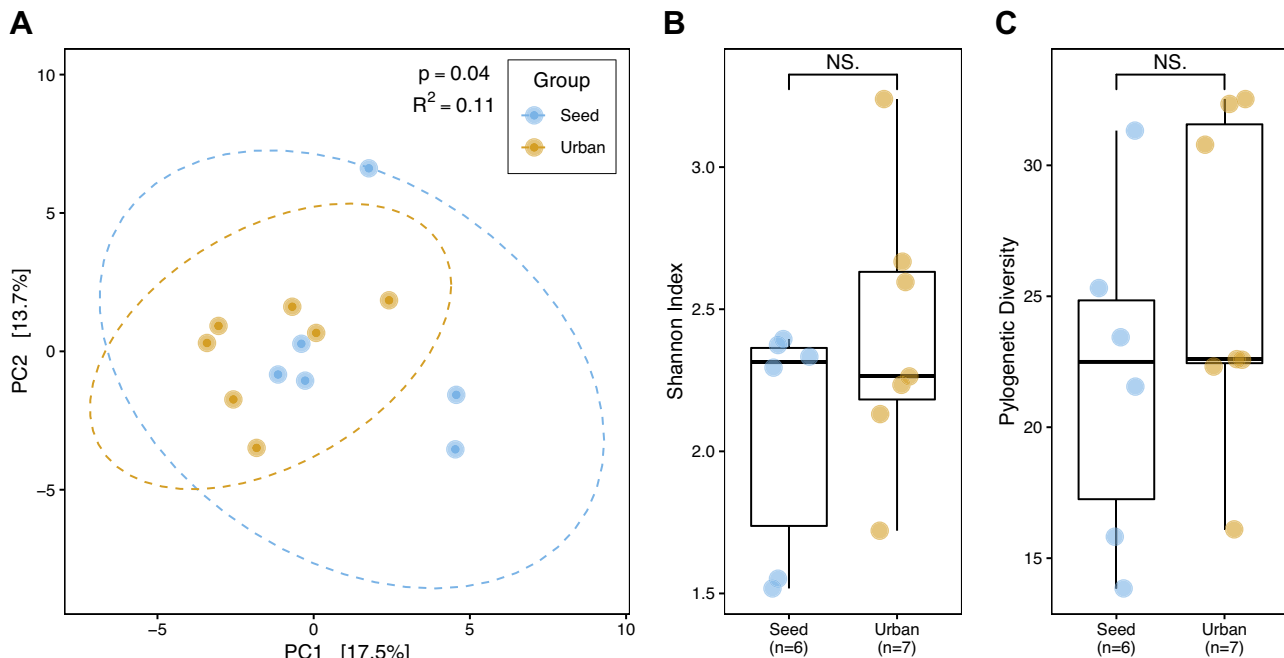


Figure 2. A: the composition of microbiome samples from the contents of the large intestine differed significantly in mourning doves fed a Seed ($n = 6$) vs Urban ($n = 7$) diet, as visualized by principal components analysis (PCA) of Aitchison distances of centered log-ratio ASV counts ($P = 0.04$). Each point represents an individual sample, and the dashed lines are the 95% confidence intervals of the two diet groups. Significance testing was performed with permutational analysis of variance (PERMANOVA; permutation $n = 999$). α -Diversity boxplots display, Shannon diversity (B) and phylogenetic diversity (C) for Seed and Urban diet groups. Boxes denote the interquartile range (IQR) between the first and third quartiles, and the horizontal line defines the median. Significance was determined by Mann–Whitney U tests. NS, not significant.

