CENTER for PRODUCE SAFETY

CPS 2020 RFP
FINAL PROJECT REPORT

Project Title
Understanding and predicting food safety risks posed by wild birds

Project Period
January 1, 2021 – December 31, 2022 (extended to February 28, 2023)

Principal Investigator
Nikki Shariat, PhD
University of Georgia
Department of Population Health, College of Veterinary Medicine
953 College Station Road
Athens, GA 30602
T: 415-640-6638
E: nikki.shariat@uga.edu

Co-Principal Investigators
William Snyder, PhD
University of Georgia
Department of Entomology
Athens, GA 30602
T: 706-542-5259
E: wesnyder@uga.edu

Laurel Dunn, PhD
University of Georgia
Department of Food Science and Technology
Athens, GA 30602
T: 706-542-0993
E: laurel.dunn@uga.edu

Objectives

1. *Assess the risk posed by wild bird feces on fresh produce plants, and the influence of proximal animal agriculture on pathogen presence in wild bird feces.*
2. *Determine the diversity of Campylobacter and Salmonella in wild bird feces and perform fine-scale tracking and source attribution using whole genome sequencing.*

Funding for this project was provided partly through the CPS Campaign for Research.

FINAL REPORT

Abstract

Wild birds can be a difficult food safety risk to manage due to their ability to travel over large distances and to avoid traditional wildlife mitigation strategies such as fences. Birds often use agricultural fields and structures as foraging, breeding, and nesting areas, which can lead to defecation on crops and transfer of foodborne pathogens. To better understand the food safety risk associated with these events, wild bird feces were collected from produce fields across the Southeast during the growing seasons in 2021 and 2022. These feces were analyzed for the detection of bird species and cultured for *Salmonella* and *Campylobacter* to identify overall and species-associated risk. A total of 773 fecal samples were collected from 45 farms across Tennessee, Georgia, South Carolina, and Florida, where 2.1% (n=16) samples were positive for *Salmonella* and all were from moist feces. No *Campylobacter* were identified in this study. From these positive samples, 10 contained multi-serovar *Salmonella* populations, including 13 total serovars and six in the CDC top 10 serovars of human concern. PCR identified an additional 59 *Salmonella*-positive feces samples and no further *Campylobacter*-positive feces. Bird species were counted using both physical and molecular identification, identifying 53 bird species in total. Birds associated with lower natural land use and higher animal agriculture land use were more commonly identified defecating in the fields. Overall, there was a low prevalence of pathogens in fecal samples, especially when feces had dried. Wild birds were not shown to play a large role in the spread of these pathogens; however, when *Salmonella* was identified, the populations often contained clinically relevant serovars. Therefore, growers should treat all feces as if they contain *Salmonella*. Additionally, growers should be aware of structures in and around produce fields that provide an area for birds to forage or nest, so that necessary mitigation can take place when significant bird intrusion occurs.

Background

Wildlife poses a risk to food safety in fresh produce by serving as vectors for pathogens from animal agriculture and the environment. Food safety risks posed by wild birds are especially challenging to mitigate, as birds can easily fly over fences that will block most other wildlife. There have been several CPS-funded studies investigating the food safety impact of wild birds (1–5) and risk mitigation strategies (6, 7). In the context of animal agriculture, pathogen transmission from cattle was most considered in those studies, and *Salmonella* and *E. coli* O157:H7 were the main pathogens. Our group recently completed a study that examined broccoli fields along the West Coast and found a significant increase in *Campylobacter* in wild bird feces on produce when the farms were in close proximity to livestock, as opposed to being embedded in natural habitats (8). In that study, 13.1% of 1217 feces samples collected from in or near broccoli rows were positive for *Campylobacter*. Molecular sequencing to define bird species determined that species carrying *Campylobacter* were most often associated with livestock operations, such as starlings and house sparrows (8). Altogether, our initial findings suggest that wild bird–associated food safety risks require three interacting links: (i) concentrated animal agriculture operations that serve as pathogen reservoirs at the landscape scale; (ii) intensively farmed landscapes that foster large populations of livestock-associated birds that move between livestock and produce fields; and (iii) simple on-farm habitats that draw invasive birds to a particular field (9). It is unknown how long pathogens deposited in bird feces can persist once delivered onto foliage.

The current study was conducted in the Southeast, where the value of fresh produce and tree nuts exceeds $4 billion (10). Food safety hazards, including those posed by wildlife and avian species, are a legitimate concern to the produce industry. Climate conditions in the Southeast (e.g., high humidity) differ dramatically from those in California and Arizona where most previous wild-bird studies have been done. This increased moisture could lengthen pathogen survival. Another difference between the West Coast and the Southeast is that animal agriculture in the Southeast is dominated by broiler production. Georgia alone produces 1.36 billion broilers a year (11). While there are a large number of cow-calf operations in the Southeast, there are few commercial beef feedlots.

To predict the risk associated with wild bird feces deposited on produce fields in the Southeast, the following objectives were established:

(i) Evaluate the prevalence of *Salmonella* and *Campylobacter* from wild bird feces and identify associations between bacterial prevalence and landscape features (i.e., animal agriculture) or bird species.
(ii) Use deep-serotyping to determine population diversity of *Salmonella* and *Campylobacter* along with whole genome sequencing to identify the source attribution of the recovered isolates from Objective 1.

Research Methods and Results

Objective 1. Evaluate the prevalence of *Salmonella* and *Campylobacter* from wild bird feces and identify associations between bacterial prevalence and landscape features (i.e., animal agriculture) or bird species.

Site selection and sample collection. Produce fields were selected throughout Tennessee, Georgia, Florida, and South Carolina to reflect a wide range of environments and native bird species in the Southeast. These fields included both small, family-owned fields as well as large commercial fields. To best measure the effect of seasonality on the prevalence of *Salmonella*, farms were repeatedly sampled, up to three times per summer. Fecal samples were collected between sunrise and 11 am to capture on-field bird activity while also limiting UV exposure and reducing the opportunity for desiccation (12). Upon arriving at a farm, sampling was conducted around the perimeter of each field, followed by a step-wise sampling through the interior of the field where possible. When a fecal sample was identified, the leaf containing the sample was removed, inserted into recovery media, and placed on ice until culturing could take place (within 24 hours). The fecal sample was visually scored for moisture as either 1 (moist) or 0 (dry) as an indicator of freshness. To test for transmission of target pathogens from the fecal sample, a surface swab was taken both from a piece of produce below the sample and from a leaf of a neighboring plant downwind from the fecal sample. Surface swabs were taken by soaking a cotton swab in recovery media and using sterile forceps to drag it across the surface of the produce or leaf. Swabs were placed in recovery media and placed on ice until culturing. For each site, the 3-day rainfall, wind, humidity, and high temperature values were determined using the closest USGS weather stations.

In two collection seasons during 2021 (May-October) and 2022 (May-September), 109 total farm visits were performed. Farms were located across the southeastern United States, including Tennessee (n=4 farms), North Georgia (n=8), South Georgia (n=20), South Carolina (n=10), and North Florida (n=3) (**Table 1**). This included 45 total farms where sites were visited between 1-6 times (average 2.4 visits/farm). Produce grown on these farms included peppers (bell, banana, and jalapeño), eggplants, cucumbers, tomatoes, squash, grapes, bananas, pole

beans, okra, and figs. Farms ranged in size from 1.6 to 233 acres and included both small or individually owned farms (range 1.6-33.3 acres), as well as large commercial farms (range 6.95-233 acres). Over the two seasons, 773 fecal samples were collected: 227 samples in the first year and 546 in the second year. In total, 43.6% (337/773) of fecal samples were scored as moist while 56.4% (436/773) were scored as dry (**Figure 1A**).

