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Sampling method influences *Salmonella* detection and quantification in pre-harvest commercial broiler production

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ABSTRACT

Effective pre-harvest *Salmonella* monitoring in broilers relies on accurate, reliable, and reproducible evaluation of pre-harvest *Salmonella*. In this study, six sampling methods were evaluated and compared to assess *Salmonella* prevalence and quantification during broiler production across three iterative experiments. In experiment one, bootsocks, electrostatic pad-rollers, feather swabs, cloacal swabs, fecal grabs, and litter grabs were collected from 24 houses across 10 farms (n = 288 samples). In the second experiment, bootsocks, bootsock-rollers, and feather swabs were collected in 16 houses on seven farms (n = 128). Bootsocks and bootsock-rollers were selected as the most reproducible sampling method. In experiment three both methods were performed in triplicate in 20 houses on 10 farms (n = 240). In all experiments, prevalence was determined by qPCR and by culture, then compared by Fisher's Exact test between and McNemar's test within methods. *Salmonella* was quantified by qPCR and Ct-values were compared using one-sided F-test. In experiment one, prevalence differed between methods by qPCR ($p = 0.0150$) only and the best performing sampling methods were bootsocks (42/48 culture and 41/48 qPCR positive), feather swabs (42/48 and 36/48), and electrostatic pad-rollers (35/48 and 34/48). In experiment 2, feather swabs differed by qPCR prevalence ($p = 0.0004$). Bootsocks (30/32 culture and 28/32 qPCR positive) and bootsock-rollers (31/32 and 32/32) performed best. In experiment 3, qPCR prevalence (210/240) was greater than culture (167/240) ($p = 0.0021$), but no differences were observed between methods or replicates. The average Ct-value for bootsocks and bootsock-rollers were 37.8 and 38.9, respectively and there was no difference in their variance ($p = 0.8061$). A linear mixed-effect model found that farm contributed 36.34 % of the variance observed while house, house-side, and replicate accounted for 24.69 %, 4.68 % and 3.85 %, respectively. This study shows that sampling methods directly influence both *Salmonella* detection and load recovery. For surveillance sampling, bootsocks and bootsock-rollers were found to best indicate pre-harvest *Salmonella*. These two methods were highly reproducible, user friendly, and provide the most reliable *Salmonella* results indicating the *Salmonella* prevalence in broiler flocks during production.

Introduction

Annually, there are approximately 1.35 million human salmonellosis cases per year in the United States, and *Salmonella* is a leading bacterial cause of human foodborne illness (Centers for Disease Control and Prevention 2023). Poultry integrators apply antimicrobial interventions during processing and between 2016-2022 successfully reduced *Salmonella* incidence in broiler parts by 53.3 % (16.7 % positive in 2016 to 7.8 % in 2022) (United States Department of Agriculture – Food Safety and Inspection Service 2023; Zhang et al. 2019). However, the proportion of annual human illnesses attributed to the consumption of contaminated poultry has not substantially decreased, with the Interagency Food Safety Analytics Collaboration reporting that 18.6 % of cases in 2021 are attributed to chicken meat (Interagency Food Safety Analytics Collaboration, 2023). This suggests that current interventions, which are mainly applied during broiler processing, are not sufficient to reduce human salmonellosis. It also highlights the need to monitor pre-harvest *Salmonella* to understand the load coming into the processing facility. Incoming *Salmonella* load could become a useful indicator for the processing plant, as it could help tailor interventions to guide directed

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processing efforts (Chavez-Velado et al. 2024; De Villena et al. 2022). This naturally raises the question of whether current surveillance and sampling programs are adequate to comprehend the dynamics of *Salmonella* during poultry production.

To better understand and mitigate *Salmonella* associated food safety risks in broiler meat products, poultry integrators monitor this pathogen both in live production to predict incoming *Salmonella* status and at the processing facility to monitor process controls. *Salmonella* surveillance in broiler production has multiple critical objectives. First, it provides information about the *Salmonella* status (e.g., prevalence, load, serovar (s)) of individual broiler flocks. As part of a risk assessment analysis, this information could help predict incoming *Salmonella* prior to the arrival of broilers at the processing facility and could guide directed processing efforts. Second, it also enables monitoring of the efficacy of on-farm *Salmonella* control programs (e.g., vaccination), and third, it could influence decisions regarding antimicrobial intervention application to specific flocks.

Salmonella screening can consist of determining *Salmonella* prevalence as well as quantifying *Salmonella* load. Prevalence can be measured by conventional culture and isolation or by molecular assays such as qPCR. Conventional culture and isolation are the gold standard for *Salmonella* isolation but are labor and time intensive, taking at least four to five days. Conversely, molecular assays can indicate *Salmonella* prevalence in approximately one day (Lungu et al. 2012). In addition, molecular detection offers the added benefits of heightened sensitivity and specificity as compared to conventional culture-based detection, however it can capture both dead and viable but not culturable cells that may overestimate *Salmonella* prevalence (Li et al. 2020; Lloyd et al. 2020; Lungu et al. 2012; Schoonbroodt et al. 2022; Thilakarathna et al. 2022). In addition to allowing more high throughput analyses, the greatest advantage of a molecular assay is that it is rapid. A quick result provides time to implement actions to reduce *Salmonella* or to make processing decisions based on *Salmonella* status. In turn rapid results could also allow samples to be collected closer to processing age, which may better predict the *Salmonella* status of a flock at processing.