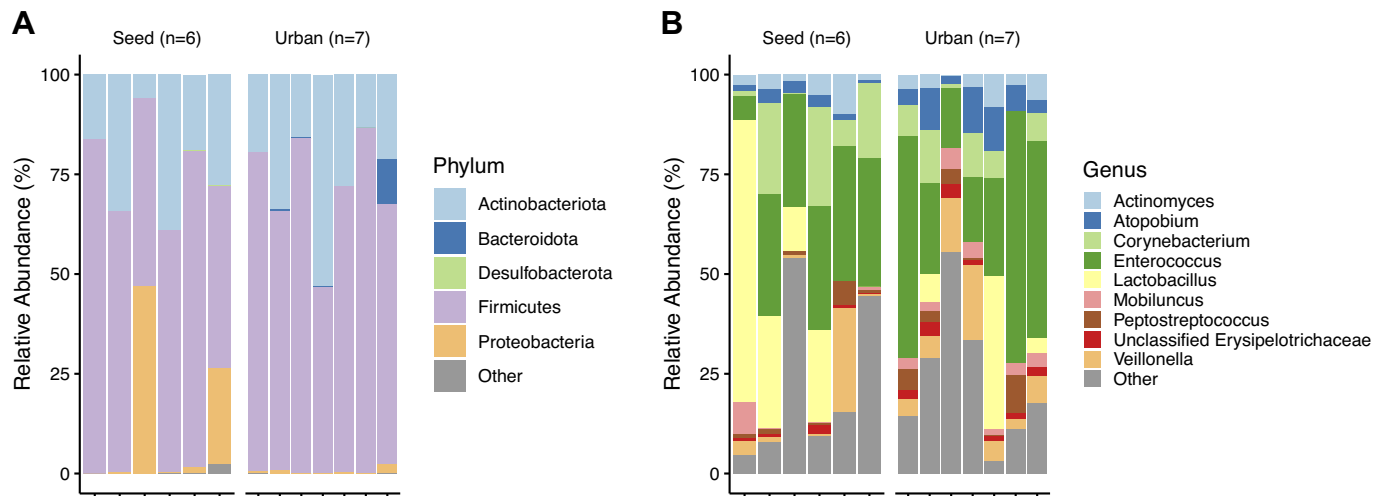


Figure 3. A: the relative abundance (%) of bacterial phyla in microbiome samples from the contents of the large intestine in mourning doves separated by Seed and Urban diet groups. Each vertical bar represents a separate individual sample. Phyla with a median relative abundance of less than 1% are collapsed into the category "Other." B: the relative abundance (%) of bacterial genera in microbiome samples from the contents of the large intestine in mourning doves separated by Seed and Urban diet groups. Each vertical bar represents a separate individual sample. Genera with a median relative abundance of less than 1% are collapsed into the category "Other."

pathway enrichment analyses yielded numerous pathways per plasma and tissue samples (plasma: $n = 49$; pectoralis muscle: $n = 50$; liver: $n = 44$; kidney: $n = 54$; gastrocnemius muscle: $n = 47$; adipose: $n = 45$). Similarly, although nine pathways were initially significantly altered between diet groups, after adjusting for multiple hypothesis testing, only three pathways within pectoralis muscle remained significantly downregulated in the urban diet group (Table 2; Supplemental Fig. S3).

Plasma LPS Was Elevated in Seed-Fed Compared with Urban-Fed Mourning Doves

Plasma LPS concentrations were significantly higher in the Seed diet group compared with the Urban diet group (51.01 ± 1.18 ng/L vs. 45.11 ± 1.02 ng/L; t test: $P = 0.003$; Table 3). No significance was detected for the tested inflammatory markers ($P \geq 0.161$). Note, IFN α , IL-21, MIP-1 β , and

MIP-3 α were not detected in many of the plasma samples and were therefore not included in the analysis. Furthermore, one animal in the Seed group displayed outlying values for nearly all of the inflammatory markers (Z -scores ≥ 2.5) and was removed from the analysis.

DISCUSSION

Urbanization influences food quality and availability for many wild species. In the current study, we hypothesized that such a diet could significantly influence the GM, host metabolome, and circulating LPS and inflammatory markers in urban mourning doves as many avian species have increased access to human refuse and food subsidies in urban areas. The GM of doves in the Urban and Seed diet groups showed significant dissimilarity in terms of microbial composition and significant differences in microbial

