frontiers in Animal Science

ORIGINAL RESEARCH
published: 29 October 2021
doi: 10.3389/fanim.2021.761930

Salmonella Diversity Along the Farm-to-Fork Continuum of Pastured Poultry Flocks in the Southeastern United States

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Greater consumer demand for all natural, antibiotic-free poultry products has led to an increase in pastured poultry operations. Given the increased level of environmental interaction, and the potential increase in exposure to foodborne pathogens, a greater understanding of the prevalence and diversity of *Salmonella* populations inherent within pastured poultry flocks. To achieve this, 42 pastured poultry flocks from 11 farms were sampled using a farm-to-fork strategy and *Salmonella* was isolated and characterized through pre-harvest (feces, soil) to post-harvest (ceca, whole carcass rinse) to the final product (whole carcass rinse) the consumer would purchase. *Salmonella* was isolated from 353 of a total of 2,305 samples, representing an overall prevalence of 18.1%. By far the most prevalent serotype was Kentucky (72.7% of all isolates), with <16% of all *Salmonella* representing a top serotype of concern for human health according to the CDC. Even though these flocks were raised antibiotic-free, *Salmonella* isolates exhibited resistances to a variety of antibiotics, with the two most common resistances being toward tetracycline and streptomycin (68.8 and 64.4% of all isolates, respectively); however, almost 98% of the multidrug resistant isolates were serotype Kentucky. *Salmonella* prevalence and diversity (both in terms of serotypes and antibiotic resistance profiles) were related more to the farm location than to the type of sample from which the *Salmonella* was isolated from along the farm-to-fork continuum. Based on these data, while *Salmonella* prevalence was similar to that from conventional poultry operations, serotypes of lesser concern to human health (Kentucky, Indiana) tended to fill the ecological niche for *Salmonella* species throughout the farm-to-fork continuum in these pastured poultry flocks. The diversity of these *Salmonella* populations tended to be farm specific, indicating the need for more tailored intervention strategies to continue to enhance the safety of these products.

Keywords: *Salmonella*, farm-to-fork, pastured poultry, antibiotic resistance, serotyping

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Animal Nutrition, a section of the journal Frontiers in Animal Science

Received: *20 August 2021*
Accepted: *20 September 2021*
Published: *29 October 2021*

Citation:
Rothrock MJ Jr, Guard JY and Oladeinde A (2021) Salmonella Diversity Along the Farm-to-Fork Continuum of Pastured Poultry Flocks in the Southeastern United States. Front. Anim. Sci. 2:761930. doi: 10.3389/fanim.2021.761930

INTRODUCTION

Salmonella is a persistent cause of foodborne illness with potential for resurgence because it can colonize many food production environments (Silva et al., 2014; Magossi et al., 2019). Poultry and poultry products are strongly linked to ∼50% of reported cases of salmonellosis (Gu et al., 2015). Uncooked poultry meat, especially comminuted or mechanically separated meat, continues to be an

important source of *Salmonella* infections according to the USDA-Food Safety Inspection Service (USDA-FSIS) (USDA-FSIS, 2020a). From 1999 to 2008, 33% of the 621 known-source outbreaks caused by *Salmonella* were attributed to contaminated poultry products, and contaminated poultry products caused ∼336,000 illnesses, ∼5,000 hospitalizations, and ∼150 deaths annually (Batz et al., 2012). Different serotypes vary in their association with poultry and foodborne disease (Gould et al., 2013; Crim et al., 2014), and poultry products are considered a primary reservoir of salmonellosis for adults and people living in urban areas (Mughini-Gras et al., 2014).

Recently, increased demand for antibiotic-free, "natural" products has pushed consumers toward the organic food market (Dimitri and Oberholtzer, 2009; Reisch et al., 2013). This has impacted the poultry industry, where broiler meat harvested from alternative poultry farming production facilities, such as organic and pastured, have increased in demand (Van Loo et al., 2011; Rothrock et al., 2016). Organic poultry farms are characterized by farms that rear birds without the use of antibiotics and allow the birds access to the outside, while pastured poultry operations require moveable pens/housing that are moved daily to fresh pasture. The microbial safety of conventionally-raised poultry and their products throughout the entire farm-to-fork continuum has been studied in the US (Rodriguez et al., 2006; Pitesky et al., 2012), but organic/pastured production studies related to food safety issues are limited. A comparison between alternative production practices and conventional practices are problematic because of vast differences inherent in these production systems (Van Loo et al., 2012), but some recent studies have worked to statistically compare the two types of farms (Siemon et al., 2007; Alali et al., 2010; Peng et al., 2016; Kassem et al., 2017). Siemon et al. (2007) and Alali et al. (2010) both found that *Salmonella* contamination of fecal matter and bird feed was significantly lower in organic farms. However, there is no scientific consensus since others have shown no difference (Bailey and Cosby, 2005; Lund, 2006; Lestari et al., 2009), or that conventional practices yielded significantly lower *Salmonella* levels (Cui et al., 2005). Therefore, more work needs to be done to better understand the ecology of *Salmonella* in these emerging poultry management systems, especially since they have been shown to be a member of the pastured broiler core microbiome along the farm-to-fork continuum (Rothrock et al., 2019b).