Salmonella and *Campylobacter* culturing. Fecal samples were homogenized by hand in Buffered Peptone Water (BPW) recovery media. For *Salmonella* isolation, 750uL of the homogenate was transferred into a culture tube containing BPW and incubated at 37°C for 24 hours. Then, this was sub-inoculated into Tetrathionate (TT, VWR) and Rappaport-Vassiliadis (RV, VWR) selective enrichment broths in parallel and incubated for 24 hours at 37°C before being streaked onto Xylose Lysine Tergitol-4 agar plates (XLT-4, VWR). The plates were incubated at 37°C for 24 hours and inspected for black colonies as an indicator of presumptive *Salmonella* colonies. If no H2S-positive colonies were present, the plates were re-incubated for another 24 hours. Colonies were re-streaked onto XLT-4 for isolation, if needed. *Salmonella* isolates were grown in Luria Broth (LB, VWR) where aliquots were used to make frozen glycerol stocks and for DNA isolation. For *Campylobacter* culturing, 750uL of fecal homogenate was transferred into a culture tube containing 8.5 mL of Bolton's Broth (Neogen) and incubated at 42°C for 48 hours under microaerophilic conditions, before being streaked onto Cefex agar plates (Neogen) and incubated for an additional 48 hours at 42°C. Presumptive positive colonies were identified as small, opaque colonies and were screened by PCR to isolate *Campylobacter* (8).

During the two years of the study, presumptive *Campylobacter* isolates were identified but did not confirm as *Campylobacter*. *Salmonella* was identified in 16 samples (16/773 total samples; 2.1%); 15 were identified in the first year of collection (15/227; 6.6%) and one was identified in the second year (1/546; 0.2%). All 16 *Salmonella* samples were identified in moist fecal samples (**Figure 1B**). *Salmonella*-positive samples were found in South Georgia (n=10), Florida (n=4), and North Georgia (n=2). This data shows that the risk of *Salmonella* and *Campylobacter* contamination from wild bird feces in produce environments is lower than has been observed in other studies (1–5). Importantly, there was no incidence of transmission from fecal samples to produce below the leaf with feces, nor to neighboring plants downwind. Together, our results indicate that, while *Salmonella* did not survive in drier fecal samples, feces should be handled as if they contain foodborne pathogens to prevent spread to produce.

PCR assays for *Salmonella* and *Campylobacter*. In addition to culturing viable *Salmonella* and *Campylobacter*, molecular identification was used to confirm the presence of these pathogens when viable cells were not present. The total genomic DNA was isolated from the remaining fecal homogenate used in the culturing protocols with a Promega Genome Wizard kit (Promega), with the additional step of grinding the fecal pellet with a sterile mortar and pestle to disrupt the fecal particles. The first molecular assay was an internal amplification control (IAC) used to identify the presence PCR inhibitors which could prevent molecular amplification (13). The primers for IAC PCR were IAC_F (5'-AGTTGCAGTGTAACCGTCATGT-3') and IAC_R (5'-TCGACGAGACTCTGCTGTTAAG-3') and the IAC template control sequence was IAC (5'-AGTTGCAGTGTAACCGTCATGTACCAGTAATCTGCGTCGCACGTGTGCACCTAGTCTA ATCACTTATGACTCAGATAACTTAACAGCAGAGTCTCGTCGA-3'). For each reaction, the following components were mixed into a (48uL) reaction: 39.5uL sterile water, 5uL 10x Taq Buffer, 0.5uL 10uM forward primer, 0.5uL 10uM reverse primer, 0.3uL 100mM dNTPs, and 1U Taq polymerase. To this mix, 2uL of diluted template control DNA along with 2uL of bird fecal DNA was added. PCR products were visualized by gel electrophoresis. Where there was no amplification, a 1:10 dilution of the bird fecal sample DNA was made, and the PCR repeated. To identify *Salmonella* in the fecal samples, an *invA* PCR was used (14). In this PCR, primers –

InvA_F1 (5'-AACGTGTTTCCGTGCGTAAT-3') and InvA_R1 (5'-TCCATCAAATTAGCGGAGGC-3') were mixed with the following mastermix (50uL per reaction): 38.5uL sterile water, 5uL 10x Taq Buffer, 2uL of 0.00625g/mL bovine serum agglutinate, 1uL 10uM forward primer, 1uL 10uM reverse primer, 0.25uL 100mM dNTPs, 2uL of bird fecal sample DNA, and 1U Taq polymerase. Identifying *Campylobacter* was completed similarly, where a 16S PCR was performed by mixing primers – 16S_F (5′-GGATGACACTTTTCGGAG-3′) and 16S_R (5′-AATTCCATCTGCCTCTCC-3′) – with the following mastermix (50uL per reaction): 40.5uL sterile water, 5uL 10x Taq Buffer, 1uL 10uM forward primer, 1uL 10uM reverse primer, 0.25uL 100mM dNTPs, 2uL of bird fecal sample DNA, and 1U Taq polymerase (15). Confirmed *Salmonella enterica* subspecies *enterica* serovar Enteritidis and *Campylobacter jejuni* stocks were used as positive controls for the PCRs listed above.

Previous publications have indicated wild bird feces contain PCR inhibitors (16). In this study, almost 10% (n=75) of samples contained PCR inhibitors, as shown by the IAC PCR. These inhibitors were resolved when the template was diluted 10-fold in molecular grade water, and this dilution used for all subsequent PCRs. Following analysis with the 16S PCR, no samples containing *Campylobacter* were identified. For *Salmonella,* 59 additional samples were found to be positive using the *invA* PCR, bringing the total *Salmonella*-positive samples to 75 (9.7%) (**Table 2**). Unlike the culture-isolated *Salmonella,* these additional positives were identified in both moist and dry feces; 74.6% (44/59) were from moist feces, and 25.4% (15/59) from dry feces (**Figure 1B**). A chi-squared test supported the hypothesis that *Salmonella* was more likely to occur in moist feces rather than dry ($p < 0.01$). Collectively, our data shows that *Salmonella* survives better in moist feces, but dry feces were also positive for *Salmonella* by PCR. This suggests that drying of the feces may correlate with a reduction in viable *Salmonella*.

Wild bird identification. Wild birds were identified in two ways: physical identification (i.e., point counts) of birds present around and in fields, and molecular identification from feces. Point counts were conducted at all field locations on sample days between 6 and 10 am. One point count was done for every 10 hectares (ha) of sampled field when field conditions and harvesting schedules allowed. Points on the same farm were at least 200 m apart. Points were positioned near the edge of fields to overlap with bacterial sampling areas while still capturing birds moving in and out of produce. All birds seen and heard within a 100-m radius during a 10-min period were recorded, along with distance, detection method, and habitat. Birds flying overhead were excluded unless they were a species that forages aerially (e.g., swallows), in which case a note was made that they were "aerial foraging". The same observer conducted all counts for both years of sampling. Due to adverse weather conditions, some farm locations changed from year 1 to year 2. The subset of farms that were visited at least twice (n=26) were retained for bird community analysis.