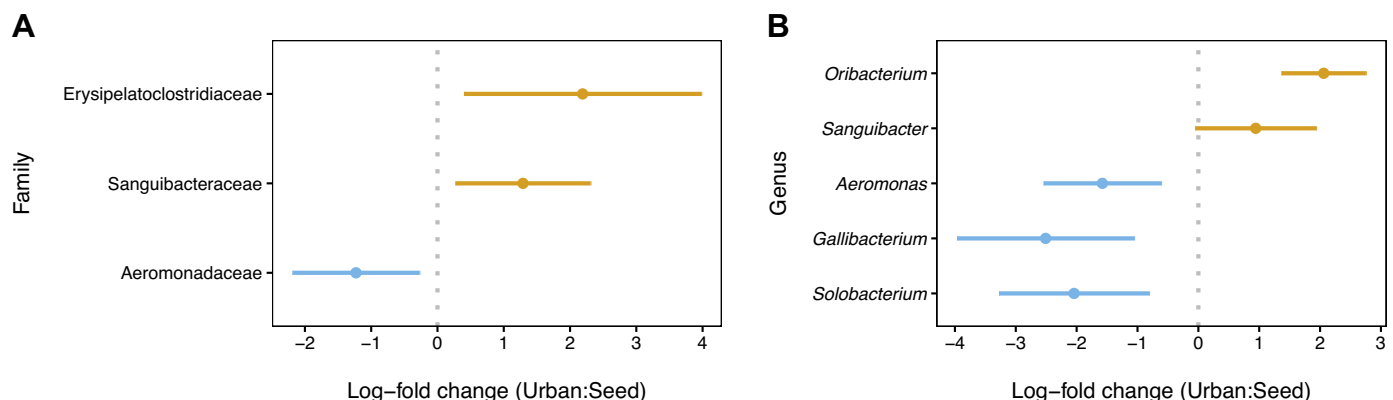


Figure 4. A: differentially abundant features at the family level in the microbiome samples from the contents of the large intestine of mourning doves in Seed ($n = 6$) and Urban ($n = 7$) diet groups. B: differentially abundant features at the genus level in the microbiome samples from the contents of the large intestine of mourning doves in Seed ($n = 6$) and Urban ($n = 7$) diet groups. Points represent the log-fold-change (effect size) of individual bacterial features with only those with significant effect sizes ($q < 0.05$) are shown. A positive log-fold-change indicates that a feature is more abundant in individuals in the Urban diet group (orange), and a negative log-fold-change indicates a higher abundance in individuals in the Seed diet group (Blue). Bars represent standard error derived from the ANCOMBC model.