An additional topic of interest is that organic and pastured poultry broilers are both reared antibiotic-free, which could potentially affect the antimicrobial resistance (AR) profile and antibiotic resistance genes (ARG) of the *Salmonella* populations along the farm-to-fork continuum. There have been conflicting results from studies comparing the abundance of AR foodborne pathogens and ARG in antibiotic-free vs. conventional broiler farms (Sapkota et al., 2011; Zhang et al., 2011; Millman et al., 2013; Garcia and Teixeira, 2017; Davis et al., 2018; Rovira et al., 2019; Pesciaroli et al., 2020). Given the recent decline in clinically important antibiotics due to the rapid appearance of resistant foodborne pathogen strains (Alanis, 2005; Smith and Coast, 2013), it is imperative that the AR potential of *Salmonella* inherent within alternative poultry production system is better studied and understood.

Therefore, the objective of this study was to better understand *Salmonella* prevalence and diversity along the pastured poultry farm-to-fork continuum, and characterize those isolates based on the AR profiles to assess the baseline *Salmonella* AR inherent within these antibiotic-free management systems. From 42 pastured broiler flocks covering eleven farms in the southeastern US, *Salmonella* was isolated from pre-harvest (feces, pasture soil) and post-harvest (ceca, end of processing and final product whole carcass rinses) samples. Isolates were serotyped and characterized using the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) protocols (CDC, 2015) to determine the AR profiles, as well as the prevalence of multidrug resistance (MDR) among these isolates. This information will provide vital *Salmonella* ecology data (prevalence, diversity, AR) from these types of alternative, antibiotic-free poultry management systems to not only help improve on-farm poultry food safety, but also the growing number of customers that are consuming these poultry products.

MATERIALS AND METHODS

Farm Sites

Forty-two flocks from eleven pastured poultry farms located in the southeastern U.S. were sampled between 2014 and 2017. The major broiler management parameters for each farm within the study can be seen in **Table 1**. All broilers flocks were considered all-natural or certified all-natural, pasture-raised using moveable pens and temporary fencing, and never had any antibiotics administered to them during their grow out, nor were they used historically on the farms.

Sample Collection

The following samples were collected along the "farm-to-fork" continuum for each flock: (i) feces, (ii) pasture soil, (iii) cecal content at processing, (iv) whole carcass rinse (WCR) directly after processing, and (v) final product WCR after the carcass has been stored at the correct temperature and correct time for each farm. All samples were collected in the field, and returned to the lab in a cooler packed in ice. Pre-harvest samples (feces and soil) were collected from the pasture where the flock was currently residing at the time of sampling, and these sampling occurred 3 times during grow-out: (i) within a few days of being placed on the pasture (Start), (ii) halfway through their time on pasture (Mid), and (iii) on the day which the flock was processed (End). At each sampling time, the pasture area was divided into five separate sections, and five subsamples in each section were pooled into a single sample for each section (five total fecal and five total soil samples were collected on each sampling day). The total volume of sample collected for each field sample was at least 25 g. For the post-harvest processing and final product samples (ceca, WCR), five pooled samples were collected for each sample type, with each pooled sample containing ceca or WCR from five carcasses.

Abbreviations: AR, antimicrobial resistant; ARG, antibiotic resistance genes; MDR, multidrug resistant; WCR, whole carcass rinse.

TABLE 1 | Comparison of the 11 antibiotic-free pastured broiler farms in this study.

Farm	Breed[a]	No. of flocks	Flock size	Multi-use farm?	Animal type	Processing[b]
A	FR	10	>500	Yes	Layers, swine, cattle, sheep	On plant (skin off)
B	FR, CC	5	<50	Yes	Layers, swine, goats	On farm (skin on)
C	FR	1	<50	No	NA	On farm (skin off)
D	FR	1	<50	No	NA	On farm (skin off)
E	FR, CC	5	50–100	Yes	Layers, swine, cattle, sheep	On farm (skin off)
H	FR	2	>500	Yes	Layers	On plant (skin off)
I	FR, CC	8	100–500	Yes	Layers, swine, goats	On plant (skin off)
J	FR, CC	2	50	Yes	Layers	On farm (skin off)
K	FR	4	100–500	Yes	Layers, cattle, goats	On farm & plant (skin off)
L	FR	2	>500	Yes	Layers, swine, cattle, sheep	On plant (skin off)
M	CC	2	50–100	Yes	Layers, swine	On farm (skin off)

[a] *FR, Freedom Ranger; CC, Cornish Cross.*

[b] *Information in parenthesis indicates if the final product retain the skin, or had it removed during processing.*

To prepare the environmental samples for homogenization, three g (feces, soil) or five ceca were combined within filtered stomacher bags (Seward Laboratories Systems, Inc., West Sussex, UK), and diluted 1:3 using 10 mmol L^{-1} phosphate-buffered saline (PBS). For the WCR, 100 ml of 10 mmol L^{-1} PBS were added to each carcass within the storage bag, and the bags were vigorously shaken for 60 s. Five WCRs were pooled into a single filtered stomaching bag, and this was repeated five times (n = 25 carcasses). No further dilution in 10 mmol L^{-1} PBS was required for the WCR samples. All samples were homogenized for 60 s and these homogenates were used for all downstream cultural isolations.

Salmonella Isolation

As a pre-enrichment step, the stomached homogenates remained in the filtered stomacher bags and incubated overnight at 35°C. Two different enrichments broths were used to isolate *Salmonella* spp. from these environmental samples: Tetrathionate (TT; Becton-Dickinson, Sparks, MD) broth and Rappaport-Vassiliadis (RV; Becton Dickinson) media. After overnight incubation at 42°C in both of these enrichment broths, one loopful from each enrichment broth was spread on two different differential media: Brilliant Green Sulfa with novobiocin (BGS; Becton Dickinson) agar and xylose lysine tergitol-4 (XLT-4; Becton Dickinson) agar. These plates were incubated overnight at 35°C, and on each plate, three *Salmonella*–like colonies per subsample were picked and confirmed using triple sugar iron agar (TSI; Becton-Dickinson) and lysine iron agar fermentation (LIA; Becton-Dickinson) using an incubation period of 18–24 h at 35°C. Final confirmation of suspect TSI/LIA isolates was performed using *Salmonella* polyvalent O antiserum agglutination (Becton-Dickinson), using manufacturer's specifications. Positive salmonellae were serogrouped using individual *Salmonella* poly O antisera for O groups A through I, following the Kauffman-White scheme (Popoff and Le Minor, 1997).