Molecular identification of wild bird species from fecal samples was completed by using 2uL of DNA isolated from fecal samples as part of a PCR to amplify the Cytochrome C Oxidase Subunit I (COI). The sequence variability of the *COI* gene between bird species enables species identification. Many *COI* PCR assays were attempted, following published protocols, but either did not yield amplicons or failed to produce quality sequences including (17–20). This PCR used the following primers: COI_F1 (5'-CGCYTWAACAYTCYGCCATCTTACC-3') and COI_R1 (5'-ATTCCTATGTAGCCGAATGGTTCTTT-3') (21) optimized with the following PCR parameters. For each reaction, the following were mixed into a 50uL reaction: 38.5uL sterile water, 5uL 10x Taq Buffer, 2uL 25mM $MgCl_2$, 1uL 10uM forward primer, 1uL 10uM reverse primer, 0.3uL 100mM dNTPs, and 1U Taq polymerase along with adding 2uL of DNA template. The mix was run on the following PCR program: Initial melting of 95°C for 4 minutes was followed by 5 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 68°C for 45 seconds. This was followed by 40 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 68°C for 45 seconds and a final 2-

minute elongation step. Appropriately sized amplicons were sequenced in the forward and reverse direction by Eton Bioscience Inc. (Research Triangle Park, NC). SeqMan (Lasergene, DNA Star) was used to assemble the forward and reverse reads into a single sequence, which was then compared to two databases: NCBI BLAST, and the Barcode of Life Database (Meiklejohn et al., 2019), with a 97% nucleotide identity threshold.

Point count data identified 859 individuals within a 100-m radius. This included 47 species, with the most common being the mourning dove (n=108) and the rock pigeon (n=107) (**Table 5**). Due to the challenges of amplifying host targets from feces, COI sequence analysis was completed on 161 (20.8%) samples. This resulted in the identification of 24 species, with the most common being the eastern bluebird (n=36), followed by the northern mockingbird (n=19) (Table 5). As the majority of samples did not yield COI data, conclusions based on the subgroup of positive samples are incomplete. We classified the birds identified in this study into three groups: Group 1 birds associated with agricultural structures (e.g., barns, fences) such as the rock pigeon (n=107), barn swallow (n=67), European starling (n=16), and house finch (n=61); Group 2 birds commonly found in-field, such as the mourning dove (n=108), chipping sparrow (n=41), eastern phoebe (n=28), and northern cardinal (n=51); and Group 3 birds more commonly found in the surroundings than in the field, such as the Carolina wren (n=38), white-eyed vireo (n=11), woodpeckers (n=11), red-shouldered hawk (n=6), and eastern towhee (n=12). These three groupings of birds can present different food safety risks based on their locations and activities, and require different management approaches (**Figure 2**). For example, birds in Group 1 may pose a higher risk of pathogen transmission to produce because they often forage near or with livestock, while birds in Group 3 pose a lower risk of pathogen transmission because they were infrequently observed interacting with produce. Developing an effective and efficient mitigation strategy largely depends on the species being targeted and the specific risks they pose.

Grower survey. To gauge growers' attitudes towards birds and common bird prevention methods used by growers in the Southeast, a 14-question anonymous survey was constructed on Qualtrics and distributed to growers via email, physical copy, and at growers' association meetings. A combination of closed and open-ended questions provided both demographic data as well as a farm-by-farm comparison of the birds identified as pest species and efficacy of different management strategies. To assess how grower concern about food safety was influenced by both farm characteristics (i.e., size, diversity, and management practices) and birds commonly observed, a series of ordinal logistic regressions were performed using the "polr" function in the R package "MASS" (23). Model assumptions (i.e., proportional odds and multicollinearity) were assessed using the "poTest" and "vif" functions in the R package "car", respectively; models that did not meet model assumptions were excluded from further testing. Models were ranked according to AIC_c, and top-competing models were identified as those within $\Delta AIC_c<2$.

Nearly half (22/48 [45.8%]) of surveyed growers indicated they used some kind of preventative measure to discourage birds. The most common type of preventative measure was reflective surfaces or mirrors (10/22 [45.5%]), followed by netting (7/22 [31.8%]) and decoy birds or predators (6/22 [27.3%]). More than half of growers who used preventative measures only used one kind (12/22 [54.5%]), although other growers used up to three. Songbirds were the most common type of bird targeted by preventive measures (8/19 [42.1%]), followed by crows (5/19 [26.3%]) and raptors (4/19 [21.1%]). Songbird species targeted were either frugivorous species (e.g., cedar waxwing, cardinal) or colonial roosting species known to colonize farm buildings (e.g., starlings, barn swallows). Survey data was combined with point counts and molecular identification of wild birds through fecal analysis (**Figure 3**) to visualize the overlap between these three sources of information. While the primary reason growers discouraged birds on their farms probably has to do with protecting crops and livestock, survey results

indicate that concern about birds as food safety hazards does play a role in growers' attitudes towards bird management. Further, growers indicate that they are spending large amounts of money attempting to manage birds, with sometimes ineffective results. Clarifying the role wild birds play in vectoring bacteria onto crops may help relieve some of the financial pressure growers face in managing wild birds on farms.

Bird community analysis. Point count data was analyzed in conjunction with landscape and livestock data to test associations between "risky" species and landscape factors. A radius of "landscape relevancy" around each farm was calculated by weighing the home range of each species (24) by the relative abundance of recorded individuals, which resulted in a "relevancy" radius of 1.61 km around each farm. Land cover (25) and livestock density (26) in a 1.61-km radius were used in subsequent statistical models. Species abundances were averaged for each farm across multiple points and sample dates. A non-metric multidimensional scaling (NMDS) with a Bray-Curtis dissimilarity matrix from the R package "vegan" (27) was used to examine how observed bird communities varied across gradients of land cover and livestock density.

The bird community NMDS had a three-axis solution (stress = 0.13). Species that were molecularly identified as defecating in fields were more closely associated with less natural landscapes and a higher density of both chicken and mammalian livestock. Species that were identified as defecating in fields were clustered around lower natural landscape values.

Landscape-bacterial prevalence analysis. Livestock were recorded as "present" or "absent" on each farm on the day of sampling. "Present" livestock were those that were directly adjacent to crops or visible from fields. Livestock included chickens, cows, horses, pigs, goats, ducks, and llamas. A series of generalized linear mixed models (GLMMs) from the R package "glmmTMB" (28) were used to examine relationships between livestock factors and proportions of landcover within 4.5 km of farms. Because *Salmonella* prevalence was significantly higher in moist than dry samples ($\chi 2 = 21.14$ [1, $n = 773$], $p < 0.01$), only moist samples were considered in these models. Visit nested inside farm was chosen as the random effect for all models; year was included as a fixed effect in all models. Continuous variables were standardized prior to analysis. Multicollinearity was assessed with the "performance" package in R, and all models not meeting assumptions were discarded. Models were assessed using AIC_c with the R package "AICcmodavg" (29). We considered "top models" as those with $\Delta AIC_c \leq 2$.

Because some of the *Salmonella* serovars identified from fecal samples are associated with open water, "% wetlands" was tested as a fixed effect along with "% open water", "% natural (wetlands, forest, scrub, grasslands)", "% developed", "% agriculture (crop + pasture)", "cow (present/absent)", "chicken (p/a)", "other livestock (p/a)", and "all livestock (p/a)".

We identified four top models (**Table 3**). Proportion (%) of wetlands was present in two of these four models, along with presence of chickens, % developed land, % agricultural land, and year. *Salmonella* prevalence was positively associated with % wetlands, presence of chickens, and % agricultural land, and negatively associated with % developed land. However, the only statistically significant factor was the year ($p < 0.05$), which fits our observational data as *Salmonella* prevalence was significantly higher in year 1 than in year 2 amongst moist samples. This might be because year 2 (2022) was hotter than year 1 (2021). Overall, the lack of association with landscape and livestock factors may also be due to the overall low prevalence of *Salmonella* recovered from fecal samples, which limits the analyses.

