Management and environmental factors influence the prevalence and abundance of food-borne pathogens and commensal bacteria in peanut hull-based broiler litter

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ABSTRACT In this study, we conducted a longitudinal sampling of peanut hull-based litter from a farm under a "no antibiotics ever" program. Our objective was to determine broiler management practices and environmental factors that are associated with the occurrence of food-borne pathogens (*Salmonella* and *Campylobacter*) and the abundance of commensal bacteria (*Escherichia coli, Enterococcus* spp., and *Staphylococcus* spp.). Litter (n = 288) was collected from 4 broiler houses over three consecutive flocks, starting with a complete house clean-out and fresh peanut hull. Litter was sampled at the beginning of each grow-out cycle and at the end of the cycle. Logistic and linear regression models were used to model the relationships between pathogen prevalence, commensal abundance and management practices, and environmental factors. The number of flocks raised on litter, grow-out period, broiler house, litter pH, litter moisture, and house temperature were associated with the prevalence of pathogens and the abundance of commensal bacteria in litter. The final logistic model for pathogens showed that a higher probability of detecting *Salmonella* in litter was associated with the number of flocks raised on litter and the grow-out period. A higher probability of detecting *Campylobacter* in litter was associated with the number of flocks raised on litter, broiler house and the sections of the house, and the pH of litter. Our results suggest that management practices and environmental factors affect *Salmonella* and *Campylobacter* differently and suggest that each pathogen will require its own tailored intervention to stop their persistence in broiler litter.

Key words: broiler litter, salmonella, campylobacter, indicator bacteria, management and environment

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INTRODUCTION

Broiler litter is a mixture of bedding material, feces, uric acid, feathers, spilled feed, and water. A wide variety of materials are used as bedding in poultry houses, including wood shavings, sawdust, peanut hulls, rice hulls, paper pulps, and sand. Pine shavings and coarse pine sawdust are the most common bedding materials used (Grimes et al., 2002; Ritz et al., 2005). In the United States, it is a common practice to reuse the same litter for raising multiple flocks of chickens. Although the practice of litter reuse offers fiscal and environmental benefits, litter can harbor food-borne pathogens like *Salmonella* and *Campylobacter*. The pecking and coprophagic behavior of chickens makes the litter one of the first broiler sourced material ingested after chick placement. Therefore, litter is a major route for chickens to get exposed to bacterial pathogens. Understanding broiler management factors that could be used to predict the occurrence of food-borne pathogens in reused litter is vital to limiting their prevalence in post-harvest chicken production. Although there are studies on the microbiology of reused litter, these studies have been limited to wood shavings and rice-hull based litter

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(Kelley et al., 1995; Brooks et al., 2010; Roll et al., 2011; Volkova et al., 2011; Chinivasagam et al., 2012; Eberle et al., 2013; Roberts et al., 2013; Wei et al., 2013; Brooks et al., 2016; Rauber Wurfel et al., 2019; Voss-Rech et al., 2019; Valeris-Chacin et al., 2021; Chinivasagam et al., 2022).

Roberts et al. (2013) demonstrated that commensals (*Staphylococcus* spp., *Enterococcus* spp., and *Clostridium perfringens*) and pathogens (*Salmonella* and *Listeria monocytogenes*) were spatially and temporally distributed in 8 broiler houses. *Salmonella* was found at a greater frequency in the beginning of the first flock and were commonly associated with the "end walls" of the house. Furthermore, bird age was the most significant factor affecting bacteria abundance. In another study, commensals were reported to be higher in areas with high litter moisture levels (e.g., water lines) than areas with lower litter water content (e.g., feed lines) (Winkler et al., 2017). Brooks et al. (2016) used naïve/pristine broiler houses to investigate bacterial colonization of litter from flock to flock. The authors demonstrated that litter commensal populations (*Staphylococcus* spp., *Enterococcus* spp., *Clostridium perfringens* and *Escherichia coli* [*E. coli*]) stabilized after ~ 4 flocks, however, the source of *Salmonella* and *Listeria* was unknown. These studies on litter reuse have improved our understanding of the microbial ecology of litter and revealed the management and environmental factors that allow pathogens to proliferate.

The state of Georgia produces up to 50% of the United States peanuts (https://www.nationalpeanutboard.org/peanut-info/peanut-country-usa.htm) and ~35% of crop producers in South Georgia reported using peanut hull-based broiler litter as fertilizer/manure (Ritz et al., 2005). Therefore, peanut hull is an attractive choice for farmers in the United States because it is affordable and accessible. However, there is a dearth of studies that have investigated the bacterial profile of peanut hull-based litter after chick placement and during consecutive reuse. Therefore, we sought to investigate the pathogenic (*Salmonella* and *Campylobacter*) and indicator/commensal (*E. coli, Enterococcus* spp., and *Staphylococcus* spp.) bacterial populations in peanut hull-based litter from 4 broiler houses over 3 consecutive flocks, starting with a complete house cleanout and fresh peanut hull application. We found that the number of flocks raised on litter and the grow-out period/age of chickens were the management factors associated with the prevalence of the pathogens and the abundance of indicator bacteria.

MATERIALS AND METHODS

Description of Sampled Farms and Litter Management

Four integrated commercial broiler houses located on the same farm in South Georgia were selected for this study. Each house has the capacity to grow between 22,000 and 24,000 broilers per flock. All chicks on this farm were being raised under a newly adopted "No Antibiotics Ever" program, and copper sulfate was given via drinking water. Three cohorts of broiler flocks were raised in succession in the same four houses between February and August 2018. A complete litter cleanout was done in each house prior to the start of the study and fresh peanut hull were spread in each house before the first cohort of broiler flock was introduced to the houses. Following the grow-out of the first broiler cohort, the second and third cohorts of broiler flocks were raised in succession on the same litter used for the first broiler cohort. The length of time the houses were empty after the harvest of earlier flocks (i.e., downtime prior to sampling) ranged from 20 to 28 d. During this downtime, the litter was mechanically conditioned by removing the caked portions and was treated for ammonia control (usually 1 wk before sampling) by topical application of a commercial litter acidifier. The integrator practices half house brooding house that is, chicks were placed in the front section of the house for the first 14 days of the grow-out. These management procedures are within the scope of routine practices for the industry.

Litter Sample Collection

A total of 288 litter samples were collected from four broiler houses during the grow-out of 3 consecutive flocks. For each broiler flock cohort, litter samples were collected during early (i.e., chicks were between 4 and 14 days old) and late grow-out (32−38 days old chickens) for a total of 6 sampling times per broiler house that is, each flock (n = 3) was sampled 2 times. For sampling purposes, each broiler house was divided into 4 sections from the front of the house to the back (front, mid-front, mid-back and back) and each section was divided into 3 subsections from the left of the house to right (area 1, area 2, and area 3; Figure 1). From each subsection, 3 litter grab samples were collected and pooled into one Whirl Pak bag. The pooled litter samples (n = 12 per house) were transported in a cooler with icepacks to the United States National Poultry Research Center for processing (Figure 1).