In addition to prevalence, *Salmonella* quantification is an important metric to determine whether the incoming *Salmonella* load is too high for the in-place antimicrobial interventions, and therefore may overwhelm interventions used during processing. The draw of this approach to evaluate *Salmonella* is that quantification of *Salmonella* in all levels of vertically integrated production could be reproducibly measured and *Salmonella* reductions can be directly and numerically estimated and evaluated (Chaney et al. 2022). Traditional *Salmonella* quantification has been assessed using most probable number (MPN) approaches, but these are cumbersome and labor intensive, which can limit sample throughput (Berghaus et al. 2013; Dunn et al. 2022; Gutierrez et al. 2020; Payne et al. 2006). Enumeration of *Salmonella* by qPCR approaches are an emerging technology; the rapidity and high throughput capacity makes them attractive alternatives for quantifying *Salmonella* (Applegate et al. 2023; Chaney et al. 2022; Eriksson and Aspan 2007; Hyeon et al. 2019; Ibrahim et al. 2014; Kasturi and Drgon 2017; Löfström et al. 2004; Lungu et al. 2012; Thilakarathna et al. 2022; Xin et al. 2021). Molecular quantification of *Salmonella* during production could be used to measure the efficacy of on-farm pathogen management and it could be utilized to guide directed processing efforts to reduce *Salmonella* cross-contamination between flocks at the processing facility (Obe et al. 2023).

While there is currently no standardized sampling method for *Salmonella* surveillance in broiler flocks, there are many sampling methods that have been and are currently being used to detect and quantify *Salmonella*. Commonly employed non-invasive sampling methods have included bootsocks, drag swabs, fecal grabs, cecal samples, and litter grabs (Bailey et al. 2001; Berghaus et al. 2013; Buhr et al. 2007; Byrd et al. 1997; Fanelli et al. 1970; Galton et al. 1955; Hacking et al. 1978; Hayes et al. 2000; Kingston 1981; Olesiuk et al. 1969; Renwick et al. 1992; Rigby and Pettit 1980; Roberts et al. 2013; Skov et al. 1999; Snoeyenbos et al. 1967; Velasquez et al. 2018). At present, bootsock sampling is the most commonly used *Salmonella* surveillance sampling method employed by integrators and researchers (Bourassa 2016; Buhr et al. 2007; United States Department of Agriculture – Animal and Plant Health Inspection Service 2024). The efficacy of bootsocks at detecting *Salmonella* from broiler house samples has been well-documented, and this sampling method has consistently performed equivalently or superior to other methods when reporting *Salmonella* prevalence (Berghaus et al. 2013; Buhr et al. 2007; Caldwell et al. 1998; Lungu et al. 2012; McCrea et al. 2005; Skov et al. 1999; Talorico et al. 2021). Additionally, bootsock sampling requires minimal labor and training to collect, making it a feasible sample for an integrator to apply during production. Historically, drag swabs have been one of the main alternatives to bootsocks, though this method does not reflect the same proportion of *Salmonella* prevalence samples in a house as bootsocks (Berghaus et al. 2013; Buhr et al. 2007; Caldwell et al. 1998; McCrea et al. 2005). Cloacal swabs and fecal grabs facilitate the detection of *Salmonella* that is actively being shed by the broilers (Buhr et al. 2007; Olesiuk et al. 1969; Snoeyenbos et al. 1969), while litter samples can indicate both the historic (Bhatia et al. 1979; Hacking et al. 1978) and current (Buhr et al. 2007; Poppe et al. 1991; Snoeyenbos et al. 1969) *Salmonella* status of broilers in a house. Feather swabs are a sampling method that can be used to determine the external carriage of *Salmonella* on broiler feathers and may therefore indicate the serovars of *Salmonella* that are more likely to be detected in the processing plant at pre-scald (Slader et al. 2002; Zeng et al. 2021). Feather and cloacal swabs are attractive sample types as they are evaluating *Salmonella* on and in the bird and will more likely represent *Salmonella* found at processing. Nonetheless, a disadvantage to both these methods is the need for broiler handling, which may induce added distress on the sampled broilers (Kannan and Mench 1997). Similarly, cecal samples are used for studies evaluating microbiota challenges and *Salmonella* colonization (Arsenault et al. 2007; Chaney et al. 2022; Robinson et al. 2022; Sheets et al. 2022; Stern 2008). These samples can provide an overview of current *Salmonella* shedding, however cecal analysis requires culling the birds. For sample types where individual birds are sampled (i.e., ceca, and feather and cloacal swabs), a further disadvantage is not knowing how many samples are required that would represent the whole flock. Lastly, roller sampling has emerged as an alternative to bootsocks, as it can be used to collect *Salmonella* from the broiler environment (Talorico et al. 2021). Modified roller samples utilizing electrostatic pads have additionally been used in farm and clinical veterinary environments to detect *Salmonella* (Burgess and Morley 2018; Burgess et al. 2004; Ruple-Czerniak et al. 2014; Zewde et al. 2009).

New molecular-based (PCR) detection methods have facilitated rapid and feasible methodologies for *Salmonella* detection and enumeration in broiler production but with these advancements have come the realization that gold-standard sampling methods for *Salmonella* may need to be reevaluated. For example, a recent study assayed 80 broiler commercial broiler houses using bootsocks and found that in the 53 houses where both pairs of boot socks were positive for *Salmonella*, 71.7 % (38/53) of the houses had *Salmonella* quantities differing by ≥ 1 $\log_{10}$ CFU using a commercially available qPCR assay (Obe et al. 2023). This discrepancy in *Salmonella* load from paired samples within the same house suggests a need to evaluate different sampling methods using new molecular tools for detection and quantification. This data is critical for both developing a standardized method of surveying *Salmonella* and to effectively and meaningfully screen pre-harvest broiler flocks to predict incoming load prior to processing.