Salmonella Serotyping

All recovered *Salmonella* isolates were serotyped using the *dkgB*-linked intergenic space region (*dkgB*-ISR) PCR method (Guard et al., 2012). Single colonies for each isolate were grown in 10 ml of brain heart infusion (BHI) broth (Difco BD, Franklin Lakes, NJ) at 37°C for 16 h. Bacterial cells were pelleted in a Sorvall RC5B Plus centrifuge at 5,000 × g for 15 min in a Sorvall Super-lite SLA 600TC rotor. The DNA from all *Salmonella* isolates were extracted and purified using the PureLink Genomic DNA Mini Kit (Invitrogen, Grand Island, NY). Spectrometer readings of DNA samples were obtained using a NanoDrop 1000 (ThermoScientific, Wilmington, DE) to assure $OD_{260/280}$ ratios are >1.7 and that DNA concentrations are above 20 ng ml^{-1}. The PCR protocol and primers targeting the *dkgB*-linked ISR region (including the entire 5S ribosomal gene) have been described previously (Morales et al., 2006). To determine serotype, amplicon sequence trimmed to the aforementioned ISR region were aligned to reference sequences deposited at NCBI by DNASTAR Lasergene SeqMan Version 8.0.2 using default project assembling parameters except as follows: minimum match percentage 100, minimum sequence length 100. Only perfect matches can be used to call serotype. ISR reference sequences that define serotype have GenBank accession numbers JN105119-JN105125 and JN092293-JN092328.

Antimicrobial Sensitivity Testing

Recovered isolates were sub-cultured on Blood Agar Plates (BAP) overnight at 36 ± 1°C, twice sequentially. One to two colonies were used to inoculate five mL of demineralized water to achieve a 0.5 McFarland equivalent using the Sensititre nephelometer (ThermoScientific, TREK Diagnostics, Inc., Cleveland, OH). Following vortexing, 10 μL of the cell suspension was transferred to 11 mL of Sensititre Cation adjusted Mueller-Hinton Broth with TES, followed by thorough vortexing. Fifty microliters of the inoculum was transferred to each well of the Sensititre® NARMS Gram-Negative Format CMV3AGNF plate (Trek Diagnostic Systems). These AST plates contained varying concentrations of the following antimicrobials: cefoxitin, azithromycin, chloramphenicol, tetracycline, ceftriaxone, amoxicillin/clavulanic acid (2:1), ciprofloxacin, gentamicin, nalidixic acid, ceftiofur, sulfisoxazole, trimethoprim/sulfamethoxazole, ampicillin, and streptomycin.

TABLE 2 | *Salmonella* prevalence based on sample type or farm of origin.

	No. of + samples	No. of samples	Prevalence[a]
	Sample Type		
Feces	124	691	0.152^{B}
Soil	94	721	0.115^{B}
Ceca	27	183	0.129^{B}
Processing WCR	67	168	0.285^{A}
Final product WCR	41	189	0.178^{AB}
	Farm		
A	107	398	0.212^{AB}
B	35	220	0.137^{A}
C	3	42	0.067
D	1	44	0.022
E	45	241	0.157^{A}
H	24	86	0.218^{AB}
I	101	289	0.259^{A}
J	0	110	0.000
K	8	272	0.029^{C}
L	0	150	0.000
M	29	101	0.223^{AB}

[a]*Superscript letters indicated significantly different prevalence values based on ANOVA analyses using the Tukey's post-test at a significance level of $p < 0.05$. For the Farm data, only farms that were followed over multiple years were included in the statistical analyses.*

Plates were sealed and incubated at 36±1°C for 24 h. Quality control strains *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853 were included in susceptibility tests as controls (Clinical and Laboratory Standards Institute, 2010). The published NARMS protocol and breakpoints were used for characterization and antibiotic resistance determination for each isolate (CDC, 2015).

Statistical Analyses

A generalized linear model with a binomial distributed outcome and a log link function was used to determine if there are significant differences in *Salmonella* prevalence (presence/absence) and the presence of multidrug resistant *Salmonella* (≥ 3 antibiotics) between farms and sample types. The significance of the model was established using a likelihood ratio test (R function ANOVA with argument test set to "Chisq"). Multiple comparisons of means, i.e., regression coefficients for farms and sample types were done using the multcomp package in R. The mcp function was used to specify linear hypotheses and the glht function was used to make Tukey contrasts. Statistical analyses were performed using R (v4.0.3) and a $p < 0.05$ was used for all analyses to determine significance.