Bacterial Isolation and Identification

All samples were processed within 24 h of collection. Thirty grams from each pooled litter grab sample was mixed with 120 ml phosphate buffered saline and shook with a hand wrist shaker (Boekel Scientific, Model 401000, Feasterville, PA) for 10 min. For *Campylobacter* spp. detection, appropriate dilutions of the litter eluate were direct plated to Cefex agar (Remel, Lenexa, KS). Plates were incubated in a microaerobic, hydrogen enriched atmosphere (7.5% H_2, 2.5% O_2, 10% CO_2, and 80% N_2) (Lynch et al., 2010) at 42°C for 48 h. Additionally, aliquots of the eluate (4 × 50 μL drops) were placed onto a 0.65-μm cellulose acetate filter placed on Cefex agar. Filters were allowed to dry 30 min before being

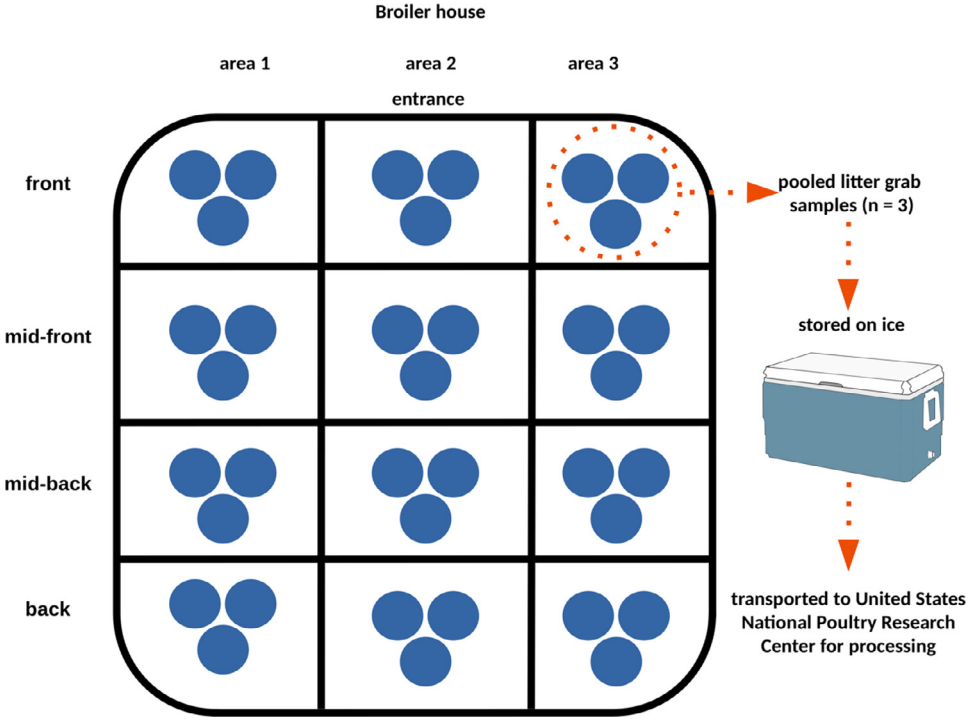

Figure 1. Broiler house sampling design. Diagram of broiler house sampling scheme. For each litter sampling, three litter grabs (shown in blue) were pooled (indicated by orange dotted line) for each floor section at each area (n = 12). Pooled samples were stored in a cooler with icepacks and subsequently transported to the United States National Poultry Research center.

removed and plates were incubated as above. Enrichment was also performed by adding 1 mL of litter eluate to 9 mL Bolton's broth and incubated in a microaerobic atmosphere at 42°C for 48 h before being transferred to Cefex agar and incubated as above. Presumptive positive colonies were selected based on typical cellular morphology and motility using phase contrast microscopy. Isolates (n = 44) were confirmed using the *Campylobacter* BAX real-time PCR Assay (Hygiena; Wilmington, DE) according to manufacturer's directions except that an isolated colony was added to the lysis reagent instead of enrichment broth.

For *Salmonella* culture, litter eluate (100 μL) was direct plated to both Brilliant Green Sulfur (**BGS**) and Xylose Lysine Tergitol-4 (**XLT-4**) agars. Plates were incubated 18 to 24 h at 37°C. Additionally, aliquots (1 mL) of the eluate were enriched in buffered peptone water (9 mL) for 18 to 24 h at 37°C. Enrichments were plated to BGS and XLT-4 agars and transferred to GN Hajna and Tetrathionate broths and incubated 24 h and 48 h, respectively at 37°C. Afterward, 100 μL of GN Hajna and Tetrathionate broth Broths were then transferred to Rappaport-Vassiliadis R10 (**RV**) broth (BD; Franklin Lakes, NJ) and incubated at 37°C for 18 to 24 h. Thereafter, 10 μL of RV broth was plated to both BGS and XLT-4 agars. Isolated colonies characteristic of *Salmonella* was struck onto triple sugar iron and lysine iron agar slants and incubated at 37°C for 18 to 24 h. Presumptive *Salmonella* isolates (n = 55) were serogrouped with antisera (Becton Dickinson, Franklin Lake, NJ) and then cryopreserved.

To determine the abundance of commensal bacteria, serial dilutions of the litter eluate were prepared, and appropriate dilutions plated onto CHROMagar ECC (DRG International, Inc.; Springfield, NJ), mEnterococcus agar (Neogen; Lansing, MI), and mannitol salt agar (**MSA**) (BD-Fisher Scientific; Pittsburgh, PA) for the isolation of *E. coli, Enterococcus* spp., and *Staphylococcus* spp., respectively. CHROMagar ECC was incubated for 18 to 24 h at 37°C and blue-green colonies typical of *E. coli* were counted. mEnterococcus agar was incubated for 24 to 48 h at 37°C and pink to dark red colonies indicative of *Enterococcus* spp. were recorded. For *Staphylococcus* spp., MSA was incubated at 37°C for 18 to 24 h and yellow colonies with yellow halos were counted (clear pink to red colonies with no yellow color change indicative of *S. epidermidis* were not present). Representative bacterial isolates from each sample were cryopreserved in 30% glycerol Luria-Bertani broth. For statistical and calculation purposes, if no colonies grew on the lowest plate dilution ($10^{-3} - 10^{-5}$), 1×10^5 was recorded for *E. coli*, 1×10^6 for *Enterococcus* spp., and 1×10^7 for *Staphylococcus* spp. Likewise, if too many colonies grew on the highest dilution plate ($10^{-5} - 10^{-7}$), 1.5×10^8 was recorded for *E. coli*, 1.5×10^9 for *Enterococcus* spp., and 1.5×10^{10} for *Staphylococcus* spp.

Determination of Litter Moisture, Litter pH, and Broiler House Temperature

Litter moisture content was determined gravimetrically by drying approximately 1 g of litter at 107°C for 24 h. Litter pH was determined using a Thermo-Scientific Orion probe submerged into a hand massaged litter

slurry (10 g litter in 20 mL nanopure water). The temperature reading inside each house at the time of sampling was recorded from the thermostat installed in the control room of each house.