Objective 2. Use deep-serotyping to determine population diversity of *Salmonella* and *Campylobacter* along with whole genome sequencing to identify the source attribution of the recovered isolates from Objective 1.

Deep serotyping. To identify the populations of *Salmonella* within wild bird feces, 1mL of selective enrichment broths corresponding to positive samples were centrifuged at 14000 rpm for 3 minutes. Total genomic DNA was isolated from the resulting pellet using a Promega Genome Wizard Kit and resuspended in 200uL of molecular-grade water. A total of 2µL of this template was used in the PCR for CRISPR-SeroSeq with primers targeting the conserved direct repeat sequences within *Salmonella* CRISPR arrays (30, 31). Primers also included index sequences which facilitated multiplexed, high throughput sequencing. PCR products were purified using the Ampure system (Beckman Coulter, Indianapolis, IN) and pooled in approximate equimolar ratios. Pooled libraries were sequenced on the Illumina NextSeq platform with 150 cycles, single-end reads. A water negative-control and a positive control containing *Salmonella* serovar Enteritidis genomic DNA with a known CRISPR profile were included in the library. CRISPR-SeroSeq analyses were performed using a R script that scans sequence reads and uses BLAST to match sequence reads to a database of over 150 serovars, before writing the output directly to Excel. Serovars were called only if they contained multiple CRISPR spacers that were unique to that serovar.

Of the 14 samples that yielded data, 13 different serovars were identified (**Figure 4**). In these samples, 71% (n=10) had *Salmonella* populations that consisted of multiple serovars, with an average of 2.6 serovars per sample (range 1-7 serovars per sample). Serovars included Saintpaul (n=6), Hadar (n=5), Newport (n=4), Kentucky (n=4), Enteritidis (n=4), Braenderup (n=4), Give (n=3), Rubislaw (n=2), Heidelberg (n=1), Infantis (n=1), Muenchen (n=1), Typhimurium (n=1), and Mississippi (n=1). Serovars Newport, Enteritidis, Braenderup, Infantis, Muenchen, and Typhimurium are all included in the CDC top 10 serovars due to their common association with human illness. Additionally, serovars Hadar, Heidelberg, and Saintpaul have all been linked to human illness outbreaks associated with produce or animal products (32). Importantly, serovars Enteritidis, Infantis, and Braenderup were always outnumbered by other serovars when they were present (thinner connecting lines in Figure 4). Often, samples collected from the same sites on the same days contained similar *Salmonella* populations. For example, two of the three fecal samples collected from South Georgia farm 18 (SGA F18) had nearly identical *Salmonella* profiles (serovars Saintpaul, Rubislaw, and Give) with respect to the serovar identity and relative abundance within the sample. Interestingly. Three fecal samples across two farms in Florida (farms 1 and 2, very close together) also had similar profiles. This data indicates that individual fecal samples from wild birds typically contain multiple *Salmonella* serovars, including those that are associated with human illness. The similarity between fecal samples collected on the same or nearby farms indicated that these birds likely acquired *Salmonella* from common sources. In summary, the diversity of *Salmonella* populations in individual fecal samples was high and included serovars associated with human illness. This data shows the need to have a deeper understanding of *Salmonella* populations within wild birds to identify and subsequently mitigate sources of pathogenic serovars.

Whole genome sequencing. Total genomic DNA from *Salmonella* isolates was extracted and sequenced on an Illumina MiSeq. The sequence reads were assembled using SPAdes de-novo assembly (33) and the serovar determined using SeqSero 2.0 (34). Sequences were uploaded to Enterobase where sequence types (ST) could be predicted and used to identify related isolates. Phylogenetic relatedness was visualized through GrapeTree and SNP differences were used to identify the closest related source type.

In total, whole genome sequencing was completed on 16 isolates, resulting in eight serovars identified through SeqSero 2.0 (34). These serovars included Typhimurium (1), Kentucky (1), Hadar (5), Give (4), Muenchen (1), Newport (2), Saintpaul (1), and Mississippi (1) (**Table 4**). Using the allele profile sequence types (ST) from Enterobase, a related isolate was identified for all samples with the exception of JSBird12 (serovar Muenchen). Four serovar Hadar isolates were very closely related to each other (JSBird3-5, and JSBird10) and were

collected from farms within eight miles on two consecutive days. These isolates were related to isolates from commercial turkeys, though it should be noted that there is no commercial turkey production in northern Florida or southern Georgia. The fifth serovar Hadar isolate was more closely related to a Hadar isolated from chicken. Serovars Typhimurium and Kentucky were both isolated from the same fecal sample and both isolates are closely related to isolates from chicken. While there is chicken production in the Southeast, further research is needed to determine how birds acquired these *Salmonella* (e.g., from foraging on poultry farms, or from coming into contact with improperly treated poultry manure on produce farms, or other reasons). Serovar Newport was isolated twice, and these were very closely related to each other. Interestingly, though these two isolates most closely matched to human isolates, they were also closely related to Newport isolates collected from fresh water sources in Georgia. For serovars Give, Muenchen, Mississippi, and Saintpaul, there were significant differences between the isolates found in birds and the most closely aligned isolates in Enterobase (>23 SNPs), which limits assessment of potential sources. In summary, our data points to diverse sources of *Salmonella* in wild birds*,* rather than a single reservoir.

Microbiome analysis. A total of 773 DNA extracts were received for 16S rRNA processing. All 16S rRNA Illumina-tag PCR reactions were performed on DNA extracts per the Earth Microbiome Project protocol (35). Negative controls (molecular grade water) were processed in parallel with the samples for PCR amplification. PCR products were pooled in batches of ~200 samples each and gel purified on a 2% agarose gel using the QIAquick Gel Purification Kit (Qiagen, Frederick, Maryland, USA). Before sequencing, purified pools were quality checked using an Agilent 2100 BioAnalyzer and Agilent DNA High Sensitivity DNA kit (Agilent Technologies, Santa Clara, California, USA). The purified pools were stored at -20°C, then sequenced using an Illumina MiSeq. Raw data were processed, analyzed, and quality checked with QIIME2 (36) before forward and reverse reads were merged and chimeras removed with DADA2 (37). DADA2 was also used to assign sequences to amplicon sequence variants (ASVs) using a pre-trained Silva 132 Database (38). MAFFT (39) and FastTree (40) were used to create a rooted phylogenetic tree using representative ASVs. Additionally, a biomarker analysis was completed to identify taxonomic groups that were differentially abundant within groupings of samples (*Salmonella* Culture, *Salmonella* PCR, and No *Salmonella*) using LEfSe (41) by normalizing the ASVs with the counts per million method and a differential abundance p-value of < 0.05 and a log(LDA) score of at least 1.0.