The objectives of this study were to determine the most effective pre-harvest *Salmonella* sampling method for the industry to apply in day-to-day production, by comparing *Salmonella* prevalence screening via conventional culture and molecular detection, and by assessing *Salmonella* load using a commercial qPCR assay. This was accomplished in three iterative experiments, starting with evaluation of six different sample methods and culminating in two that performed best.

Materials and methods

Study overview

The present study employed an iterative process to identify the most optimal sampling methods for assessing *Salmonella* prevalence and load in commercial broiler houses. This process encompassed three distinct and independent experiments. Given the iterative nature of the current study, each experiment had its own experimental design based on the findings of the previous experiment. The broiler houses evaluated in each experiment were unique and not resampled. Broiler flocks in all three experiments were aged 27-29 days at the time of sample collection. This time point was selected based on the industry average of birds being processed at 42 days. For pre-harvest *Salmonella* testing by industry, this timing would coincide with AI and pesticide residue testing 14 days before harvest which means sampling could be completed as part of that visit and not necessitate an additional farm visit. Within each house, left and right house sides were assigned by standing at the evaporative cooling cell end of the house and looking across the length of the house towards the tunnel fan end of the house.

Experimental design

Experiment 1. For the first experiment, six sampling methods were evaluated across 10 farms and 24 houses. The sampling methods evaluated were bootsocks, cloacal swabs, electrostatic pad-rollers (ESP-rollers, these were dry Swiffer pads; Swiffer, Proctor & Gamble, Toronto, Ontario, Canada), feather swabs, and fecal grabs, litter grabs. For each sampling method, one sample was collected independently on each side of every house (see details below). In the first experiment, a total of 48 samples were collected of each type, for a total of 288 samples obtained across the entire experiment. For some sampling methods, the middle point of each house needed to be determined, and this was defined by where the feed hoppers and antechamber were located. Supplemental Figure 1A depicts where samples of each type were collected in each house as part of the first experiment.

Experiment 2. In the second experiment, three sampling methods were evaluated across seven farms and 16 houses. The sampling methods evaluated included bootsocks, bootsock-rollers, and feather swabs. One sample was collected from both sides (left and right) of the house for bootsocks and bootsock-rollers, whereas for feather swabs, one sample was collected from each quadrant of every house. In this experiment a total of 32 samples were collected of both bootsocks and bootsock-rollers and 64 feather swab samples were collected across the entire experiment. Supplemental Figure 1B depicts where samples of each type were collected in each house as part of the second experiment.

Experiment 3. In the third experiment, two sampling methods – bootsocks and bootsock-rollers – were evaluated across 10 farms and 20 houses. Each sampling method was collected from each side of a house in triplicate, for a total of six samples per house of each method, resulting in 240 total samples across both methods in the third experiment. Supplemental Figure 1C depicts where samples of each type were collected in each house as part of the third experiment.

Sampling methods

Bootsock Sampling. For all three experiments, pairs of sterile boot swabs in twirl tie bags pre-moistened with 20 mL buffered peptone water (Romer Labs Inc., Newark, DE) were utilized in this study for bootsock sampling. To sample each side, a single bootsock from a pair was placed on one foot of the sample collector, who then walked slowly on the litter in a straight line with the covered foot under the inner water line of that side of the house. At the middle point of the house, the bootsock was removed and returned to the bag. The second bootsock of the pair was then put on and used to sample the final length of the side and returned to the same bag. This process was repeated on the opposite side of the house with a fresh pair of bootsocks.

Roller Sampling. In the first experiment, ESP-roller samples were collected using an unscented dry sweeping cloth (Swiffer, Proctor & Gamble, Toronto, Ontario, Canada). The ESP was placed around a 25 cm long, 5 cm diameter PVC pipe (Charlotte Pipe, Charlotte, NC) fitted to a paint roller on a 1.21 m stick and secured using disinfected rubber bands. The ESP-roller was run over the litter directly under the inner water line of each house side. The process was repeated on the opposite side of the house with a fresh ESP. In the second experiment, a bootsock (as above, pre-moistened with buffered peptone water) was used in place of the ESP. A single bootsock was secured to the PVC pipe using disinfected rubber bands. At the halfway point down the house, this was replaced with the second bootsock of the pair. Both bootsocks were placed together in a single bag. This process was repeated on the opposite side of the house. For the third experiment, the process was identical, with the addition that samples were collected in triplicate. In all three experiments, within a single house, the same person performed the bootsock sampling and roller sampling.