Statistical Analyses

Data management was done using Microsoft Excel (2016, Microsoft Corp., Redmond, WA) and then imported into R v4.1.1 software for analysis. *E. coli, Enterococcus* spp. and *Staphylococcus* spp., abundance were normalized by litter dry weight, while *Campylobacter* and *Salmonella* occurrences were calculated as proportions that is, presence/absence. Initial exploratory analyses revealed that bacterial abundance data was skewed to the right, therefore, logarithmic transformation (logCFU/g) was performed before any further statistical analysis was done. To determine if there was correlation between the concentration of *E. coli, Enterococcus* spp., and *Staphylococcus* spp., we performed a Pearson's pairwise correlation analysis. Afterwards, we performed a multivariate analysis of variance (**MANOVA**) to account for the possible correlation in the abundance of the different commensal bacteria and to explore the statistical differences in abundance.

We explored the statistical differences between the commensal bacterial abundances for each category of independent variables (litter moisture, litter pH, broiler house temperature, broiler flock cohort, grow-out period broiler house, and broiler house floor section) using One-way ANOVA with Bonferroni post-hoc adjustment for multiple comparisons. To determine which independent variables could predict commensal bacterial abundance we used multivariable linear regression models. Independent variables that were unconditionally associated with bacterial abundance at a liberal *P*-value of ≤0.25 in the One-way ANOVA analyses were included in subsequent multivariable regression models. For *Salmonella* and *Campylobacter* species binary outcomes, multivariable logistic regression models were performed. Multicollinearity between the independent variables was assessed using the variance-covariance matrix and variance inflation factor estimates.

Since we were interested in how bacterial abundance differed between the independent variables, all the independent variables were included as fixed effects in the multivariable regression models and interactions between the independent variables were taken into account. Likelihood ratio test was used to examine if the model was appropriate for the intended comparisons. The model was assessed by checking the residuals, identifying influential observations, and evaluating its reliability and predictive power (Dohoo et al., 2010). We selected the best fitting model based on Akaike's information criterion (**AIC**). Statistically significant independent variables using Likelihood ratio test or Wald test at *P*-value ≤0.05 were retained in the final model.

RESULTS

Prevalence of Salmonella *and* Campylobacter *in Litter Differed Between Houses, Flock Cohorts, and Grow-Out Period*

To determine the prevalence of *Salmonella* and *Campylobacter* in litter collected from four broiler house floors (Figure 1), we performed direct and selective enrichment plating of litter eluate onto relevant selective solid agar media ("Methods"). *Salmonella* was isolated from 10.41% (30/288) of litter samples (95% CI: 7.14 −14.54). The proportion of litter samples positive for *Salmonella* differed between broiler flock cohorts, broiler houses, and grow-out period ($P < 0.001$; Table 1). Litter samples positive for *Salmonella* were higher for cohort 1 compared to cohort 2 and 3. Furthermore, litter samples from houses 2 and 3 had higher *Salmonella* positivity rates than houses 1 and 4. Litter samples collected during late grow-out (bird ages 32−38 d) had higher number of *Salmonella* positives than litter collected during

Table 1. Proportion (95% Confidence Interval) of occurrences of *Salmonella* spp. and *Campylobacter* spp. for each level of category for different management variables.

Variable	Category (Number of litter samples)	*Salmonella* % (95%CI)	*Campylobacter* % (95%CI)
Flock cohort	Cohort 1 (n = 96)	23.95 (15.83−33.74)	17.71 (10.67−26.83)
	Cohort 2 (n = 96)	6.25 (2.33−13.10)	3.13(0.64−8.86)
	Cohort 3 (n = 96)	1.04 (0.00−5.67)	7.29 (2.98−14.44)
	P-value	<0.001	0.002
Grow-out period	Early (n = 144)	4.16 (1.54−8.84)	1.39 (0.17−4.93)
	Late (n = 144)	16.67 (10.98−23.78)	17.36 (11.56−24.55)
	P-value	0.001	<0.001
House	House 1 (n = 72)	0.00 (0.00−4.99)	2.78 (0.33−9.67)
	House 2 (n = 72)	15.27 (7.88−25.69)	5.56 (1.53−13.62)
	House 3 (n = 72)	23.61 (14.39−35.09)	16.67 (8.91−27.30)
	House 4 (n = 72)	2.77 (0.33−9.67)	12.50 (5.88−22.41)
	P-value	<0.001	0.015
House floor section	Front (n = 72)	9.72 (3.99−19.01)	16.67 (8.91−27.30)
	Mid-Front (n=72)	11.11 (4.92−20.72)	9.72 (3.99−19.01)
	Mid-Back (n = 72)	8.33 (3.11−17.26)	5.56 (1.53−13.61)
	Back (n = 72)	12.50 (5.87−22.41)	5.56 (1.53−13.61)
	P-value	0.863	0.091

Table 2. *Salmonella* serogroups and *Campylobacter* species found in litter.

	Number of isolates	Flock	House	Broiler age	Grow-out period	Serogroup/Species
Salmonella	34	1, 2, 3	2, 3, 4	4−38	Early, Late	C2-C3
	10	1	2, 3	32−36	Late	C2-C3, D1-D2
	3	1	3	32	Late	C2-C3, E
	6	1, 2	2, 3, 4	14−36	Early, Late	D1-D2
	2	1	3	4	Early	E
Campylobacter	6	1, 2	2, 3, 4	14−36	Early, Late	*C. coli*
	38	1, 3	1, 2, 3, 4	32−38	Late	*C. jejuni*

early grow-out (bird ages 4−14 d) (Table 1). The proportion of litter samples positive for *Salmonella* was not different between the floor sections of the houses (Table 1).

Eighty percent of the isolates (44/55) (1−8 isolates per *Salmonella* positive litter sample (n = 30) were serogrouped) were classified as to serogroup C2-C3, while 16% of the isolates were assigned to D1D2 (Table 2, Table S1). The remaining 2 isolates (4%) belonged to serogroup E. Isolates belonging to serogroup C2-C3 were found in each flock, while D1-D2 were present in flock 1 and 2. Serogroup E was found only in flock 1. Serogroups C2-C3 and D1-D2 were found in house 2, 3, and 4, while serogroup E was found in only house 3 (Table 2, Table S). No *Salmonella* was found in house 1.

Campylobacter was recovered from 9.38% (27/288) of litter samples (95% CI: 6.23−13.35). Like *Salmonella*, the number of litter samples positive for *Campylobacter* differed between broiler flock cohorts, broiler houses, and grow-out period ($P < 0.05$; Table 1). Litter samples from cohort 1 had a higher proportion of *Campylobacter* positives than cohort 2 and 3, and broiler houses 3 and 4 had a higher rate of *Campylobacter* positives than houses 1 and 2 (Table 1). *Campylobacter* positivity rate was higher during late grow-out than early grow-out (Table 1). Eighty-six percent (38/44) of the *Campylobacter* isolates ((1−3 isolates per *Campylobacter* positive litter sample (n = 27) were confirmed by PCR) were determined to be *C. jejuni* while 14% (6/44) were identified as *C. coli* (Table 2, Table S2). *C. jejuni* was found in flock 1 and 3, while *C. coli* was found in flock 1 and 2. *C. jejuni* was found in each house, while *C. coli* was present in houses 2, 3, and 4.