When comparing measured continuous metadata variables (3-day rainfall, wind, humidity, and high temperature), there were no factors with a strong positive or negative (± 0.30) association to a change in alpha diversity (data not shown). *Salmonella* was not found to affect species richness when comparing "*Salmonella* culture" or "*Salmonella* PCR" to the "No *Salmonella*" group (data not shown). Within "*Salmonella*-Culture" samples, the family Enterobacteriaceae was found to be significantly enriched compared to the other two data sets (**Figure 5A-B**). Within this family, there was a significant increase in the Escherichia-Shigella genera (these cannot be separated using 16S) in the *Salmonella* culture group (data not shown). Conversely, *Enterococcaceae* and *Erwiniaceae* families were relatively reduced in the "*Salmonella*-Culture" group compared to the other two groups (data not shown) This suggests a potential relationship between *Salmonella* and other *Enterobacteriaceae* (e.g., *Escherichia-Shigella* and others) and these may support the viability of *Salmonella* within bird feces. Nonetheless, the significant enrichment of *Enterobacteriaceae* in the *Salmonella*-culture group suggests that species belonging to this family (which includes *Salmonella*) can be considered a *Salmonella* risk factor. This data also suggests that *Enterococcaceae* and *Erwiniaceae* may have a negative impact on the survival of *Salmonella* within this environment. Due to the low number of *Salmonella*-Culture positive samples (n=16), these relationships need to be further explored.

Outcomes and Accomplishments

All objectives of this project were completed. This was the first study investigating the prevalence of *Salmonella* and *Campylobacter* from bird feces in produce fields within the southeastern United States. A total of 773 fecal samples was collected from 45 farms during 109 site visits. Over the two years of these collections, *Salmonella* was cultured from 16 bird fecal samples and was not identified in any instance of transmission to neighboring produce or plants. *Campylobacter* was not identified in any fecal samples. Both *Salmonella* and *Campylobacter* prevalence were lower than expected based on previous studies on the West Coast, prompting us to compare viable culturing to PCR identification of the pathogens. A *Salmonella*-specific PCR resulted in almost 10% *Salmonella* prevalence, which more closely matched these prior studies. A *Campylobacter*-specific PCR did not identify additional positive samples.

No significant relationships were identified between *Salmonella* prevalence and more natural or developed landscapes. Bird species identified through molecular analysis were more closely associated with less natural landscapes and higher densities of animal agriculture. However, *Salmonella* prevalence was not associated with any one landscape or livestock factor. While it is therefore difficult to predict which bird species' activity or which surrounding landscapes are more likely to result in higher shedding of foodborne pathogens, some bird species are more likely to defecate on produce in less natural habitats. Identification of these "risky" bird species is helpful to growers because they can tailor deterrents to these species, saving themselves time and money and reducing the risk of pathogen transmission. It was also found that *Salmonella* was only identified in moist fecal samples, which suggests that a less moist environment reduces the viability of *Salmonella* in feces.

In addition, the relative proportions of *Salmonella* populations within individual wild bird feces were identified for the first time. In 14 samples, 13 distinct serovars were identified, including six in the CDC top 10 serovars of human concern. In almost three-quarters of positive samples, multiple serovars were found, including one sample containing seven serovars. Genetically, some *Salmonella* isolates from fecal samples were related to isolates recovered from humans, indicating the possibility that clinically relevant strains could be found in wild bird feces. Isolates closely related to animal agriculture (turkey and chicken) and environmental sources (river water) were also identified.

Finally, an additional goal was completed to investigate the microbiome of bird feces to identify associations between the presence or absence of *Salmonella* with any organisms. The *Enterobacteriaceae* family was enriched in the presence of *Salmonella* (PCR and culture), while *Enterococcaceae* and *Erwiniaceae* families were significantly reduced when viable *Salmonella* was found. Further studies can address whether these organisms play a competitive or cooperative role in the presence of *Salmonella*.

Summary of Findings and Recommendations

Key Findings –

- The overall prevalence of culturable *Salmonella* and *Campylobacter* in wild bird feces was low, and zero, respectively.
- There was no evidence of pathogen transmission from contaminated feces to produce on the same plant or the leaves of neighboring plants.
- Viable *Salmonella* was only identified in moist fecal samples.
- Molecular assays detected *Salmonella* in both moist and dry feces, with the majority found in moist samples.
- Bird species that defecated on produce plants were associated with lower natural land use and higher animal agriculture land use.
- Deep serotyping resulting in the identification of 13 serovars in 14 samples, including six in the CDC top 10 serovars of human concern.
- In almost three-quarter of positive samples, *Salmonella* was found to exist as populations of mixed serovars, including one fecal sample containing seven different serovars.
- Whole genome sequencing revealed a subset of isolates are related to isolates from a variety of sources including animal agriculture (chicken and turkey), humans, and the environment (surface water).
- Microbiome analyses showed that viable *Salmonella* was positively associated with the *Enterobacteriaceae* family and negatively associated with the *Enterococcaceae* and *Erwiniaceae* families.

Recommendations –

- While harvesting produce, care should be taken to avoid any plants or produce with feces present.
- Good Agricultural Practice (GAP) recommendations for no-harvest buffer zone distances around feces should be examined to better guide buffer distance recommendations.
- If there is significant bird intrusion, growers could consider measures to reduce wild bird presence on farms, which can include scaring (decoys, lasers, or predator sounds) and physical (netting and spikes) deterrents.
- While prevalence is low, the presence of clinically relevant *Salmonella* serovars suggests that all wild bird feces should be handled as if they contain viable pathogens.
- Growers should be aware that the use of stakes, cages, and other structures in and around produce plants can provide a space for birds to forage and may result in increased fecal deposits.
- A reduction of natural habitat may encourage bird intrusion into produce production and handling areas.

APPENDICES

Publications and Presentations

Presentations:
Dunn, L. Understanding and predicting food safety risks posed by wild birds (talk). Center for Produce Safety Research Symposium. June 2022, La Jolla, CA.

Smith, J. Do wild birds pose a risk for transmitting *Salmonella* and *Campylobacter*? (poster). Southeast Regional Fruit and Vegetable Conference. January 2022, Savannah, GA.

Smith, J. Role of wild birds in pathogen transmission and food safety (talk). Southeastern Branch-American Society for Microbiology. November 2021, Virtual.

Publications (In preparation):
Smith, J., Varriano, S., Dunn, L., Snyder, W., Shariat, N. Molecular characterization of *Salmonella* from wild bird feces on produce plants.

Varriano, S., J. Smith, L. Dunn, Z. Snipes, N. Shariat, & W.E. Snyder. Southeastern US birds pose lower food safety risk.

Dunn, L., Varriano, S. Deterring wild birds in produce. (Extension article)

Budget Summary

This project was awarded a total of $384,994 in grant funds and not all funds were spent. Total expenditures were considerably lower, partly due to finding lower prevalence of *Salmonella* and *Campylobacter* in fecal samples than expected, which meant that few samples required analysis and overall supply and sequencing expenditures were greatly reduced.

Description	Cost
Student salaries	$138,156.63
Student benefits	$1,178.93
Travel	$7,213.88
Consumables, supplies, sequencing	$56,513.27
Equipment (stomacher)	$6,157.48
Indirect cost	$11,146.88
Total	$220,367.07

Figures 1–5 and Tables 1–5 (see below)

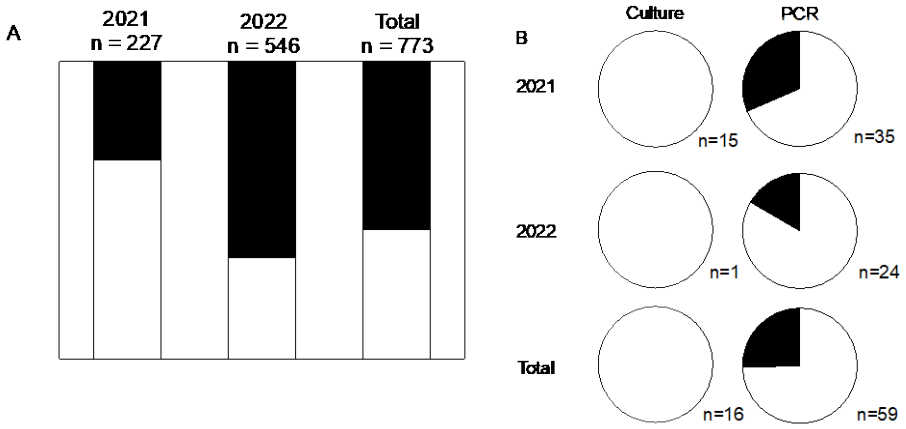

Figure 1. Moist feces support survival of *Salmonella.* (A) the distribution of moist (white) and dry (black) feces per year and in total. (B) The classification of *Salmonella*-positive samples in both culture-positive (left) and PCR-positive samples (right).