RESULTS AND DISCUSSION

Salmonella Prevalence

Overall, 353 of the 2,305 samples (18.1%) were positive for *Salmonella,* which is similar to the prevalence of *Salmonella* found in other studies focusing on pastured or free-range broiler flocks (16–31%) (Bailey and Cosby, 2005; Siemon et al., 2007; Melendez et al., 2010; Scheinberg et al., 2013; Shi et al., 2019). While this prevalence in this study is higher than what was reported for *Salmonella* from conventionally reared poultry in 2014 (3.7%) (USDA-FSIS, 2020b), several direct comparison studies have found that *Salmonella* prevalence was lower in pastured vs. conventional systems (Lestari et al., 2009; Alali et al., 2010; Van Loo et al., 2012). **Table 2** outlines the breakdown of *Salmonella* prevalence based on either sample type or farm of origin. Pre-harvest *Salmonella* prevalence (15.2 and 11.5% for feces and soil, respectively) and post-harvest cecal samples (12.9%) were significantly lower ($p < 0.001$) than processing WCR *Salmonella* prevalence (28.5%), with final product prevalence (17.8%) almost being significantly lower than processing WCR prevalence as well ($p = 0.0502$). Farm of origin had a significant effect on *Salmonella* prevalence ($p < 0.001$), with farms being categorized into 4 groups based on their *Salmonella* prevalence: >21% (A, H, I, M); 13–16% (B, E); 2–7% (C, D, K); and 0% (J, L). In general, the farms where flocks were sampled over multiple years (A, B, E, I) tended to have higher prevalence than those flock(s) sampled only within a single year (C, D, J, L), which speaks to the temporal variability of *Salmonella* on pastured farms. This variation in *Salmonella* prevalence between farms was not unexpected, considering the variability of environmental and management conditions pastured flocks are exposed to have been shown to effect pathogen concentrations, as previously reviewed (Rothrock et al., 2019a; Shi et al., 2019).

Salmonella Serotype Diversity

Sixteen isolates were not able to be recovered from frozen stocks, hence the reduced number of isolates seen between the prevalence (353) and diversity (337) analyses. In total, fifteen different serotypes were identified among the 337 *Salmonella* isolates with nine serotypes being isolated in more than one sample (**Table 3**). The dominant serotype recovered was Kentucky, representing 72.7% of all the isolates, with Indiana (9.5%) and Infantis (5.9%) representing the other most prevalent serotypes. All other recovered serotypes represented ≤ 3% of the *Salmonella* recovered throughout the study. The USDA-FSIS has identified Kentucky as the dominant *Salmonella* serotype in US poultry production consistently, with it representing an ever increasing percentage of all *Salmonella* recovered from 1998 (26.7%) to 2014 (60.1%) (USDA-FSIS, 2020b). Kentucky has also been previously shown to be the most dominant *Salmonella* serotype in pastured flocks, representing 53–95% of all serotypes recovered (Melendez et al., 2010; Rothrock et al., 2016). While there is a concern for the safety of pastured raised poultry products due to the less stringent/controlled management systems and increased environmental exposure of the flocks, it should be noted that of all the recovered *Salmonella*, only 15.7% (53/337 isolates) represented the top 32 *Salmonella* serotypes that the CDC considered of concern for human health (CDC, 2013).

When looking at serotype diversity on a sample type level (**Figure 1A**), the distribution of *Salmonella* was fairly consistent. Kentucky represented a higher percentage of the post-harvest isolates (81.2%) as compared to the pre-harvest environment

TABLE 3 | *Salmonella* serotype diversity.

	No. of *Salmonella* isolates	Serotype Prevalence[a]	CDC top 32[b]
Kentucky	245	0.7270	No
Indiana	32	0.0950	No
Infantis	20	0.0593	Yes
Enteritidis	9	0.0267	Yes
Seftenberg	7	0.0208	Yes
Braenderup/Cholareasuis	6	0.0178	Yes
Meleagridis	5	0.0148	No
Muenchen	5	0.0148	Yes
1,4,[5],12:I:-	2	0.0059	Yes
Heidelberg	1	0.0030	Yes
Javiana	1	0.0030	Yes
Mbandaka/Typhimurium	1	0.0030	Yes
Orion	1	0.0030	No
Schwarzengrund	1	0.0030	Yes
Unclassified	1	0.0030	NA

[a]*Calculated based on the No. of isolates for a given serotype divided by the total number of Salmonella isolates (337).*
[b]*Based on the CDC Atlas of Salmonella in the United States, 1968–2011.*

(68.3%). The serotype richness of *Salmonella* populations (e.g., number of serotypes recovered) decreased from the pre-harvest (10 and 7 for feces and soil, respectively) to post-harvest (5 and 8 for the ceca and processing WCR, respectively) to the final product WCR (3). It should be noted that there is a spike in *Salmonella* richness in the processing WCR, half of those serotypes represent only a single isolate for the entire study. Not only did the *Salmonella* richness decrease throughout the farm-to-fork continuum, so did the prevalence of *Salmonella* serotypes that are of the greatest concern to human health (Infantis, Enteritidis, Seftenberg, Braenderup/Cholareasuis, Muenchen, 1,2,[5],12:I:-, Javiana, Schwarzengrund, Mbandaka/Typhimurim, and Heidelberg) (CDC, 2013). While these serotypes represented 22.7% of all *Salmonella* isolated from the fecal samples, they constituted only 12.5% of the processing WCR rinse isolates and only 2.6% of the *Salmonella* recovered from the final product WCR. In fact, Infantis was the only *Salmonella* serotype of greatest concern to human health according to the CDC recovered from the final products, and represents only a single isolate from the 230 final product WCR samples in the study.