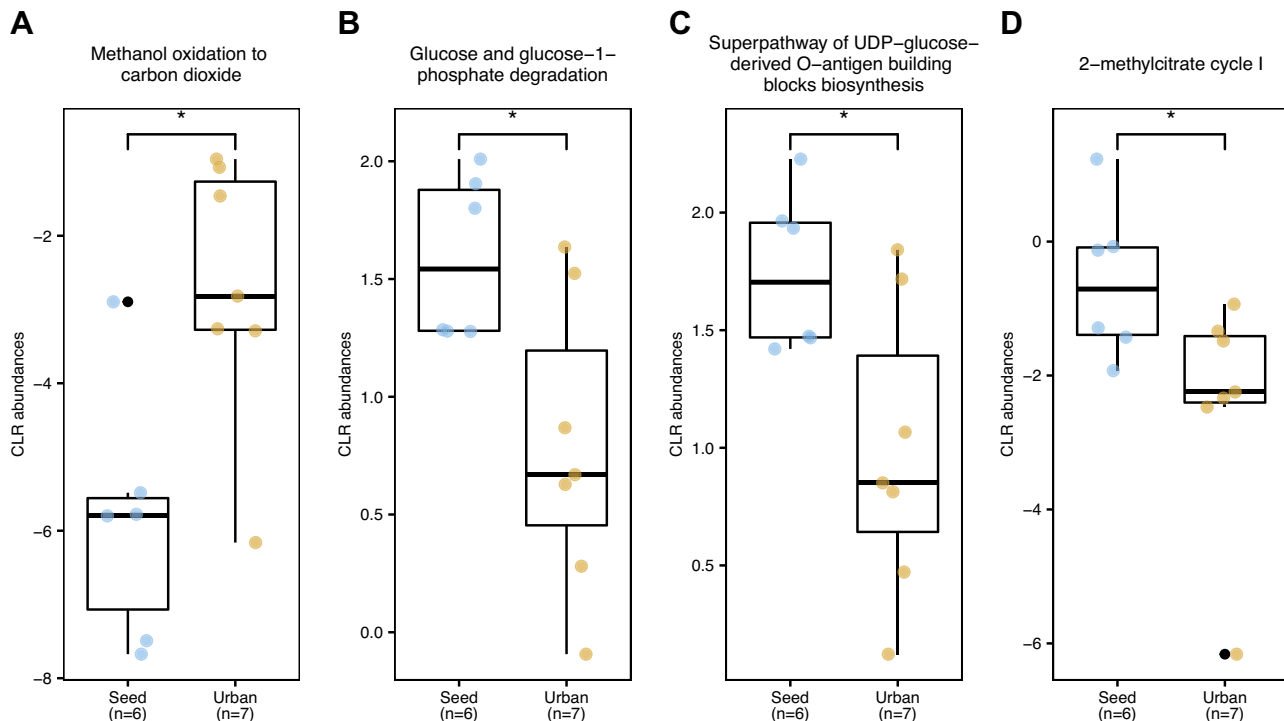


Figure 5. Boxplots displaying statistically significant differences in predicted metabolic pathways in the microbiome samples from the contents of the large intestine of mourning doves from the Seed and Urban diet groups: methanol oxidation to carbon dioxide (A), glucose and glucose-1-phosphate degradation (B), superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis (C), and 2-methylcitrate cycle I (D). Pathway abundances were transformed by centered log-ratio (CLR) and statistical testing was performed via Mann–Whitney *U* tests with a Bonferroni multiple-comparison correction. Boxes denote the interquartile range (IQR) between the first and third quartiles, and the horizontal line defines the median. **q* value < 0.05.

abundance at the family and genus level. Moreover, and in contrast to our hypothesis, the Seed-fed doves had a significantly greater concentration of circulating LPS, though there were no differences in inflammatory markers between groups. These differences between the diet models implemented in this investigation suggest an urbanized versus a seed diet can promote differential shifts in the community profile and dynamics of the GM. Though, whether these differences could potentially impact other nutritional physiology outcomes were less apparent as a comprehensive survey of the metabolomes from plasma and tissue samples revealed a single significantly altered metabolite and three pathways, all within the pectoralis muscle samples.

Previous work from our laboratory examining the GM in house sparrows from rural and urban environments reported urban animals were more enriched with class- and order-level microbes from the phylum Proteobacteria, which are implicated in several mammalian intestinal and extraintestinal diseases (23, 45). In the present work, we did not note significant differences at these higher phylogenetic levels, instead observing more subtle differences at the family and genus level between the Seed- and Urban-fed doves. This discordant finding between studies is of note and could be related to differences in the species, environmental exposure (experimental diet and laboratory housing vs. wild-caught), and the inclusion of only urban-caught doves in the present study. Indeed, the house sparrows from the previous study presumably had much longer exposure to their respective environments compared with the short-duration experimental diets in the present work. Moreover, the mourning doves

used in this study were captured from an urban environment, and it is likely the diet provided to the birds in the Seed group was a greater departure from their normal nutritional intake compared with the mixed diet provided to the birds in the Urban group. Another consideration and important feature of the avian GM is the large variation that has been noted over multiple species (31, 46). It may be that birds are more apt to acquire microbes based on their environment and this transfer may be even more variable in birds of flight as they potentially have greater exposure to different environments (46). Although we acquired mourning doves used in the current study at a confined location, central to a large urban environment, it is likely they were exposed to disparate conditions before being captured. Therefore, future work should implement longer dietary intervention periods and, importantly, dietary change with ABA study designs [e.g., 1) seed diet to, 2) urban diet, 3) to seed diet, and vice versa] to observe if potential shifts in the GM alter and/or diminish.