Flock Cohort, Grow-Out Period, House Floor Section Influenced the Abundance of Commensal Bacteria in Litter

To determine the abundance of *E. coli, Enterococcus* spp., and *Staphylococcus* spp. (collectively referred to as commensal/indicator bacteria in this study) in litter, we performed direct plating of litter eluate onto relevant selective solid agar. We chose these three bacterial groups because they are known indicator bacteria and have been shown to be part of the core microbiome of broiler chickens (Oakley et al., 2014). In addition, some strains can cause diseases in chickens and humans (Oakley et al., 2014). Therefore, there is literature available on their survival dynamics in litter.

The average *E. coli, Enterococcus* spp. and *Staphylococcus* spp. concentration in litter for the 3 broiler cohorts was 4.73 LogCFU/g, 5.65 LogCFU/g, and 6.99 LogCFU/g, respectively (Figure S1). There was a high correlation between *Enterococcus* spp. and *Staphylococcus* spp. abundance ($\rho = 0.66$, $P < 0.001$), moderate correlation between *E. coli* and *Enterococcus* spp. ($\rho = 0.39$, $P < 0.001$), and lower correlation between *E. coli* and *Staphylococcus* spp. abundance ($\rho = 0.13$, $P < 0.05$). There was no difference in the abundance of the 3 commensal bacteria in the litter samples collected from the four houses (MANOVA), but the broiler flock cohort and broiler house sections significantly affected their abundance in litter (MANOVA; $P < 0.001$, Table 3, Figure S1, Table S1). There was no collinearity between the independent variables, however, we saw a 3-way interaction between broiler flock cohort, grow-out period, and floor sections of the broiler house.

There was a gradual decrease in the abundance of *Enterococcus* spp. from the first broiler flock cohort to the third broiler cohort (Table 3, Figure S1), while *E. coli* and *Staphylococcus* spp. increased in abundance from the first cohort to the second cohort and decreased for the third cohort (Table 3, Figure S1). *E. coli* abundance was significantly higher during early grow-out compared to late grow-out ($P < 0.05$), while *Enterococcus* spp. and *Staphylococcus* spp. were higher during late grow-out compared to early grow-out (Table 3, Figure S1). The litter from the front and mid-front section of the broiler houses harbored significantly higher abundance of *Enterococcus* spp. and *Staphylococcus* spp. than the back and mid-back sections ($P < 0.05$; Table 3, Figure S1)

Environmental Conditions Affected Pathogen Prevalence and Commensal Bacteria Abundance

We determined the influence of litter moisture, litter pH and broiler house temperature on pathogen prevalence and commensal bacteria abundance. Average litter moisture and pH was 28.2 ± 0.005% (range = 11−62%) and 7.47 ± 0.05 (range = 5.34−8.83), respectively, while the average temperature inside the broiler houses was 79.49 ± 1.04°F (range = 72.30−89.10°F; Figure 2).

The litter moisture was higher during late grow-out (35.0 ± 0.5%) compared to early grow-out (20.3 ± 0.7%; $P < 0.001$; Figure 2). Litter moisture for broiler cohort 2 (28.3 ± 0.7%) and 3 (28.5 ± 0.8%) was higher than

Table 3. Mean ± SD of commensal bacterial abundance (LogCFU/g) for each level of category for different management variables.

Variable	Category	*Escherichia coli*	*Enterococcus* spp.	*Staphylococcus* spp.	*MANOVA P*-value
Flock cohort	Cohort 1	5.09 ± 0.68[a]	5.83 ± 0.84[a]	6.72 ± 1.05[a]	<0.001
	Cohort 2	5.17 ± 1.04[a]	5.77 ± 0.40[b]	7.40 ± 0.46[b]	
	Cohort 3	3.96 ± 0.52[b]	5.35 ± 0.63[c]	6.84 ± 0.77[a]	
	P-value	<0.001	<0.001	<0.001	
Grow-out period	Early	5.04 ± 1.04[a]	5.41 ± 0.71[a]	6.50 ± 0.87[a]	<0.001
	Late	4.43 ± 0.74[b]	5.89 ± 0.56[b]	7.48 ± 0.44[b]	
	P-value	<0.001	<0.001	<0.001	
House	House 1	4.77 ± 0.95	5.60 ± 0.70	7.03 ± 0.86	0.511
	House 2	4.70 ± 0.94	5.69 ± 0.69	6.98 ± 0.86	
	House 3	4.69 ± 1.00	5.70 ± 0.70	7.02 ± 0.88	
	House 4	4.79 ± 0.94	5.60 ± 0.64	6.92 ± 0.80	
	P-value	0.912	0.705	0.870	
House floor section	Front	4.86 ± 0.85	5.94 ± 0.56[a]	7.24 ± 0.71[a]	<0.001
	Mid-Front	4.86 ± 0.95	5.93 ± 0.60[a]	7.19 ± 0.77[a]	
	Mid-Back	4.61 ± 1.02	5.36 ± 0.68[b]	6.76 ± 0.90[b]	
	Back	4.59 ± 0.97	5.36 ± 0.64[b]	6.76 ± 0.89[b]	
	P-value	0.119	<0.001	<0.001	

[ab]Different superscript letters indicate statistically significant difference between the means within each category of independent variables at 5% level of significance. (*Bonferroni post-hoc adjustment*).

cohort 1 (25.7 ± 1.30%), while litter moisture of houses 1 (29.3 ± 1.00%) and 2 (27.9 ± 1.00%) were higher than litter moisture of houses 3 (26.3 ± 1.00%) and 4 (27.2 ± 1.00%). In addition, the front (28.9 ± 1.00%), mid-front (28.4 ± 1.00%) and mid-back (28.0 ± 1.00%) floor sections of the houses had nonsignificant higher moisture than the back (25.4 ± 1.00%) section. However, this difference in moisture between cohorts, houses, and floor sections were not statistically significant (Figure 2). Litter pH was higher during late grow-out (pH = 8.11 ± 0.08) compared to early grow-out (pH = 6.87 ± 0.02; $P < 0.001$). There was no significant difference in litter pH between houses, but litter pH significantly increased from cohort 1 to 3 (cohort 1; pH = 6.96 ± 0.12, cohort 2; pH = 7.72 ± 0.07, cohort 3; pH = 7.82 ± 0.06, $P < 0.001$; Figure 2). Additionally, the back (pH = 7.67 ± 0.11) and mid-back (pH = 7.65 ± 0.12) floor sections had higher litter pH than the front (pH = 7.32 ± 0.10) and mid-front (pH = 7.35 ± 0.10) sections of the house but the difference was not statistically significant (Figure 2). As expected, the houses temperature was higher during early grow-out (83.67 ± 0.97°F) than during late grow-out (75.33 ± 0.71°F; $P < 0.001$) but there was no difference in temperature between broiler cohorts and houses (Figure 2).

To assess the significance of the environmental factors on the occurrence of *Salmonella* and *Campylobacter*, we performed a univariate logistic regression model. To do this, we divided each environmental variable into 2 levels/groups. For litter moisture, samples with a moisture content greater than 25% represented one level (n = 165), while samples with a moisture content of 25% or lower formed the other group (n = 123). We chose 25% moisture because it has been suggested that a moisture content above 25% can cause litter cushioning, insulating, and water holding capacity to become compromised (Dunlop et al., 2016). For pH, litter samples with a pH higher than the average pH of 7 were assigned to one level (n = 208), while samples with a pH of 7 or lower represented the second group (n = 80). Similarly, litter samples collected when the house temperature was higher than the average temperature of 79°F were assigned to one level (n = 10), while samples collected when the house temperature was 79°F or lower formed the second group (n = 14).