As seen in the overall *Salmonella* prevalence data described above, farm of origin had a direct effect on serotype diversity (**Figure 1B**). *Salmonella* was not recovered from flocks from two of the farms (J, L); therefore, only nine farms are analyzed here. Of the fifteen serotypes identified in this study, twelve of them were not isolated from more than one farm. While serotype Indiana was the second most prevalent *Salmonella* recovered in the study across all sample types (9.5% of isolated *Salmonella*), it was only isolated from a single farm (B), highlighting how influential farm location was on *Salmonella* diversity. The three multi-farm serotypes were Kentucky (A, C, E, H, I, M), Infantis (K, M), and Seftenberg (B, H). Kentucky was the dominant serotype on 5 of the 6 farms they were isolated from, ranging from 13.0 to 100%, while Infantis was the dominant serotype recovered from both farms it was found on (87.5 and 56.5% on farms K and M, respectively). Of interest related to the Infantis isolates is the fact they were only recovered from flocks that were sampled in the final year of the study (2017). The significance of Infantis has increased in recent years, with salmonellosis cases attributed to Infantis increased by 165.8% from 2006 to 2016 (CDC, 2018), its prevalence among *Salmonella* isolated from poultry products increased by 483.6% from 1998 to 2014 (USDA-FSIS, 2020b). Additionally, it was responsible for a multi-state, 129-case outbreak associated with raw poultry products in 2018 that resulted in 25 hospitalizations and 1 death (CDC, 2019). The emergence of Infantis in these final year flocks processed months before the multistate outbreak occurred may indicate that Infantis was present in both conventional and pastured poultry management systems simultaneously, but a greater genetic characterization of the Infantis from this study would need to be performed.

Salmonella Antibiotic Resistance Profile Diversity

While currently discontinued poultry industry, the prophylactic use antibiotics during rearing provided selective environmental pressure resulting in the proliferation of antibiotic resistance within the poultry-associated microbiome, including pathogens such as *Salmonella*. Considering all of the flocks in the present study were raised with antibiotic-free/medication-free feed and were not exposed to any exogenous sources of antibiotics pre- or post-harvest, determining the AR profiles of the recovered *Salmonella* would provide valuable baseline AR data on poultry-related *Salmonella*. Even within an antibiotic-free management system, several antibiotic resistances were expressed in the recovered *Salmonella* (**Table 4**). This was not unexpected, since previous work has shown that *S. enterica* serotypes to have low level natural resistance to several antibiotics, including tetracycline, macrolides/azithromycin, and streptomycin (Stock and Wiedemann, 2000). Around two-thirds of all *Salmonella* isolates were resistant to STR (64.4%) and TET (68.8%). Resistance toward STR and TET have been previously observed in pastured poultry management systems, ranging from <40% (Griggs et al., 2006; Alali et al., 2010) to 75–90% (Melendez et al., 2010; Rothrock et al., 2016) of the recovered *Salmonella* isolates in those studies. The next set of expressed resistances (26–27% of all isolates) comprised three antibiotic classes: b-lactam/b-lactamase inhibitors (AMO), cephems (FOX, TIO, AXO), and penicillins (AMP). While similar levels of AMO and AMP resistance have been observed in previous studies (Griggs et al., 2006; Lestari et al., 2009; Alali et al., 2010), very few described *Salmonella* isolates with resistance to any of the cephem class of antibiotics (Siemon et al., 2007; Rothrock et al., 2016). Of all of the antibiotics tested against using the gram negative NARMS panel, ciprofloxacin was the only antibiotic that no *Salmonella*