Feather Swab Sampling. Feather swab samples were collected using bootsocks. In experiment 1, the feathers of six individual broilers were swabbed on each side of the house (three per quadrant) for a total of 12 broilers per house and 288 birds. Each bird was swabbed in a pattern meant to minimize distress associated with handling. A bootsock was placed on one of the sampler's hands over clean gloves and a broiler was caught and suspended by the sample collector from the shanks using the other hand. First, the ventral (breast) surface was swabbed in a motion extending from caudal to cranial and back, twice. After this, the ventral surface of the broiler was rested against the thigh of the sample collector and the left and right sides of the sagittal plane along the body of the broiler extending from the thigh to the shoulder joint of the wing were swabbed. Next, the dorsal (back) surface of the broiler was sampled, again from caudal to cranial, twice. The skin of the neck was next swabbed twice using a grabbing motion. Finally, both wings were sampled by wrapping the bootsock around the wing and swabbing along the entire wing surface from proximal to distal, twice. The same bootsock was used to sample two additional birds. The process was repeated on the opposite side of the house using the second bootsock of the pair. In experiment 2, the feathers of eight individual broilers were swabbed on each side of the house (four per quadrant) for a total of 16 birds per house and 256 birds across the experiment. Here, a pair of bootsock were used to sample four birds (two birds per swab), for a total of four samples per house, generating 64 feather swab samples in total.

Cloaca Swab Sampling. Using the same broilers that were feather swabbed, cloacal swabbing was conducted using cotton fiber tipped wooden applicators (Thermo Fisher Scientific, Waltham, Massachusetts). A new sterile swab was utilized to swab each individual broiler by inserting the cotton tip of the swab approximately 2 cm into the cloaca and rotating five times with gentle pressure to collect mucous, epithelial cells, and feces from the cloaca before releasing the broiler. Following cloaca swab collection, the swab was pooled in a 50 ml conical tube containing 3 ml of BPW with the other cloaca swabs collected on that side of the house then stored on ice. A total of six cloacal swabs made up each pooled sample, with 12 broilers sampled in each house.

Fecal Grab Sampling. From each side of the house, 10 fresh fecal grabs were collected, five per quadrant, from along the inner water lines using disinfected metal catering tongs then pooled together in a sample collection bag. The process was repeated on the opposite side of the house.

Litter Grab Sampling. Top litter grabs were collected from each side of the broiler house using disinfected metal catering tongs. Each top litter grab was collected as a 4.5 × 6 cm surface grab up to a litter depth of approximately 2 cm. For each side, 10 top litter samples were collected, five per quadrant, and pooled together for each side. After placing the sample in the sample bag, the samples were hand mixed to break the grabs up and to homogenize the pooled sample. The process was repeated on the opposite side of the house.

Salmonella assessment

Culture. Following collection, samples were transported back to the laboratory on ice and stored at 4 °C; culture began on all samples within 24 h of collection. In the laboratory, fecal and litter grab samples were hand massaged, then homogenized in a stomacher (Stomacher 400 Circulator, Seward, Worthing, UK) for 1 min at 230 rpm. Next, Buffered Peptone Water (BPW; Hardy Diagnostics, Santa Maria, CA) was added to all samples as follows: For fecal and litter grab samples, 250 mL BPW was added to 25 g homogenate. For samples that used bootsocks (bootsocks, rollers, and feather swabs), 200 mL BPW was added to each sample bag. Finally, for cloacal swabs, 80 mL BPW was added to each sample. Each sample was then stomached for 1 min at 230 rpm (Stomacher 400 Circulator, Seward, Worthing, UK) to form a homogenate, which was used for all subsequent tests. For culture analysis, 1:1 dilutions of homogenate:pre-warmed BPW were prepared for each sample. This involved combining 60 mL for fecal grab, litter grab, and all bootsock samples and 30 mL for cloacal swab samples into an equal volume of pre-warmed BPW. Next, samples were incubated for 20-24 h at 37 °C in a shaking incubator. After 24 h, 1 mL of each sample was transferred to 10 mL of tetrathionate broth (TT; Hardy Diagnostics, Santa Maria, CA) supplemented with 200 μL of iodine (Remel, Lenexa, KS) for selective enrichment, and incubated, stationary, at 37 °C for 20-24 h. Next, samples were streaked onto Xylose Lysine Tergitol-4 (XLT-4; Hardy Diagnostics, Santa Maria, CA) with Difco XLT-4 agar supplement (Becton, Dickinson and Company, Sparks, MD) and Harlequin Chromogenic Agar for *Salmonella* Esterase (CASE; Neogen, Lansing, MI) to determine culture prevalence. These plates were incubated stationary for 24 h at 37 °C then determined to be positive or negative. After streaking on to Luria-Bertani (LB; VWR Chemicals, Solon, OH) agar, isolates were confirmed using *Salmonella* antiserum poly A-I & Vi (Becton, Dickinson and Company, Sparks, MD). Any samples that did not agglutinate were screened using the *InvA* PCR as described previously (Cheng et al. 2008), to confirm whether they were *Salmonella* positive.

Molecular detection and quantification. Each BPW homogenate (see above) was diluted to 1:1 ratio of homogenate:pre-warmed (42°C) BAX System MP media (MP; Hygiena, Camarillo, CA) supplemented with novobiocin (Thermo Fisher Scientific, Waltham, MA) for a final concentration of 40 mg/L of novobiocin in each sample. For fecal grab, litter grab, and all bootsock samples, 60 mL of each sample was added to 60 mL of pre-warmed MP. For cloacal swabs, 30 mL of homogenate was added to 30 mL of MP. Samples were incubated at 42 °C for 24 h. Following the manufacturer's instructions, the BAX-*Salmonella* PCR assay kit (Hygiena, Camarillo, CA) was used to generate a lysate following 10 h of incubation and again following 24 h of culture. Lysates were stored at 4 °C for up to two days. The 24 h lysate was assessed first to determine *Salmonella* prevalence. If samples were positive, we returned to the 10 h lysate and used this to quantify *Salmonella.* As quantification curves have not been validated for some of the sampling methods under evaluation in this study (rollers, feces, litter, and feather swabs), comparisons of quantification were made by assessing variance instead of means. The Ct values were determined using version 3.6 of the SalQuant software (Hygiena, Camarillo, CA).