Litter pH and moisture did not affect *Salmonella* occurrence in litter, but litter samples with a moisture content >25% tended to have a higher proportion of *Salmonella* positives than litter with a moisture content of ≤25% (Table S3). In addition, the proportion of litter samples positive for *Salmonella* was higher when the house temperature was ≤79°F than when the temperature was >79°F (Table S3). For *Campylobacter*, all 3 environmental variables were unconditionally associated with the occurrence of *Campylobacter* in litter (Table S3). The proportion of litter samples positive for *Campylobacter* were higher 1) when litter moisture was >25% compared to ≤25%, 2) when litter pH was >7 compared to ≤7, and 3) when house temperature was ≤79°F compared to >79°F ($P < 0.01$; Table S3).

The abundance of all three commensal bacteria (*E. coli, Enterococcus* spp., and *Staphylococcus* spp.) were significantly higher when the litter pH was >7 compared to when the litter pH was ≤7 ($P < 0.05$; Table S4). Likewise, the abundance of *Enterococcus* spp. and *Staphylococcus* spp. were significantly higher when litter moisture was >25% compared to when it was ≤25% ($P < 0.05$). Moisture did not have a significant effect on *E. coli* abundance. A multilinear regression model revealed that there was a 3-way interaction between pH, moisture, and house temperature for *E. coli.* House temperature did not affect the abundance of commensal bacteria (Table S4).

A principal component analysis (**PCA**) was conducted using the abundance of the 3 commensals, litter pH, litter moisture, and house temperature revealed that the litter samples clustered by the number of flock cohorts, the age of chickens at sampling and the grow-out period. Litter samples were not separated by houses or by the floor sections of a house (Figure 3). In addition, the PCA variables could explain up to ~66.1% (Dim 1 and Dim 2) of the variability in our data (Figure 3).

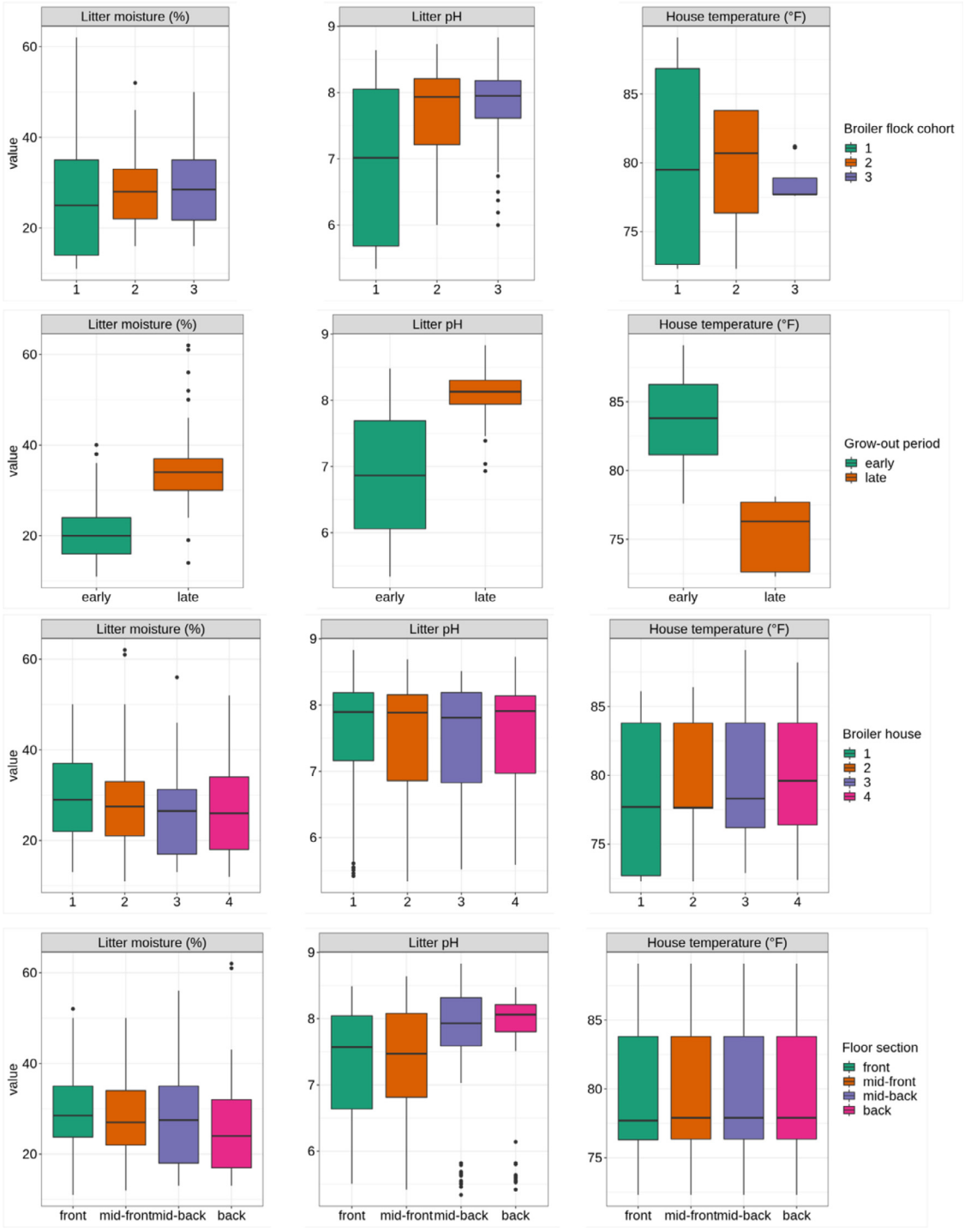

Figure 2. Box plots of environmental factors across the broiler cohorts, grow-out period, broiler houses, and house floor sections.

Probability of Detecting Salmonella *and* Campylobacter *in Litter*

To determine the management practice and environmental factors that could predict the occurrence of *Salmonella* and *Campylobacter* in litter, we performed univariate and multivariate logistic modeling. We used data on "broiler flock cohort", "grow-out period", "broiler house", and "broiler house floor section" as independent management factors, while "litter moisture", "litter pH" and "house temperature" were assigned as independent "environmental factors". Univariate logistic regression model revealed that most of the management and environmental factors were unconditionally correlated ($P < 0.25$) with *Salmonella* occurrence except for "broiler house floor section" and "litter pH" (Table 1, Table S3). The multivariable logistic model showed that only "broiler flock cohort" and "grow-out period" were significant in the final model (Table 4). The likelihood of recovery of *Salmonella* from litter was higher for broiler cohort 1 compared to cohort 2 and 3 (cohort 2 vs cohort 1; OR (odds ratio) = 0.19, cohort 3 vs. cohort 1; OR = 0.03, $P < 0.05$; Table 4). The recovery of *Salmonella* from litter was also more likely during the late