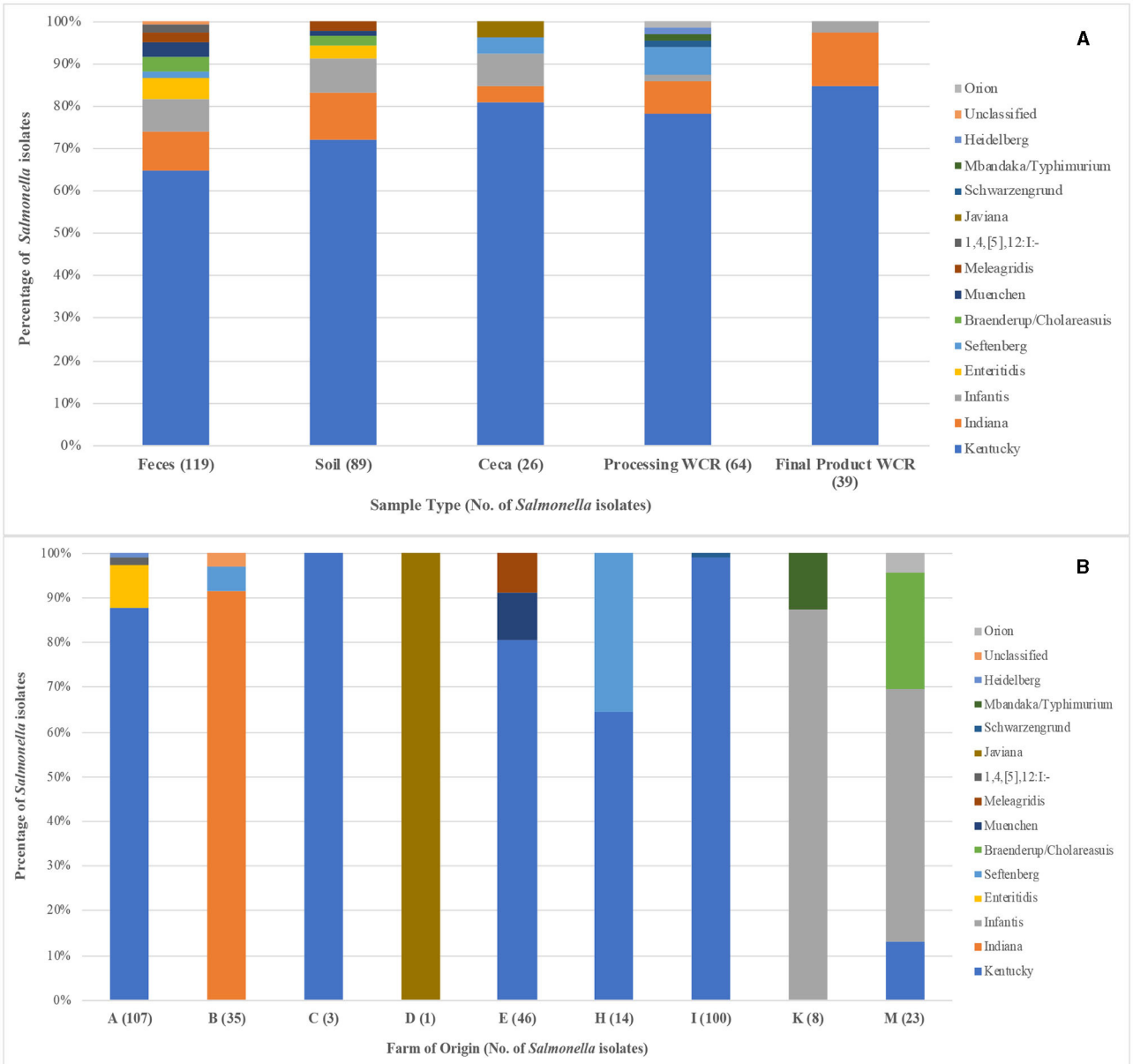

FIGURE 1 | *Salmonella* serotype diversity of the 337 isolates recovered from pastured poultry flocks as determined by the *dkgB*-ISR PCR method. **(A)** Distribution of *Salmonella* serotypes based on sample type from which the *Salmonella* was isolated. **(B)** Distribution of *Salmonella* serotypes based on the farm from which the *Salmonella* was isolated. For both graphs, the number in parentheses next to the sample type/farm indicates the number of isolates for that sample type/farm.

isolates were resistant to, while resistances were found for six other antibiotics in one (FIS, CHL, NAL) or two (GEN, SXT, AZI) isolates.

When comparing the *Salmonella* AR profiles for the seven antibiotics with the most resistances based on sample type (**Figure 2**), a similar trend was seen for all sample types. While the resistance rates varied among sample types, in general resistance to STR/TET (52.5–80.8%) was higher than to the AMO/AMP/FOX/TIO/AXO group (7.7–32.7%), as was seen with the serotype diversity above, *Salmonella* AR profile diversity was much more distinct at the farm level (**Figure 3**). Three farms were excluded from this analysis since either no *Salmonella* were recovered (J, L), or all *Salmonella* that were recovered were pan-susceptible (K). When comparing farms where more than a single flock was followed, two major trends became evident. First, most of the STR/TET resistance came from *Salmonella* isolated from 3 of the 4 farms where at least 5 flocks were followed (A, E, I). Secondly, the AMO/AMP/FOX/TIO/AXO group resistances were predominantly only found on 2 farms (A, H). One potential cause of this resistance group is carriage by the bla_{CMY-2}gene,

TABLE 4 | *Salmonella* antibiotic resistances based on NARMS gram negative panels[a].

Antibiotic Class	Antibiotic[b]	No. of isolates resistant (% total isolates)	No. of isolates intermediate (% total isolates)	No. of isolates susceptible (% total isolates)
Aminoglycosides	Gentamicin (GEN)	2 (0.6%)	0 (0.0%)	335 (99.4%)
	Streptomycin (STR)	217 (64.4%)	0 (0.0%)	120 (35.6%)
b-lactam/b-lactamase Inhibitors	Amoxicillin-Clavulanic Acid (AMO)	90 (26.7%)	1 (0.3%)	246 (73.0%)
Cephems	Cefoxitin (FOX)	89 (26.4%)	1 (0.3%)	247 (73.3%)
	Ceftiofur (TIO)	88 (26.1%)	0 (0.0%)	249 (73.9%)
	Ceftriaxone (AXO)	90 (26.7%)	1 (0.3%)	246 (73.0%)
Folate pathway inhibitors	Sulfisoxazole (FIS)	1 (0.3%)	0 (0.0%)	336 (99.7%)
	Trimethoprim-Sulfamethoxazole (SXT)	2 (0.6%)	0 (0.0%)	335 (99.4%)
Macrolides	Azithromycin (AZI)	2 (0.6%)	0 (0.0%)	335 (99.4%)
Penicillins	Ampicillin (AMP)	90 (26.7%)	1 (0.3%)	246 (73.0%)
Phenicols	Chloramphenicol (CHL)	1 (0.3%)	1 (0.3%)	335 (99.4%)
Quinolones	Ciprofloxacin (CIP)	0 (0.0%)	0 (0.0%)	337 (100.0%)
	Nalidixic Acid (NAL)	1 (0.3%)	0 (0.0%)	336 (99.7%)
Tetracyclines	Tetracycline (TET)	232 (68.8%)	0 (0.0%)	105 (31.2%)

[a] *Determination of resistant/intermediate/susceptible is based on the established NARMS breakpoints for Salmonella.*
[b] *The acronym in parenthesis is the one given by the NARMS protocol.*