Statistical analysis

To evaluate *Salmonella* prevalence in all experiments, Fisher's Exact test was used to determine differences among sampling methods, taking into consideration that each sampling method was independent from each other. To compare *Salmonella* prevalence through molecular detection against conventional culture-based prevalence within each sampling method, the Chi-Square McNemar's test was employed, as observations were not independent (Supplemental Figure 2). To evaluate *Salmonella* quantification in experiment three, it was imperative to acknowledge the hierarchical and ordered structure of the observations, which was done by constructing a linear mixed-effect model that considered the respective fixed and random effects. To assess if one sampling method was more variable than the other when reporting *Salmonella* load, one-sided F tests were performed. All statistical tests were performed in R version 4.1.1 and significant differences were established at $p < 0.05$.

Results

First Experiment: evaluating six sampling methods

Overall, the *Salmonella* prevalence across all sampling methods was 82.6 % (238/288 samples) when screened by culture and 69.1 % (199/288) when screened using qPCR. By culture, the prevalence ranged from 87.5 % (42/48, for bootsocks and feather swabs) to 72.9 % (35/48, for ESP-rollers), though there were no significant differences observed between sampling methods (Fisher's Exact test, $p = 0.4378$) (Fig. 1). However, when the same samples were screened by qPCR, significant differences emerged between sampling methods ($p = 0.0146$), and the prevalence ranged from 85.4 % (41/48, for bootsocks) to 52.1 % (25/48, for litter grabs). When comparing each sampling method, bootsocks, cloacal swabs, ESP-rollers, and feather swabs detected higher *Salmonella* prevalence compared to fecal grab and litter grab samples. By comparing culture and qPCR prevalence for each sampling method, *Salmonella* prevalence did not differ in bootsock, ESP-roller, or feather swab samples (Fig. 1; Supplemental Figure 2). The qPCR under-reported *Salmonella* prevalence when using cloacal swabs (Chi-square McNemar's test, $p = 0.0027$), fecal grabs ($p = 0.0114$), and litter grabs ($p = 0.0028$).

This experiment demonstrates that both the choice of sampling method and screening methodology influences *Salmonella* prevalence results. In the first experiment, the challenges of litter grabs, fecal grabs, and cloacal swabs to adequately report *Salmonella* prevalence via qPCR demonstrate the shortcomings of implementing these sampling methods in commercial broiler production, perhaps due to high background flora in these sample types. In contrast, the prevalence data reported by bootsocks, ESP-rollers, and feather swabs were comparable, and results did not differ from culture to qPCR.

Second experiment: evaluating bootsocks, bootsock-rollers, and feather swabs

Based on their performance in Experiment 1, we re-evaluated bootsocks, rollers, and feather swabs with some modifications. The low number of feather swabs that were qPCR positive (75 %; 36/48) compared to culture (87.5 %; 42/48) suggested that while there was no significant difference between the two detection methods, the qPCR was not efficient, which might be caused by high background microflora levels. To address this, we reduced the sample complexity on each swab: instead of swabbing six birds with each pair of swabs (three per swab), we swabbed four birds per pair of swabs, increasing the number of samples to four per house, and sampling 16 birds in each house. For the ESP-rollers, we suspected that the high moisture in the litter negated the electrostatic nature of the wipes and because the prevalence on this sampling method was lower, these were switched to bootsocks (pre-moistened in 20 mL of BPW) to try and improve *Salmonella* recovery.

Overall, the *Salmonella* prevalence observed across all sampling methods was 91.4 % (117/128) and 82 % (105/128) when screened by culture and qPCR, respectively. As in the first experiment, no significant differences were observed between sampling methods when screened via culture (Fisher's Exact test; $p = 0.3517$) (Fig. 2). For the culture-based prevalence, 96.9 % (31/32) of bootsock samples were *Salmonella* positive, compared to 93.8 % (30/32) of bootsock rollers and 87.5 % (56/64) of feather swabs. As in the first experiment, differences emerged between sampling methods when the samples were screened by qPCR ($p = 0.0004$). Bootsock-roller *Salmonella* incidence was higher, 100 % (32/32) when compared to feather swabs (70.3 %; 45/64). For both culture and qPCR, the bootsock rollers performed better than the