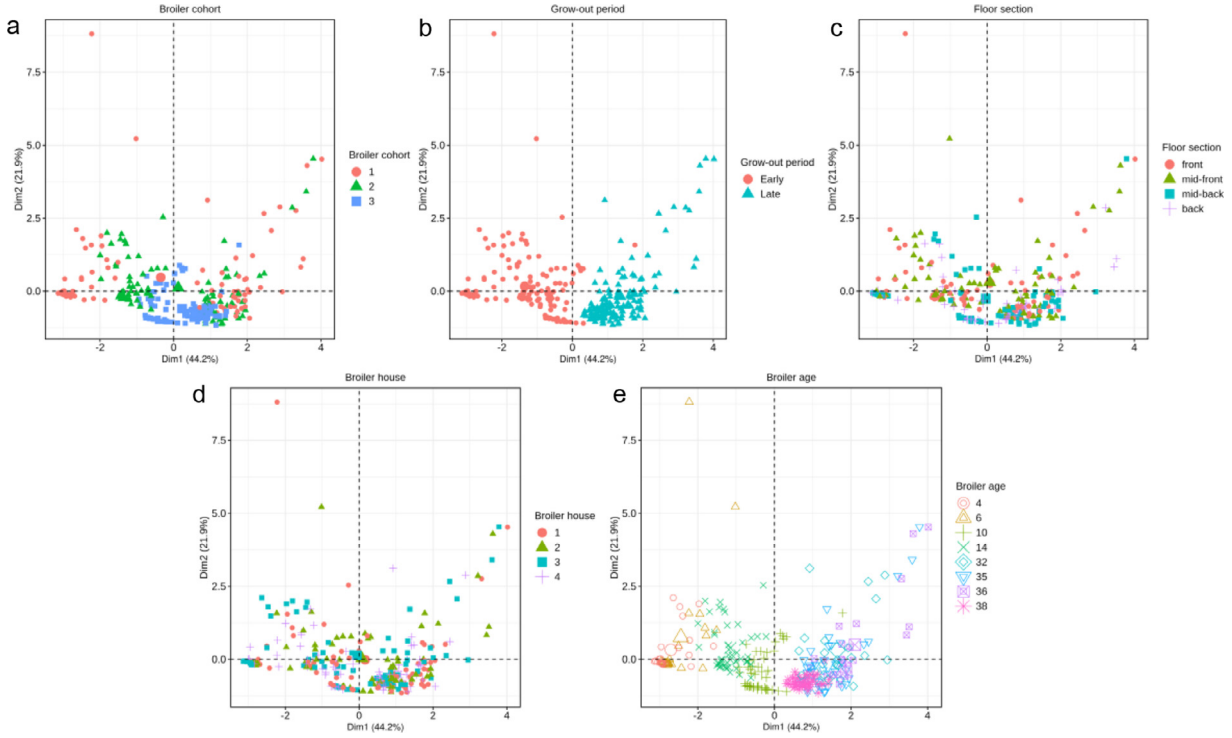

Figure 3. Principal component analysis (PCA) characterizing the differences between litter samples grouped by (A) flock cohort (B) grow-out period, (C) floor sections of broiler house (D) broiler house, and (E) broiler age. The PCA was constructed using litter moisture levels, litter pH, house temperature and commensal bacteria (*E. coli, Enterococcus* spp., *Staphylococcus* spp.) abundance. Each dot denotes one sample, and data represents 288 litter samples. The farther apart two samples are from each other, the more different they are.

grow-out period compared to the early grow-out period (OR = 3.31, 95% CI: 1.74−6.99, $P < 0.05$). The predictive power and reliability of the final model was accurate at 95% and only 5% of the observations were not correctly classified.

For *Campylobacter*, "broiler flock cohort", "broiler house", "broiler house floor section", and "litter pH" were significant factors retained in the final *Campylobacter* model ($P < 0.05$; Table 4). *Campylobacter* recovery from litter was more likely in broiler cohort 1 compared to cohort 2 and 3 (cohort 2 vs. cohort 1; OR = 0.06, cohort 3 vs. cohort 1; OR = 0.18, $P < 0.05$). Furthermore, *Campylobacter* recovery from litter was more likely in broiler house 3 (OR = 14.04, 95% CI: 2.91−110.0, $P < 0.05$) and 4 (OR = 8.4, 95% CI: 1.69−65.91, $P < 0.05$) compared to broiler house 1. *Campylobacter* was more likely to be present in the front section of the house than in the mid-front section (OR = 0.18, 95% CI: 0.06−0.49, $P < 0.05$; Table 4). Lastly, *Campylobacter* isolation from litter was more likely when the litter pH was > 7 compared to a litter pH ≤7 (OR = 5.67, 95% CI: 2.65−15.02, $P < 0.05$; Table 4). The predictive power and reliability of the final *Campylobacter* model was accurate at 92% with just 8% of the observations not correctly classified.

Table 4. Multivariable logistic regression models of independent variables associated with *Salmonella* and *Campylobacter* detection in litter.

		Salmonella			*Campylobacter*		
Variable	Category	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Broiler flock cohort	Cohort 1[a]	-	-	-	-	-	-
	Cohort 2	0.19	0.07−0.48	0.001	0.06	0.01−0.22	<0.001
	Cohort 3	0.03	0.01−0.14	0.001	0.18	0.06−0.49	0.001
Grow-out period	Early [a]	-	-				
	Late	3.31	1.74−6.99	0.001			
House	1 [a]				-	-	-
	2				2.82	0.46−23.7	0.283
	3				14.04	2.91−110	0.003
	4				8.40	1.69−65.91	0.0179
House floor section	Front [a]				-		-
	Mid-Front				0.18	0.06−0.49	0.001
	Mid-Back				1.67	0.62−4.66	0.312
	Back				1.33	0.48−3.89	0.588
Litter pH	Low (≤7) [a]				-		
	High (>7)				5.67	2.65−15.02	<0.001

[a]Reference category used in the logistic regression model.

DISCUSSION

Proper litter management before, during and after each broiler flock is critical for improved food safety and broiler health. It has been demonstrated that the *Salmonella* and *Campylobacter* status of broiler chickens at post-harvest is significantly associated with their recovery from litter and environmental samples collected at pre-harvest (Volkova et al., 2010; Berghaus et al., 2013; Schroeder et al., 2014). These studies have demonstrated that litter sampling can be useful for monitoring pathogen contamination and transmission in pre-harvest. However, none of these studies showed how management practices and the environment affect the prevalence of pathogens in litter. A few studies that have investigated the microbial ecology of reused litter have focused on litter composed of wood shavings or rice hull (Kelley et al., 1995; Brooks et al., 2010; Cressman et al., 2010; Roberts et al., 2013; Wei et al., 2013; Brooks et al., 2016; Wang et al., 2016) and no studies to the authors knowledge have reported the microbial ecology of reused peanut hull-based litter.

In this study, we found significant associations between management practices and the prevalence of *Salmonella* and *Campylobacter*, and the abundance of commensal/indicator bacteria. Using a multivariate logistic regression model, we found that the probability of detecting *Salmonella* decreased as the number of broiler flock cohorts raised on litter increased. There was a 33.3% greater chance of finding *Salmonella* in the first cohort compared to the third cohort. Also, we found that it was more likely to detect *Salmonella* during late flock litter sampling than earlier during the grow-out. The observed decrease in *Salmonella* prevalence from increased litter reuse could be due to competition with other microbes colonizing litter. For the first broiler flock cohort where fresh/naïve peanut hull was used, it is expected that the bacterial diversity and richness (i.e., number of bacterial species) would be lower compared to reused litter from flock 2 and 3.