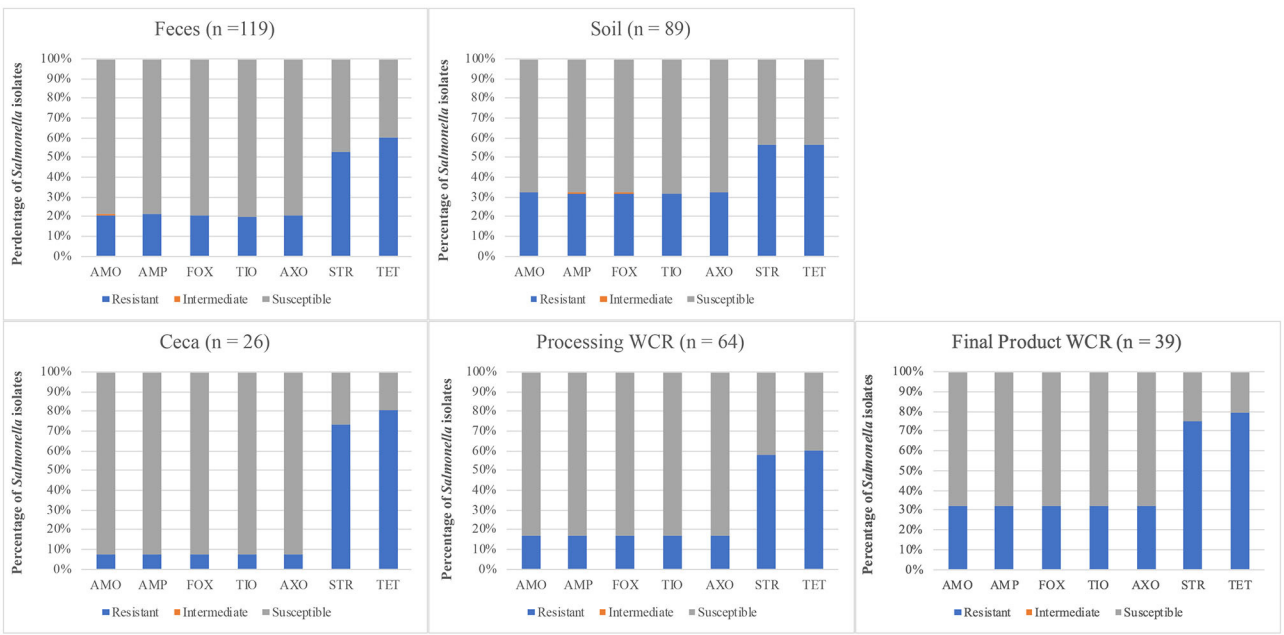

FIGURE 2 | Distribution of antibiotic resistances using the CDC-NARMS method and breakpoints for *Salmonella* isolated from pastured poultry flocks based on the sample type from which the *Salmonella* was isolated. The antibiotics include amoxicillin-clavulanic acid (AMO), ampicillin (AMP), cefoxitin (FOX), ceftiofur (TIO), ceftriaxone (AXO), streptomycin (STR), and tetracycline (TET).

which confers resistance to several antibiotic classes, including penicillins (AMP), b-lactam/b-lactamase inhibitors (AMO), and cephems (FOX/TIO/AXO) (Jacoby and Munoz-Price, 2005), and has been found to confer MDR in *Salmonella* and other bacteria (Miriagou et al., 2004; Mataseje et al., 2009). Preliminary whole genome sequencing analysis of a few of these isolates

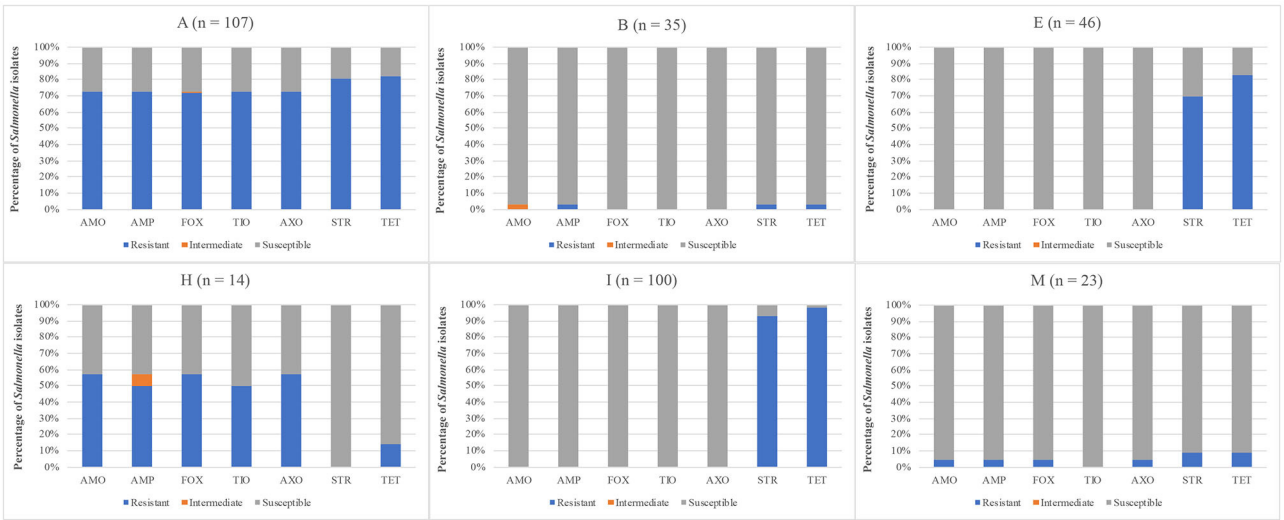

FIGURE 3 | Distribution of antibiotic resistances using the CDC-NARMS method and breakpoints for *Salmonella* isolated from pastured poultry flocks based on the farm from which the *Salmonella* was isolated. The antibiotics include amoxicillin-clavulanic acid (AMO), ampicillin (AMP), cefoxitin (FOX), ceftiofur (TIO), ceftriaxone (AXO), streptomycin (STR), and tetracycline (TET).