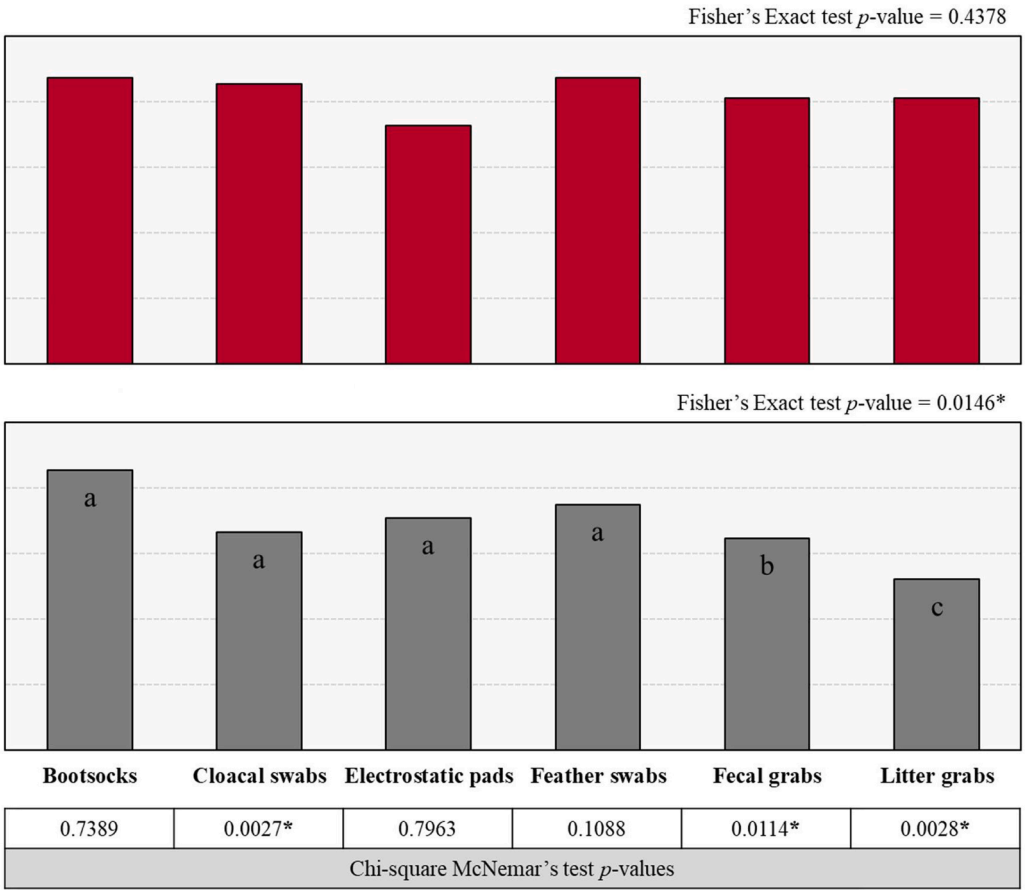


Fig. 1. *Salmonella* prevalence determined by culture and qPCR for the first experiment. In the first experiment, bootsocks, cloacal swabs, ESP-rollers, feather swabs, fecal grabs, and litter grabs were used to sample 24 houses on 10 farms. For each sampling method there were n = 48 samples. *: Significant differences were established at $p < 0.05$ for Fisher's Exact test and Chi-square McNemar test. Bars with different alphabets (qPCR prevalence) show differences across sampling methodologies by the Fisher's Exact test. There were no differences between sample types by culture. McNemar test *p*-values (bottom) compare culture and qPCR outcomes within the same sampling method.

ESP-rollers in Experiment 1. However, bootsocks did not differ from either of the other two sampling methods evaluated during the second experiment, with a prevalence of 87.5 % (28/32). In the case of bootsocks (Chi-square McNemar's test; $p = 0.1797$; Supplemental Figure 2) and bootsock-rollers ($p = 0.1573$), no differences were observed between the culture and qPCR results. Conversely, for feather swabs, we observed a significant difference between culture and qPCR ($p = 0.0076$), which is explained by lower detection by qPCR (70 %; 45/64) than culture (87.5 %; 56/64), supporting the findings from Experiment 1. The results of this experiment demonstrate that bootsocks and bootsock-rollers were higher recovery sampling methods to detect *Salmonella* prevalence by both culture and qPCR.

Third experiment: evaluating repeatability of bootsocks and bootsock-rollers and Salmonella quantification

The iterative approach of Experiments 1 and 2 resulted in selection of bootsocks and bootsock-rollers as the best sampling methods we evaluated. Therefore, the purpose of Experiment 3 was to determine the reproducibility of these two sampling methods at detecting and quantifying *Salmonella* in broiler houses. Overall, the *Salmonella* prevalence observed across both sampling methods in the third experiment was 87.5 % (210/240) and 69.6 % (167/240) when screened by qPCR and culture, respectively (Fig. 3). When examining each detection method, there was no significant difference in *Salmonella* prevalence observed between bootsocks and bootsock-rollers (Fisher's Exact test, $p = 0.5748$ and $p = 0.8456$, respectively). Also, there were no differences in *Salmonella* prevalence by culture across the three replicates for either bootsocks ($p = 0.8037$) or bootsock-rollers ($p = 0.8194$). Similarly, there were no differences across the three replicates for qPCR within each sampling method ($p = 0.679$ for bootsocks; $p = 0.8394$ for bootsock-rollers). However, when the culture-based and qPCR-based prevalence were compared within a same sampling method, differences emerged for both bootsocks (Chi-square McNemar's test; $p < 0.0001$; Supplemental Figure 2) and bootsock-rollers ($p = 0.0021$). Unlike the previous two experiments, the qPCR-based prevalence of both bootsocks (88.3 %; 106/120) and bootsock-rollers (86.7 %; 104/120) was notably higher compared to their corresponding culture-based prevalence (bootsocks: 67.5 %, 81/120; bootsock-rollers: 71.7 %, 86/120). This demonstrates that both sampling methods can effectively detect *Salmonella* prevalence from broiler houses.