Early studies on the competitive exclusion properties of reused litter have shown a lower survival of *Salmonella* in reused litter compared to fresh litter (Tucker, 1967; Fanelli et al., 1970; Gustafson and Kobland, 1984; Soerjadi-Liem and Cumming, 1984; Corrier et al., 1992). Recent studies have shown that chickens raised on reused litter harbor a different microbiome than chicks grown on fresh litter (Cressman et al., 2010; Wang et al., 2016; Oladeinde et al., 2022) and that the number of litter samples positive for *Salmonella* significantly decreases as the number of litter reuses increased compared with the first use of the litter (Roll et al., 2011; Chinivasagam et al., 2012; Muniz et al., 2014; Oladeinde et al., 2022). Roberts et al. (2013) detected *Salmonella* at a greater frequency with the first flock of broilers raised on fresh pine shavings compared to flock 2 and 3. Likewise, Brooks et al. (2016) reported 100% *Salmonella* positivity rate for all litter samples collected from the first flock of broilers raised on fresh rice hull and a 72 to 77% positivity rate for flock 2−5 raised on reused litter. In a *Salmonella* risk factor model (Volkova et al., 2011), the use of fresh wood shavings to top-dress or completely replace the litter between flocks was found to increase the probability of detecting *Salmonella* in litter. Nevertheless, the detection of *Salmonella* in all 3 flocks in our study suggests that the litter management practice in place could not stop the cycle of residual *Salmonella* contamination. Here, *Salmonella* serogroup C2-C3 was the dominant serogroup that was found in each flock which suggests that members of this group have the capability to persist in litter. This serogroup of *Salmonella* includes serovar Kentucky that has become the most dominant serovar in United States broiler chicken production (Shah et al., 2017; Rama et al., 2022).

One explanation for the higher number of litter samples testing positive for *Salmonella* during late grow-out compared to early grow-out is the restriction of access to the whole house during brooding and changes in environmental parameters. Higher *Salmonella* prevalence has been reported during late grow-out compared to early grow-out (Brooks et al., 2010; Voss-Rech et al., 2019). Voss-Rech et al. (2019) reported that none of the litter samples collected before the first flock of day-old chicks were placed on fresh litter were positive for *Salmonella* (0/10). However, *Salmonella* was found in at least 3 litter samples from each of the 5 subsequent flocks before chick placement. Furthermore, the *Salmonella* positivity rate of litter samples collected at harvest was higher (79%) than samples collected at placement (30%). Brooks et al. (2010) found higher *Salmonella* abundance at late flock compared to pre-flock and early-flock but did not report if the concentrations were significantly different.

During the early grow-out in this study, the chicks were between 4 and 14 days old and only occupied the brooding section of the house, while during late grow-out the chickens were 32 to 38 days old and had access to whole house from d 14. The low detection rates of *Salmonella* during early grow-out (6/144) suggests that *Salmonella* was not established in each house and throughout the litter at placement (Table S1). For instance, 5 of 6 samples that were *Salmonella* positive during early flock were from the front/mid-front section of houses 2, 3, and 4, while only 1 sample from the back of house 3 was positive (Table 2 and Table S1). Furthermore, after a chick is exposed to *Salmonella* it does not get colonized at once since there are multiple factors that will affect a successful colonization and shedding of *Salmonella*, including the *Salmonella* dose/strain ingested, the immune status of the chicken and innate chicken gut and litter microbiome (Cosby et al., 2015; Rogers et al., 2021). For chicks that got colonized during early grow-out, they will serve as a reservoir of *Salmonella* in litter through fecal shedding and allow *Salmonella* to spread throughout the flock during late grow-out. Since we did not investigate the prevalence of *Salmonella* in day-old chicks and the house environment before placement, we can only speculate that post-hatch chicks and the broiler house environment were the likely source of *Salmonella* during early grow-out.

Changes in litter physicochemical properties have been shown to perturb the microbiome of litter and affect the survival/proliferation of bacterial pathogens including *Salmonella* (Lovanh et al., 2007; Payne et al., 2007; Chinivasagam et al., 2012; Williams et al., 2012; Dunlop et al., 2016; Bucher et al., 2020). Here, we assessed litter physicochemical changes by determining the moisture and pH of litter samples. Litter moisture is correlated with an assembly of environmental parameters including broiler house environment (e.g., temperature, relative humidity, and ammonia) and litter properties (e.g., type, age, and pH) (http://extension.msstate.edu/sites/default/files/publications/publications/P3657_web.pdf). Litter pH and moisture were not statistically significant factors affecting *Salmonella* prevalence in litter and were not retained in the final multivariate logistic model (Tables 1 and 4). This result contradicts other studies (Pope and Cherry, 2000; Payne et al., 2002, 2007) that have shown pH and moisture to affect *Salmonella* survival but supports studies by Line and colleagues (Line, 2002; Line and Bailey, 2006) that demonstrated that acidified litter did not significantly affect *Salmonella* colonization of broilers.

The number of broiler flock cohorts raised on reused litter also affected the probability of detecting *Campylobacter* (Table 4). The odds of detecting *Campylobacter* were highest for flock 1 and lowest for flock 3, however, in contrast to *Salmonella*, where the odds decreased from flock to flock, for Campylobacters we saw a decrease from flock 1 to 2 and an increase from flock 2 to 3 (Table 4). There is no clear explanation for the observed increase in prevalence from flock 2 to 3 but the overall small number of *Campylobacter* positives (3/96 and 7/96 positive litter samples for flock 2 and 3, respectively) suggest a low detection rate of *Campylobacter* in litter. Indeed, many studies have reported low to no detection of *Campylobacter* in litter (Pokamunski et al., 1986; Bull et al., 2006; Eberle et al., 2013; Roberts et al., 2013; Thakur et al., 2013; Brooks et al., 2016).

Roberts et al. (2013) did not detect *Campylobacter* in all 192 litter samples analyzed, while Eberle et al. (2013) detected *Campylobacter* in only 5 of the 2,300 litter samples collected during the grow-out of 4 consecutive flocks. Similarly, Brooks and colleagues did not detect *Campylobacter* in 2 studies conducted to determine the prevalence of pathogens in reused litter (Brooks et al., 2010; Brooks et al., 2016). Pokamunski et al. (1986) found 75 to 100% of cecal contents collected from 4 broiler cohorts raised consecutively on reused litter to be *Campylobacter* positive, however only 2 of the 20 litter samples were positive. Likewise, Bull et al. (2006) found *Campylobacter* in only 3 of 18 samples even though the flock was confirmed to be *Campylobacter* positive by fecal/cloacal sampling. The authors of these earlier studies argued that the litter environmental conditions may not be favorable for *Campylobacter* growth (Eberle et al., 2013) and the presence of high microbial background inhibited the occupation of *Campylobacter*—litter niches and/or prohibited detection using cultivation techniques (Brooks et al., 2016). Furthermore, it was suggested that *Campylobacter* is in a viable but nonculturable (**VBNC**) state in litter and this prevented its recovery from litter (Roberts et al., 2013; Kassem et al., 2017; Plumblee Lawrence et al., 2021). In fact, Kassem et al. (2017) detected culturable *Campylobacter* in only 7 of 288 litter samples collected over 2 grow-out cycles and downtime, but when the authors used qPCR, they found *Campylobacter* in all pooled litter samples. Together, these findings further support the notion that Campylobacters are in a VBNC state in litter and makes it difficult to compare results across studies.