Overall, diet-focused GM research indicates the gut microbes in adult animals are resilient, both in wild and controlled conditions (47–49). However, as Bodawatta and colleagues recently highlighted, the avian GM does appear to possess a greater degree of malleability to environmental and dietary changes, as compared with other species (50). The avian GM is also of particular interest due to the unique physiology of these animals. In the case of the doves used in our study, the capability of powered flight likely plays a large role in the contents and assembly of their GM. However, Song et al. (2020) recently assessed the

Table 1. Altered plasma and tissue metabolites between diet groups

	P Value	q Value (FDR)	Fold-Change Urban/Seed	
Plasma				
Acrylamide	0.001*	0.220	0.54	Down
1-Methylhistidine	0.008*	0.609	0.64	Down
Epinephrine	0.009	0.609	0.68	Down
Pantothenic acid	0.019	0.883	1.61	Up
3-Hydroxybutyric acid	0.029	1.000	0.72	Down
Choline	0.034	1.000	1.50	Up
Pectoralis muscle				
Myoinositol	<0.001	0.017	0.80	Down
Leucic acid	0.016	0.822	0.62	Down
Malate	0.036	0.822	1.27	Up
Liver				
Norvaline	0.005*	0.511	0.69	Down
Benzoic acid	0.008	0.511	0.79	Down
Valine	0.008*	0.511	0.69	Down
Proline	0.014*	0.658	0.77	Down
Homoserine/threonine	0.034	0.955	0.77	Down
Threonine	0.034	0.955	0.77	Down
Isoleucine	0.045	0.955	0.88	Down
Muconic acid	0.046	0.955	0.41	Down
TMAO	0.049	0.955	1.33	Up
Kidney				
2-deoxyadenosine	0.001	0.212	1.24	Up
Allopurinol	0.019	0.929	1.41	Up
Urocanic acid	0.027	0.929	2.12	Up
Phenylacetic acid	0.034	0.929	1.11	Up
F6P/G6P	0.035*	0.929	2.03	Up
L-(−)-arabitol	0.035*	0.929	0.96	Down
Succinate	0.035*	0.929	1.19	Up
Uridine	0.041	0.929	0.67	Down
Gastrocnemius muscle				
2-Hydroxybenzoic acid	0.018	0.884	0.84	Down
Citrate	0.026	0.884	0.64	Down
Betaine	0.035*	0.884	0.88	Down
Adipose				
4-Aminobutyric acid	0.001	0.224	0.51	Down
Carnitine	0.007	0.663	0.61	Down
Suberic acid	0.020	1.000	0.60	Down

*P value determined via Wilcoxon Mann–Whitney *U* test; bold values are $P < 0.05$.

gut microorganisms of ~900 vertebrate species, including 315 mammals and 491 birds, and reported that bird's GMs are only weakly correlated with host phylogeny and not correlated with host diet (31). Whereas mammals have a strong correlation to both diet and phylogeny. Song and colleagues speculated that the lack of correlation between

Table 3. Plasma markers related to inflammation between groups

Marker	Urban Diet	Seed Diet	P Value
LPS, ng/dL	45.11 ± 1.01	51.01 ± 1.18	0.003
IFN γ , pg/mL	19.66 ± 12.01	7.24 ± 11.66	0.893
IL-2, pg/mL	1548.24 ± 200.61	1553.41 ± 175.58	0.989
IL-6, pg/mL	63.10 ± 41.34	19.45 ± 14.63	0.161
IL-10, pg/mL	11.28 ± 9.75	1.06 ± 1.05	0.283
IL-16, pg/mL	6.96 ± 2.08	4.29 ± 1.73	0.256
M-CSF, pg/mL	3.66 ± 2.55	1.46 ± 0.95	0.329
RANTES, pg/mL	5.55 ± 2.93	7.98 ± 3.83	0.620
VEGF, pg/mL	3.66 ± 2.54	1.45 ± 0.95	0.322

Data displayed as means ± SE; bold values are $P < 0.05$. IFN γ , interferon- γ ; IL, interleukin; LPS, plasma lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; RANTES, regulated on activation, normal T cell expressed and secreted; VEGF, vascular endothelial growth factor.

host factors and microbial composition in birds may be partly a consequence of an overall lower level of specificity between microbial and host taxa than is found in mammals. This may be related to flight, as birds' intestinal tracts are much shorter than those of comparably sized land mammals and carry far fewer bacteria, reducing the burden of holding extra weight (31). There is also the possibility that their digestive tracts are not providing the necessary food to bacteria to maintain the symbiotic (mutually beneficial) exchange that would make living there beneficial to bacteria. Although these findings partly align with our nonsignificant findings for microbial richness between groups, null findings do not appear to be the rule as previous observational research has implicated diet as a potential modulator of the avian GM, including animals from rural and urban environments (23, 25, 26, 28). More recent experimental work with house sparrows found that birds fed urban or rural-modeled diets for 6 wk significantly altered the diversity and taxonomic composition of the GM (27).