To evaluate *Salmonella* load, only the samples that were *Salmonella* positive by qPCR (i.e., from the 24-hour lysate) were analyzed with the same qPCR kit using the 10-hour lysate to assess differences in *Salmonella* amounts between samples. For this study, only samples that generated a Ct value were included in the subsequent analysis, resulting in n = 101 for bootsocks (out of 106 qPCR positive bootsock samples) and n = 93 for bootsock-rollers (of 104 qPCR positive bootsock-roller samples). We used a linear mixed-effect model where Ct value was the dependent variable and sampling method (bootsocks and bootsock rollers) was considered a fixed effect. Farm, house, and replicate were considered hierarchical (farm>house>replicate) and ordered (replicates: 1, 2, or 3) random effects. The model showed that the two sampling methods reported different Ct values ($p = 0.000408$). The average

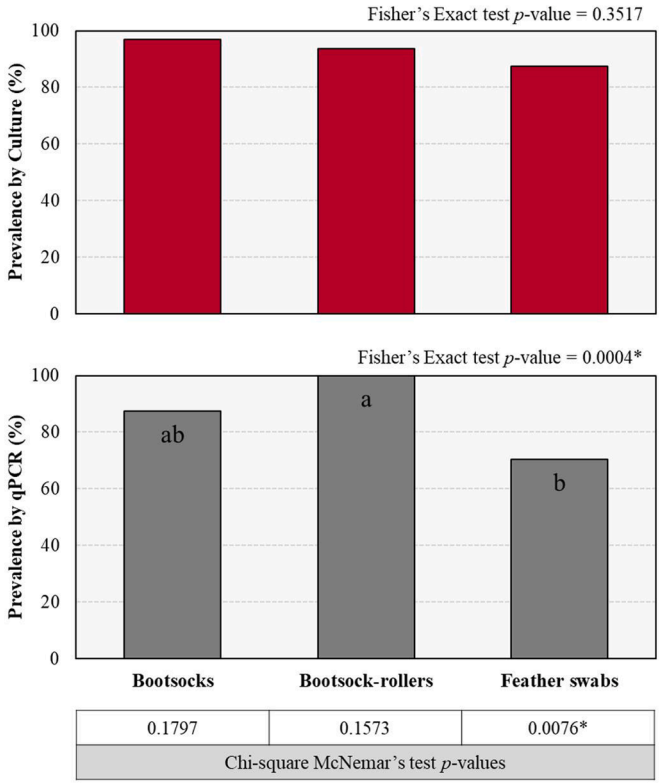


Fig. 2. *Salmonella* prevalence determined by culture and qPCR for the second experiment. In the second experiment, bootsocks, bootsock-rollers, and feather swabs were used to sample 16 houses on seven farms. For each sampling method there were n = 32 for Bootsocks and Bootsocks-rollers, and n = 64 for Feather swabs. *: Significant differences were established at $p < 0.05$ for Fisher's Exact test and McNemar test. Bars with different alphabets (qPCR prevalence) show differences across sampling methodologies by the Fisher's Exact test. There were no differences between sample types by culture. McNemar test *p*-values (bottom) compare culture and qPCR outcomes within the same sampling method.

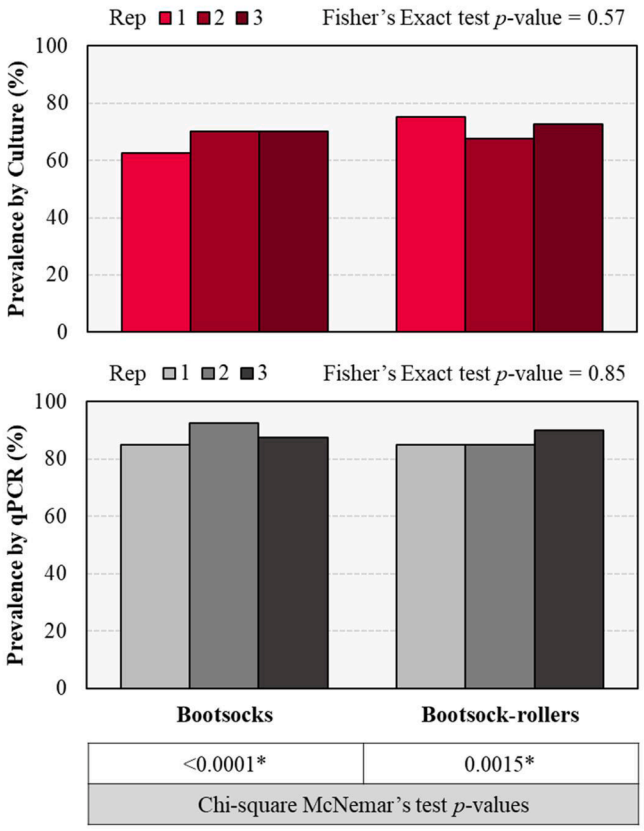


Fig. 3. *Salmonella* prevalence determined by culture and qPCR for the third experiment. In the third experiment, bootsocks and bootsock-rollerswere used to sample 20 houses on 10 farms in triplicate. For each sampling method there were *Salmonella* prevalence screened by culture and qPCR for the third experiment, n = 40 for Bootsocks and Bootsocks-roller per replicate. *: Significant differences were established at $p < 0.05$ for Fisher's Exact test and McNemar test. McNemar test *p*-values (bottom) compare culture and qPCR outcomes within the same sampling method.