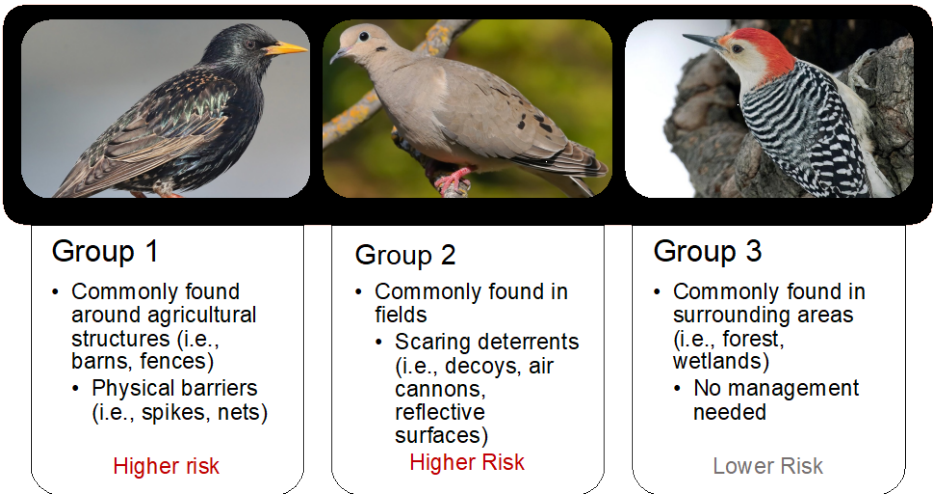

Figure 2. Birds found around and in agricultural fields pose a higher associated risk. Wild bird species can belong to one of three categories of interaction with produce plants. These categories have different levels of management necessary based on the risk members of these groups introduce.

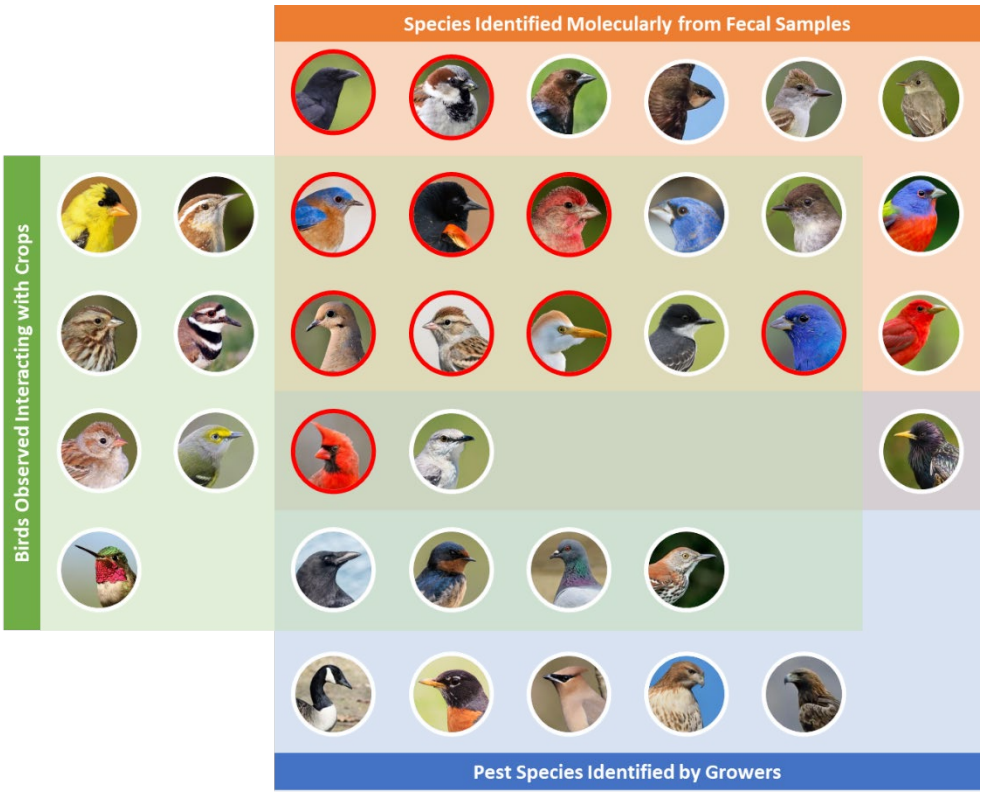

Figure 3. Bird species linked to *Salmonella* and those of grower concern do not always align. Species identified in this study are categorized into three groups: species identified through molecular analysis of fecal samples (orange), species observed interacting with crops (green), and pest species identified by grower surveys (blue). Species that were identified through molecular analysis and found in samples positive for *Salmonella* were circled in red.

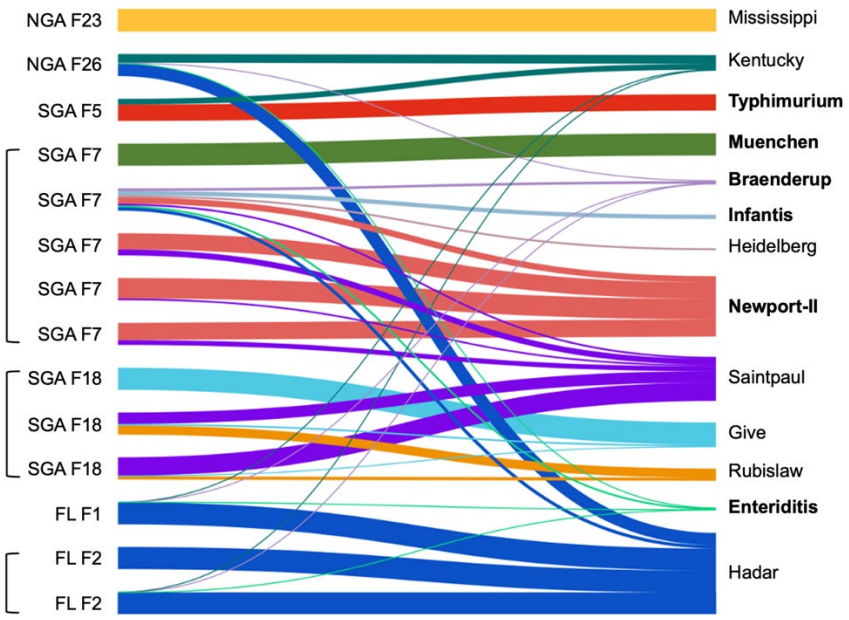

Figure 4. Multi-serovar *Salmonella* populations exist in wild bird feces. A Sankey plot showing the sample (Left, indicated by region including North Georgia (NGA), South Georgia (SGA), and Florida (FL)) and the *Salmonella* serovar population within each sample. The colored bars represent different serovars (right) and the thickness of the bars represent the relative rate of the serovar in each population, with the thicker bars indicating a larger relative rate in the sample. Bolded serovar names indicate a CDC top 10 serovar of human concern. Brackets around samples indicate that samples were collected from the same farm on the same day.