TABLE 5 | Multidrug resistance among *Salmonella* isolated from this study[a].

	No. of isolates	MDR isolates	MDR rate
	Sample type		
Feces	119	30	0.252
Soil	89	34	0.382
Ceca	26	2	0.077
Processing WCR	64	13	0.203
Final product WCR	39	13	0.333
	Farm		
A	107	80	0.748
B	35	1	0.029
C	3	3	1.000
D	1	0	0.000
E	46	0	0.000
H	14	8	0.571
I	100	0	0.000
J	0	0	0.000
K	8	0	0.000
L	0	0	0.000
M	23	1	0.043

[a]*Isolates were considered multidrug resistant (MDR) if the expressed resistance to ≥3 antibiotics on the gram negative NARMS protocol and breakpoints.*

shows the presence of the bla_{CMY-2}gene (data not shown), supporting this hypothesis. Interestingly, farm A relocated to a new location during the study, and all flocks from the new location were labeled under Farm H after a full year passed between samplings. Therefore, the farmer, management system, and farm equipment were the same, but the physical farm location was completely different (including soil type, composition of pasture, and climate). It has been previously demonstrated that flock management could significantly impact the farm environment (Sossidou et al., 2011; Sánchez-Casanova et al., 2020), so potentially the physical management structures (e.g., housing, farm equipment) have to potential to not only shape new farm environments physically, but also biologically in terms of being potential vectors for pathogens expressing specific AR profiles, or harboring plasmids containing specific ARGs.

Even within these antibiotic-free management systems, multidrug resistant (MDR) *Salmonella*, isolates exhibiting three of more resistances, were prevalent (**Table 5**). Overall, 27.3% (92/337) of the *Salmonella* isolates were considered MDR, with 82.6% (76/92) of the MDR isolates exhibiting resistances to seven antibiotics. This MDR rate is in line with what NARMS has found in conventional poultry samples from 2104 to 2017 (8.3–24.5%) (FDA, 2021). Aside from cecal samples (7.7%), the MDR rate across sample types was consistent (20.3–38.2%), and there were no significant differences between the MDR prevalences for any pairwise comparison, although cecal and soil prevalences were nearly significant ($p = 0.0615$). Conversely, when characterizing MDR *Salmonella* based on farm of origin, the MDR rates ranged from 0.0% (D, E, I, K) to 100% (C), although 87.0% (80/92) of the MDR *Salmonella* were recovered from farm A. It should be noted that in terms of serotypes of public health concern, of the 92 MDR *Salmonella* isolates from this study, 90 (97.8%) were Kentucky, with only a single MDR isolate representing a CDC top 32 serotype (Braenderup/Cholerasuis). Since Kentucky has been shown to be genetically more similar to common environmental *E. coli* than human pathogenic *Salmonella* serotypes (Morales et al., 2006), the results from this study demonstrate that Kentucky represents more of a biological vector to transfer MDR

to the more pathogenic *Salmonella* serovars, than an actual threat to public health themselves.

CONCLUSIONS

While *Salmonella* was recovered from pre-harvest, post-harvest, and final product samples from these pastured poultry farms at prevalence rates similar to those seen from conventional poultry operations, only ∼16% of all of the *Salmonella* isolates were serotypes of greatest concern to human health (CDC, 2013). The prevalence of these human health-related serotypes decreased along the farm-to-fork continuum (<3% of *Salmonella* recovered from the final product samples), indicating that the increased environmental exposure in these flocks did not result in a major health risk to the consumers of these products. Even though these pastured flocks were raised antibiotic-free, resistances to several antibiotic classes were expressed consistently among the *Salmonella* isolates, although >98% of the MDR isolates were serotyped as Kentucky. Only a single MDR *Salmonella* serotype of human health importance was isolated (∼0.04% of all samples tested in this study), further indicating the relative safety of these pastured poultry products to the consumer. Additionally, these data suggest that *Salmonella* diversity is driven largely by the farm of origin, rather than the type of sample from which the *Salmonella* was isolated within the production cycle for that farm. Therefore, while general *Salmonella* intervention strategies may be successful in pastured poultry systems, due to the diversity in management options based on the need of and availability to these farmers, more targeted understanding of the environmental and management variables on these farms is vital.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material. Further inquiries regarding the data can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because while we sampled from active pastured poultry farms, we did not manage the production or processing of the birds. We only collected the pre-harvest samples (soil, feces) after the broilers were moved on the pasture, and the post-harvest samples (ceca, whole carcass rinses) were collected after the farmer or processing facility culled the broilers. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MR designed the experiment, oversaw the processing of the samples, analyzed the results, and wrote the manuscript. JG performed the serotyping and edited the manuscript. AO assisted in the analysis of the antibiotic resistance data, performed the statistical analyses, and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

These investigations were supported by the Agricultural Research Service, USDA CRIS Projects Genetic Analysis of Poultry-Associated *Salmonella enterica* to Identify and Characterize Properties and Markers Associated with Egg-Borne Transmission of Illness #6040-32000-007-00 and Molecular Approaches for the Characterization of Foodborne Pathogens in Poultry #6612-32000-059-00.

ACKNOWLEDGMENTS

The authors thank Katelyn Griffin, Ariella Perry, Hellen Noh, Sarah Khorramzadeh, and Manju Amin for assistance in sample acquisition and pathogen isolation and Laura Lee Rutherford, Cheryl Gresham-Pearson, and Latoya Wiggins for performing the NARMS panels.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.