In the present study, we noted the GM from Urban-fed doves was significantly more abundant in Erysipelatoclostridiaceae and Sanguibacteraceae at the family level and *Oribacterium* and *Sanguibacter* at the genus level. Of note, Erysipelatoclostridiaceae is a butyrate-producing family of bacteria and has been reported to be enriched in Pekin ducks fed diets with high levels of dietary fiber (51). The genus *Oribacterium*, which is also from a butyrate-producing family (Lachnospiraceae),

Table 2. Altered metabolic pathways between diet groups

	Matched Compounds	P Value	Holm Adj. P Value	Impact
Plasma				
Pantothenate and CoA biosynthesis	3/19	0.020	0.988	0.025
Synthesis and degradation of ketone bodies	1/5	0.029	1.000	0.000
Glycerophospholipid metabolism	1/35	0.034	1.000	0.030
Pectoralis muscle				
Inositol phosphate metabolism	1/30	<0.001	0.007	0.080
Phosphatidylinositol signaling system	1/28	<0.001	0.007	0.024
Ascorbate and aldarate metabolism	1/10	<0.001	0.007	0.000
Liver				
Valine, leucine, and isoleucine biosynthesis	5/8	0.039	1.000	0.000
Adipose				
Butanoate metabolism	4/15	0.012	0.533	0.032
Purine metabolism	5/62	0.049	1.000	0.036

Kidney and gastrocnemius muscle revealed no significant pathways; bold values are $P < 0.05$.

has been reported to be associated with obesity in humans (52). The other genus found to be more abundant in the Urban-fed group, *Sanguibacter*, was found to be among the dominant genera in wintering black-necked cranes (*Grus nigricollis*) (53). Increased abundance of these taxa is suggestive of greater energy extraction potential by the GM, though this was not reflected in physiological data previously described (e.g., plasma glucose and triglyceride concentration) (24). In comparison, the Seed diet group had greater abundance of the family, Aeromonadaceae, and genera, *Aeromonas*, *Gallibacterium*, and *Solobacterium*. The genus, *Aeromonas*, often found in aquatic environments and in soil, comprises mostly pathogenic species (54) and has been detected in several birds of flight, particularly in seabirds (55, 56). As part of the family, Aeromonadaceae, these bacteria are Gram-negative and contain LPS on their surface (54). Similarly, the Gram-negative *Gallibacterium* has been found to contain species that are pathogenic to chickens (e.g., *Gallibacterium anatis*) (57) and has been proposed to be an indicator of microbiome dysbiosis in songbirds (58). Overall, reconciling and understanding the differential findings between diet groups is difficult with the nonsignificant physiological data. In addition, the composition of the GMs between diet groups only showed modest separation. It may be that many of these microbes are symbionts or regular inhabitants in the GI tract of urban mourning doves. To better understand these differences, longer intervention durations are needed, as well as ABA study designs as noted previously. Such designs may reveal the observed differences in abundances at the family and genus levels are more transitory and therefore influence the host to a very small degree. When compared with mammals, the avian GM appears to have less complexity and specificity (31). Ultimately, these traits may make the avian GM more sensitive to changes in bacterial abundance and more apt to harbor transitory taxa (31, 59, 60). Regardless, our findings are of interest considering birds in built environments subsisting off urban-type foods may carry differential abundance of specific microbes compared with their rural counterparts.

Contrary to our initial hypothesis, the doves fed the Seed diet had significantly greater levels of circulating LPS in comparison to the Urban diet birds. Although a relatively unexplored area in avian research, prior work in goslings supported our original hypothesis as a relationship was found with increased circulating LPS from so-called dysbiosis and intestinal permeability and kidney injury (61). An important consideration with the birds in the present study may be related to prior environmental exposure. Although we did have a 2-wk control period before the animals were randomized into their respective diets, further work may consider longer control periods. Regardless, we did not find an increase in inflammation that might be expected with increased circulating endotoxin. The reason for this is unclear, however, the GM of doves in the Seed diet did display increased abundance of several Gram-negative taxa and the predicted metabolic pathway, “superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis,” which forms specific sugar residues used by several taxa for incorporation in the repeated subunit O-antigen in the LPS structure (62). Another notable predicted pathway was “glucose and glucose-1-phosphate degradation,” suggestive of an enhanced ability to utilize sugars and their modes of