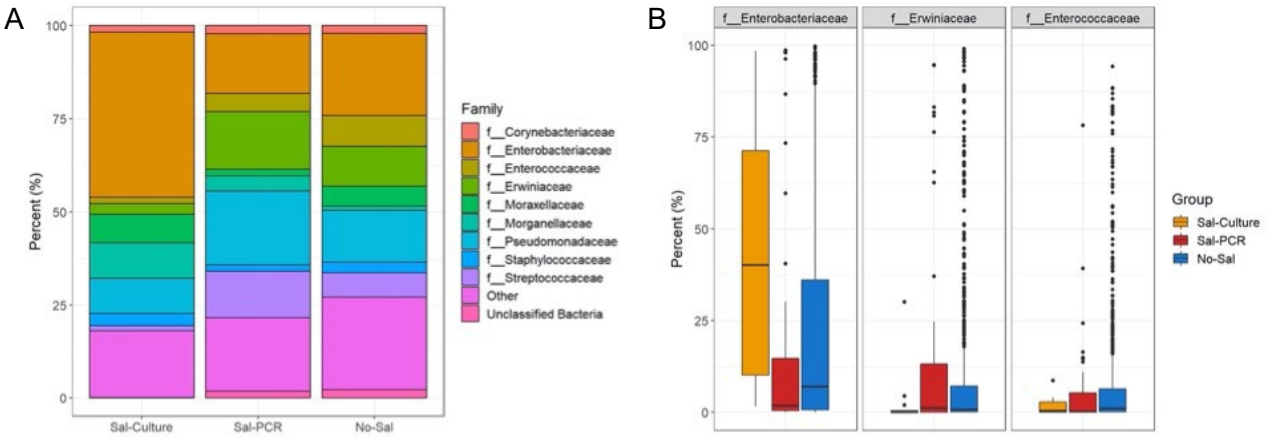

Figure 5. **16S sequencing of bird feces shows differences in composition in *Salmonella* culture positive samples.** (A) 100% bar graph of mean abundances of the 10 most prominent families identified across the entire dataset are displayed when summarized by *Salmonella* group (*Salmonella* Culture Positive, *Salmonella* PCR Positive, and No *Salmonella*). All taxa outside the top 10 taxa are classified as “other”. (B) Differential relative abundance boxplots of prominent Enterobacteriaceae, Erwiniaceae, and Enterococcaceae are displayed with significantly ($P < 0.05$) differential pairwise relationships displayed when considering the Wilcoxon Rank Sum test.

Table 1. Sampling distribution across the Southeast.

State	Number of farms (Number of visits)	Number of fecal samples collected
TN	4 (16)	218
SC	10 (29)	225
GA-N	8 (31)	235
GA-S	21 (27)	76
FL	3 (6)	19
Total	**45 (109)**	**773**

Table 2. *Salmonella* prevalence increases with inclusion of molecular detection.

Year	Fecal Samples	*Salmonella* isolated by culture (%)	Additional *Salmonella* detected by PCR (%)	Total (%)
2021	227	15 (6.6)	35 (15.4)	50 (22)
2022	546	1 (0.2)	24 (4.4)	25 (4.6)
Total	**773**	**16 (2.1)**	**59 (7.6)**	**75 (9.7)**

Table 3. Landscape use within bird radius was not shown to be a driving factor of *Salmonella* presence.

Model	Wetlands	Chicken	Developed	Ag	Year	ΔAIC_c	AIC_c Weight
Wet + Year	0.38 [0.10]				-1.056 [0.04]	0	0.147
Wet + Chick + Year	0.48 [0.06]	0.75 [0.22]			-1.04 [0.06]	0.495	0.115
Develop + Year			-0.37 [0.18]		-1.3 [0.01]	0.817	0.098
Ag + Year				0.26 [0.42]	-1.3 [0.01]	1.947	0.056

ΔAICc and Akiake weight (ω) for *Salmonella* and landscape/livestock factor models at 1.61 km scale. Only models with ΔAIC_c <2 are shown. Columns 2-5 indicate model estimates [*p* values].

Table 4. Bird *Salmonella* isolates are related to isolates from a variety of sources.

Sample ID	Serovar	Most closely related source type (SNP differences)
JSBird1	Typhimurium	Chicken (3)
JSBird2	Kentucky	Chicken (3)
JSBird3	Hadar	Turkey (2)
JSBird4	Hadar	Turkey (2)
JSBird5	Hadar	Turkey (2)
JSBird6	Give	River Water (24)
JSBird7	Give	River Water (24)
JSBird8	Give	River Water (23)
JSBird9	Give	River Water (23)
JSBird10	Hadar	Turkey (2)
JSBird11	Hadar	Chicken (5)
JSBird12	Muenchen	No Similarity
JSBird13	Newport	Human (8)
JSBird14	Saintpaul	Human (29)
JSBird15	Newport	Human (7)
JSBird16	Mississippi	Human (46)

Table 5. Bird species can be grouped through field interactions. All COI positive samples categorized into the associated bird species along with a point count data showing the occurrence of bird sightings on the farm, in the field, and *Salmonella* culture and PCR positives.

Species	On Farm Sigtings	In-field sightings	Count (COI)	*Salmonella* culture positive	*Salmonella* PCR positive
Rock pigeon	102	5	0	0	0
Barn swallow	55	12	0	0	0
House finch	53	8	11	0	1
Mourning dove	43	65	9	0	1
Northern mockingbird	36	13	19	0	0
Carolina wren	35	3	0	0	0
Northern cardinal	29	22	14	0	1
American crow	26	4	12	0	0
Chipping sparrow	26	15	9	1	2
Eastern bluebird	23	10	36	1	0
European starling	16	0	3	0	0
Eastern phoebe	14	14	5	0	1
Blue Jay	14	0	0	0	0
Eastern towhee	12	0	0	0	0
Carolina chickadee	11	0	0	0	0
White-eyed vireo	10	1	0	0	0
Black vulture	9	0	0	0	0
Song sparrow	8	23	0	0	0
Blue grosbeak	7	1	7	0	0
Red-winged blackbird	7	5	7	0	0
Chimney swift	7	0	1	0	0
Tufted titmouse	7	1	0	0	0
Field sparrow	6	3	1	0	0
Red-shouldered hawk	6	0	0	0	0
Cattle egret	5	13	1	1	0
American robin	5	0	0	0	0
Red-bellied woodpecker	5	0	0	0	0
American goldfinch	4	3	0	0	0
Downy woodpecker	4	1	0	0	0
Northern rough-winged swallow	4	0	0	0	0
Brown thrasher	3	1	0	0	0
Common grackle	3	0	0	0	0
Painted bunting	2	0	1	0	0
Europeean collared-dove	2	4	0	0	0
Common ground dove	2	2	0	0	0
Eastern meadowlark	2	2	0	0	0
Yellow-throated vireo	2	0	0	0	0
House sparrow	1	0	5	1	0
Eastern kingbird	1	3	2	0	0
Northern parula	1	0	0	0	0
Pileated woodpecker	1	0	0	0	0
Pine warbler	1	0	0	0	0
Red-tailed hawk	1	0	0	0	0
Ruby-throated hummingbird	1	0	0	0	0
Tree swallow	1	0	0	0	0
Killdeer	0	8	0	0	0
Indigo bunting	0	4	10	0	1
Brown-headed cowbird	0	0	2	0	0
Fish crow	0	0	2	2	0
Great creasted flycatcher	0	0	1	0	0
Summer tanager	0	0	1	0	0
Eastern wood pewee	0	0	1	0	0
Blue-gray gnatcatcher	0	0	1	0	0
Total	**613**	**246**	**161**	**6**	**7**

Literature Cited

1. Franklin A., Bevins S., Bisha B., Chandler J., VerCauteren K. 2016. Contamination of leafy green crops with foodborne pathogens: are wildlife a problem? Center for Produce Safety final report.