Compared to *Salmonella*, the grow-out period was not included in the final regression model for *Campylobacter* (Table 4), however, *Campylobacter* prevalence has been reported to be higher during late grow-out than early grow-out (Stern et al., 2001; Chinivasagam et al., 2016; Rauber et al., 2019). Chinivasagam et al. (2016) found only 1 *Campylobacter* positive litter sample on d 7 and 9 of grow-out while 33.3 to 86% of the litter samples were positive later in the grow-out cycles (d 27–51). Rauber Wurfel et al. (2019) and Vaz et al. (2012) monitored 3 flocks of broilers and found no *Campylobacter* positive in litter samples collected on d 7 and 14 whereas 90% of samples at d 21 to 42 were *Campylobacter* positive. These results suggest that a litter sample that is negative for culturable *Campylobacter* during early grow-out cannot be used to predict the occurrence of *Campylobacter* in litter at harvest.

In contrast to *Salmonella*, litter pH affected *Campylobacter* prevalence in litter and was kept in the final logistic model (Table 4). Lowering pH of litter has been shown to reduce the rate of *Campylobacter* colonization in broilers (Line, 2002; Line and Bailey, 2006; Rothrock Jr et al., 2008). Line and Bailey (2006) revealed that amending reused litter with acidifiers such as aluminum and sodium bisulfate (litter pH <5 after acidifier was applied) delayed the colonization of chicks by Campylobacters but found no effect on *Salmonella*. Another study showed that aluminum sulfate significantly decreased *Campylobacter* population in litter (Rothrock Jr et al., 2008). The bactericidal effect of litter acidification has been linked to low pH, moisture, and water activity (Line, 2002; Payne et al., 2002, 2007; Line and Bailey, 2006). Contrastingly, Kassem et al. (2017) reported that pH and moisture had no significant effect on *Campylobacter* occurrence in litter. In our study, we found that the probability of detecting *Campylobacter* increased with a higher pH (pH >7; Table 4). This ambiguous result may be attributed to the low detection of Campylobacters in litter and the differences in matrices examined. For instance, Kassem et al. (2017) found *Campylobacter* in only 2.4% of litter samples, while Line (2002) confirmed the presence of *Campylobacter* in whole carcass rinsates only. Nonetheless, the significant associations found between environmental parameters and *Campylobacter* prevalence (Table S3) indicates they affect the ecology of *Campylobacter*. Taken together, our results suggest that management practices and environmental factors interact in complex ways with *Salmonella* and *Campylobacter* that cannot be fully explained from the data available.

Commensal bacteria like *E. coli, Enterococcus* spp., and *Staphylococcus* spp. are widely used as indicators for the microbiological safety of broiler litter (Brooks et al., 2010; Winkler et al., 2017). Fecal indicator bacteria have been reported to be present in varying concentrations in litter (Lu et al., 2003; Winkler et al., 2017). Furthermore, longitudinal studies have shown that the grow-out period, the number of flocks, broiler age, and intrahouse sampling location significantly affect the ecology of indicator bacteria in litter (Roberts et al., 2013; Wei et al., 2013; Winkler et al., 2017). Here, we provide new data on the effect management practices have on the ecology of indicator bacteria. We observed significant interactions between the management factors investigated suggesting that these parameters collectively affected the abundance of commensal bacteria in litter.

Broiler age has been reported as the most significant factor associated with the abundance of indicator bacteria in reused litter collected from 3 flocks of broilers (Roberts et al., 2013). For each grow-out cycle, the authors (Roberts et al., 2013) reported a significant gradual increase in abundance per week for *Enterococcus* spp and *Staphylococcus* spp. In this study, *Enterococcus* and *Staphylococcus* abundance in litter was higher when the chickens were 32 to 38 days old (late grow-out) than when the chicks were 4 to 14 days old (early grow-out), while *E. coli* were higher during the early grow-out period (Table 3). Williams, (2012) also showed that *E. coli* abundance in litter increased from d 1 to 21 of grow-out and decreased from d 35 to 49. The significant difference in abundance between *Enterococcus/Staphylococcus* spp. and *E. coli* suggest that the relative abundance of these bacterial species changes as the chicks developed a matured gut microbiome.

The brood end of the broiler house (front/mid-front) harbored significantly higher abundance of indicator bacteria than the back/mid-back. This result supports other findings that have shown that indicator bacteria are spatially distributed across the litter in a broiler house (Roberts et al., 2013; Brooks et al., 2016). The practice of partial/half house brooding involves the partitioning off half of the broiler house for the first 2 wk of grow-out. As the broiler chicks get bigger, the partitions are removed and the whole house becomes available to the entire flock. Thus, the non-brood end houses the broilers from 2-wk-old until harvest, while the brood end has broilers from placement through harvest (Roberts et al., 2013). Therefore, it is not surprising that commensal bacteria abundance in litter will differ between the 2 halves of the house.

Brooks et al. (2016) reported that the proximity of the litter sampling location to water lines, feeder lines, and the eastern side wall of the broiler house was significantly associated with indicator bacteria abundance and were highest among locations and suggests that higher moisture in these areas may allow for specific bacterial populations to proliferate in these areas. Roberts et al. (2013) reported no significant difference in abundance for any of the bacterium surveyed when comparing brood versus non-brood halves of the house but found significant differences in moisture levels between sampling locations. In this study, litter moisture was significantly associated with *Enterococcus* and *Staphylococcus* spp. abundance while litter pH was associated with the abundance of all three indicator bacteria (Figure 2, Table S4). Litter from the front/mid-front sections of the house had higher moisture than the back section of the house (Figure 2). Contrastingly, litter from the back/mid-back had higher pH than front/mid-front section of the house (Figure 2). The difference in litter physicochemical properties could explain the differential abundance of commensal bacteria between sampling locations.

We have provided new data on the prevalence of pathogens and commensal bacteria in peanut-hull based litter and our results are slightly comparable to findings on pine shaving and rice hull-based litter. In addition, we demonstrated that management practices and environmental factors affected *Salmonella* and *Campylobacter* differently and suggest that each pathogen will require its own tailored intervention to reduce its occurrence in broiler litter. Nonetheless, there are several limitations of this study that could have biased our interpretation of the results including the small number of flocks monitored and the overall low prevalence of *Salmonella*. The salmonellacidal effect of litter reused was modeled to diminish after it has been reused $\sim$ 6 times (Machado Junior et al., 2020). Here, we only investigated 2 consecutive litter reuse events. Furthermore, the inherent limit of detection of the methods used may have underestimated the prevalence of pathogens in the litter samples. Lastly, the results from this one farm might not be representative of all farms that reuse peanut hull as bedding for broiler chicks.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that

could have appeared to influence the work reported in this paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102313.

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