utilization. In comparison, the Urban group displayed greater abundance in the “methanol oxidation to carbon dioxide” pathway. This finding was of note as the GM is now recognized as an important source of metabolic methanol (63). The water-soluble fiber pectin, found in fruits and vegetables (including potatoes), is degraded by the gut microbes to produce methanol (63). Although this analysis offers an exploration of the differences in potential functionalities of these two diet types, the data are inherently predictive. Moreover, although significant, the number of significant pathways were quite small and should be interpreted with caution.

With the exception of pectoralis muscle samples, metabolomics analyses revealed no significant differences in individual metabolites or pathways in plasma or several tissue samples (liver, kidney, adipose, and gastrocnemius muscle) between diet groups following multiple hypothesis testing. Of note, the metabolite myoinositol was decreased in pectoralis muscles from doves fed the Urban diet as compared with the seed diet. Myoinositol is involved in numerous metabolic pathways in poultry (lipid signaling, osmolarity, glucose, and insulin metabolism) and thus, it is possible that the decrease of myoinositol observed in the Urban diet pectoralis muscle could affect glucose metabolism in the muscle (64). Since seeds are a rich source of dietary myoinositol (65), the observed decrease in muscle myoinositol could be related to the decreased proportion of seeds provided in the Urban diet. Similarly, the pathway enrichment analyses revealed only three low-impact pathways that were downregulated in pectoralis muscles from doves fed the Urban diet as compared with birds fed the seed diet. Moreover, since all three pathways only had one metabolite matched to them for the analyses, which was also the only altered metabolite (myoinositol), this further demonstrates that the main metabolite altered by the Urban diet is myoinositol and its associated pathways in pectoralis muscles. The minimal differences observed in the metabolites align with the initial results from this study (24), where body mass and numerous nutritional physiology markers were likewise not altered between diet groups.

Perspectives and Significance

In summary, data generated from this study show a diet modeled after what birds in urban environments plausibly consume significantly alters the community of microbes harbored in the large intestine. The Urban diet fed doves had greater abundance of potentially energy harvesting taxa; however, alterations as a result of this were not reflected in metabolomic analyses from several tissues, in addition to circulating LPS and inflammatory markers. Indeed, contrary to our hypothesis, doves fed the diet of seeds had a significantly greater concentration of circulating LPS in comparison to the Urban diet fed doves. This may have been due to prior environmental exposures and deserves future investigation as this elevation did not appear to negatively impact the health of these animals. In addition, metabolites and metabolite-produced metabolic pathway analysis revealed largely no difference between diet groups, with the exception of decreased pectoralis myoinositol in the doves fed the Urban diet. Although our analysis was limited by a smaller

sample size and study length, our research supports the notion that the microbial composition in doves is impacted by diet, though may only weakly associate with host physiology.

DATA AVAILABILITY

Raw 16S rRNA gene sequencing data for all samples have been deposited in the open-source repository, Sequence Read Archive (SRA), under the NCBI BioProject number, PRJNA849645 (<https://www.ncbi.nlm.nih.gov/sra>).

SUPPLEMENTAL DATA

Supplemental Figs. S1 and S2 and Supplemental Tables S1–S3: <https://doi.org/10.6084/m9.figshare.20084090>.

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DISCLOSURES

A.E.M. is employed by Isagenix International LLC. All authors have no financial interests regarding the content of this manuscript. None of other authors have any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

A.E.M., A.J.B., and K.L.S. conceived and designed research; A.E.M., A.J.B., and K.L.S. performed experiments; A.E.M. and K.L.S. analyzed data; A.E.M., A.J.B., and K.L.S. interpreted results of experiments; A.E.M. and A.J.B. prepared figures; A.E.M. and K.L.S. drafted manuscript; A.E.M., A.J.B., and K.L.S. edited and revised manuscript; A.E.M., A.J.B., and K.L.S. approved final version of manuscript.

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