2. Gordus A., Mandrell R., Atwill R. 2011. Wildlife survey for *E. coli* O157:H7 and *Salmonella* in the central coastal counties of California. Center for Produce Safety final report.

3. Gorski L, Parker CT, Liang A, Cooley MB, Jay-Russell MT, Gordus AG, Atwill ER, Mandrell RE. 2011. Prevalence, distribution, and diversity of *Salmonella enterica* in a major produce region of California. *Appl Environ Microbiol* 77:2734–2748.

4. Jay-Russel MT, Justice-Allen A. 2014. Reducing the risk for transfer of zoonotic foodborne pathogens from domestic and wild animals to vegetable crops in the southwest desert. Center for Produce Safety final report.

5. Navarro-Gonzalez N, Wright S, Aminabadi P, Gwinn A, Suslow TV, Jay-Russell MT. 2020. Carriage and subtypes of foodborne pathogens identified in wild birds residing near agricultural lands in California: a repeated cross-sectional study. *Appl Environ Microbiol* 86:e01678-19.

6. Jay-Russell MT, Navarro-Gonzalez N., Suslow TV. 2016. Evaluation of falconry as an economically viable co-management strategy to deter nuisance birds in leafy green fields. Center for Produce Safety final report.

7. Rivadeneira P. 2017. Use of raptors to prevent wild bird and rodent intrusion into fresh produce fields. Center for Produce Safety final report.

8. Smith OM, Edworthy A, Taylor JM, Jones MS, Tormanen A, Kennedy CM, Fu Z, Latimer CE, Cornell KA, Michelotti LA, Sato C, Northfield T, Snyder WE, Owen JP. 2020. Agricultural intensification heightens food safety risks posed by wild birds. *J Appl Ecol* 57:2246-2257.

9. Smith OM, Kennedy CM, Owen JP, Northfield TD, Latimer CE, Snyder WE. 2020. Highly diversified crop–livestock farming systems reshape wild bird communities. *Ecol Appl* 30:e02031-14.

10. United Fresh Produce Association. 2020. State profiles of produce across America.

11. Commodity breakdown by state. United States Department of Agriculture -National Agriculture Statistics Service. 2022. https://quickstats.nass.usda.gov/. Accessed March 14th, 2023.

12. Fahimipour AK, Hartmann EM, Siemens A, Kline J, Levin DA, Wilson H, Betancourt-Román CM, Brown G, Fretz M, Northcutt D, Siemens KN, Huttenhower C, Green JL, Van Den Wymelenberg K. 2018. Daylight exposure modulates bacterial communities associated with household dust. *Microbiome* 6:175.

13. Rosenstraus M, Wang Z, Chang S-Y, Debonville D, Spadoro JP. 1998. An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. *J Clin Microbiol* 36:191–197.

14. Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galán JE, Ginocchio C, Curtiss R, Gyles CL. 1992. Amplification of an InvA gene sequence of *Salmonella* Typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol Cell Probes* 6:271–279.

15. Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A. 2004. Development of an extensive set of 16s rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 97:1166–1177.

16. Lurdes M, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R. 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori. Model. J Clin Microbiol* 35:995–998.

17. Hebert PDN, Cywinska A, Ball SL, DeWaard JR. 2003. Biological identifications through DNA barcodes. *Proc R Soc Lond B Biol Sci* 270:313–321.

18. Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN. 2007. Universal primer cocktails for fish DNA barcoding. *Mol Ecol Notes* 7:544–548.

19. Kerr KCR, Stoeckle MY, Dove CJ, Weigt LA, Francis CM, Hebert PDN. 2007. Comprehensive DNA barcode coverage of North American birds. *Mol Ecol Notes* 7:535–543.

20. Joo S, Park S. 2012. Identification of bird species and their prey using DNA barcode on feces from Korean traditional village groves and forests (*Maeulsoop*). *Anim Cells Syst (Seoul)* 16:488–497.

21. Patel S, Waugh J, Millard CD, Lambert DM. 2010. Conserved primers for DNA barcoding historical and modern samples from New Zealand and Antarctic birds. *Mol Ecol Resour* 10:431–438.

22. Meiklejohn KA, Damaso N, Robertson JM. 2019. Assessment of BOLD and Genbank – their accuracy and reliability for the identification of biological materials. *Plos One* 14(6):e0217084.

23. Venables WN, Ripley BD. 2002. Modern applied statistics with S-plus, 4th Ed. Springer Press.

24. Birds of the World Online. 2022. https://birdsoftheworld.org/bow/home. Accessed March 18th, 2023.

25. National Land Cover Database. 2019. https://www.mrlc.gov/data?f%5B0%5D=category%3Aland%20cover&f%5B1%5D=region%3Aconus. Accessed January 18th, 2023.

26. National Agriculture Statistics Service. 2017 https://www.nass.usda.gov/. Accessed March 18th, 2023.

27. Oksanen J. 2010. Vegan: community ecology package. *R-Project*. 2:1-295.

28. Brooks M. 2022. Generalized linear mixed models using template model builder. *The R J* 378–400.

29. Mazerolle MJ. 2020. Aiccmodavg: model selection and multimodel inference based on (Q)AIC(C). *R J*.

30. Siceloff AT, Waltman D, Shariat NW. 2022. Regional *Salmonella* differences in United States broiler production from 2016 to 2020 and the contribution of multiserovar populations to *Salmonella* surveillance. *Appl Environ Microbiol* 88(8):e00204-22.

31. Thompson CP, Doak AN, Amirani N, Schroeder EA, Wright J, Kariyawasam S, Lamendella R, Shariat NW. 2018. High-resolution identification of multiple *Salmonella* serovars in a single sample by using CRISPR-Seroseq. *Appl Environ Microbiol* 84(21):e01859-18.

32. Centers for Disease Control and Prevention. 2023. Reports of selected *Salmonella* outbreak investigations. https://www.cdc.gov/salmonella/outbreaks.html. Accessed March 5th, 2023.

33. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. Spades: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput. Biol.* 19:455–477.

34. Zhang S, Den Bakker HC, Li S, Chen J, Dinsmore BA, Lane C, Lauer AC, Fields PI, Deng X. 2019. Seqsero2: rapid and improved *Salmonella* serotype determination using whole-genome sequencing data. *Appl Environ Microbiol* 85(23):e01746-19.

35. Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, Gilbert JA, Jansson JK, Caporaso JG, Fuhrman JA, Apprill A, Knight R. 2016. Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. *Msystems* 1:e00009-15.

36. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, *et al*. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 37:852–857.

37. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 13:581–583.

38. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 41:D590–D596.

39. Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30:772–780.

40. Price MN, Dehal PS, Arkin AP. 2010. Fasttree 2 – approximately maximum-likelihood trees for large alignments. *Plos One* 5:E9490.

41. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. 2011. Metagenomic biomarker discovery and explanation. *Genome Biol* 12:R60.