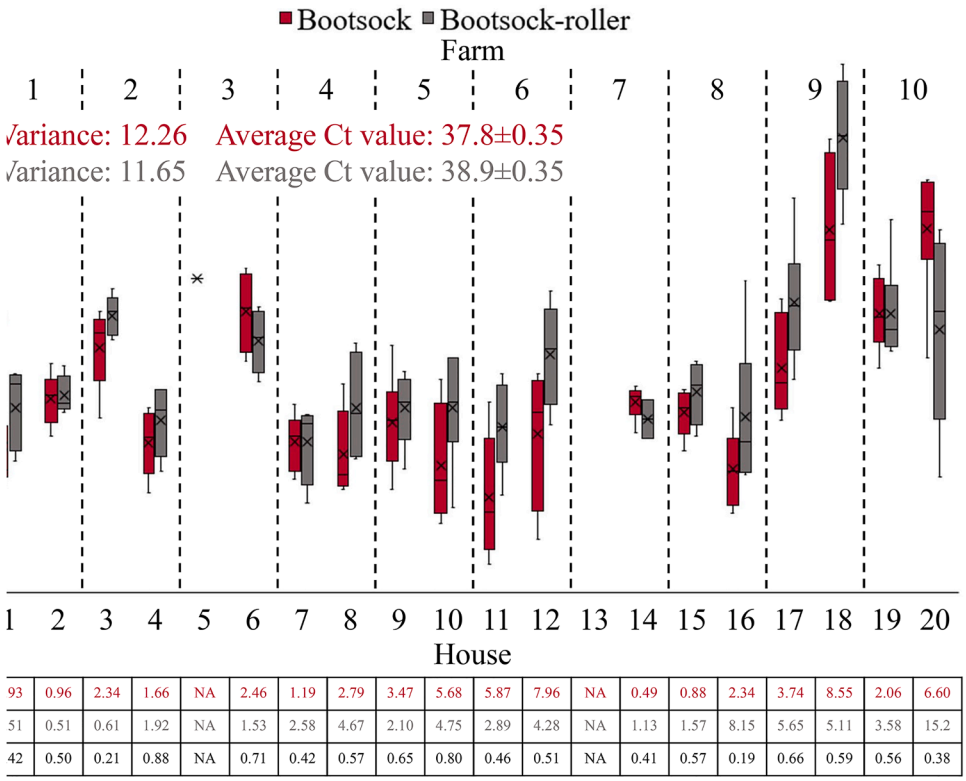
Ct for bootsocks and for rollers (37.8 ± 0.35 and 38.9 ± 0.35, respectively). In the model, the overall total variance was 12.39, which was the sum of total variance for all random effects plus residuals. For the individual random effects, farm best explained *Salmonella* quantity (36.3 %; 4.39) and had nearly 1.5-fold higher influence on the variance of *Salmonella* quantity than house (24.7 %; 2.99; nestled after farm). Side of house explained 4.68 % (0.57) variance and replicate explained the least variance in *Salmonella* quantity in this model (3.85 %; 0.47; nestled after farm then house). This can be interpreted as individual farms are more different to each other than are houses on the same farm. Further, while side of house and replicate introduce some variance, their effect is less impactful.

The model above established a difference in the Ct values means between sampling methods; next, a one-sided F-test was used to determine if the variance differed between sampling methods (Fig. 4). Overall, bootsock variance (var: 12.26, range: 0.49-8.55) was comparable to the bootsock-roller variance (var: 11.65, 0.51-15.2), as their Ct values did not differ significantly (one-sided F-test; $p = 0.8061$). In addition, comparisons of variance within each house were performed also using one-sided F tests, and no houses of the 20 sampled in this experiment reported a difference in variation between bootsocks and bootsock-rollers. This evidence supports that variance observed in Ct values is not inherited from the sampling methods used in this study (bootsocks and bootsock-rollers).

Discussion

To evaluate interventions for improving food safety in broiler production, there is a need to effectively monitor pre-harvest *Salmonella*. This requires a reliable, standardized, and feasible sampling method that can indicate *Salmonella* prevalence via both traditional and molecular means and which can also reproducibly represent the *Salmonella* load in each broiler house. The objective of this study was to evaluate *Salmonella* surveillance sampling methods in commercial broiler production to address this need and to determine the method that best indicates the prevalence and load of *Salmonella* within commercial broiler houses between 27-29 days of age. To be feasible, due to the high volume of samples a surveillance program could generate, the sampling method must be straightforward to collect and affordable to implement. For the latter reason, we did not include cecal samples in our study because collecting enough samples to represent a flock would require culling a large number of birds. In this iterative study, a total of six methods were evaluated across 60 unique houses from 27 farms.

In the first experiment, *Salmonella* prevalence did not differ between sample types when assessed by culture. However, litter and fecal grabs had significantly lower prevalence by qPCR compared to other sample types. Past studies that used culture approaches have shown that *Salmonella* prevalence in broiler litter and feces was low (Berghaus et al. 2013; Buhr et al. 2007; Hacking et al. 1978; Rigby and Pettit 1980), with Buhr and colleagues also demonstrating that both feces and litter significantly underperformed compared to bootsocks at detecting *Salmonella*. One of the possible contributors to this effect could be the



93	0.96	2.34	1.66	NA	2.46	1.19	2.79	3.47	5.68	5.87	7.96	NA	0.49	0.88	2.34	3.74	8.55	2.06	6.60
51	0.51	0.61	1.92	NA	1.53	2.58	4.67	2.10	4.75	2.89	4.28	NA	1.13	1.57	8.15	5.65	5.11	3.58	15.2
42	0.50	0.21	0.88	NA	0.71	0.42	0.57	0.65	0.80	0.46	0.51	NA	0.41	0.57	0.19	0.66	0.59	0.56	0.38

Fig. 4. *Salmonella* load in experiment three samples. *Salmonella* was quantified from bootsock and bootsock-roller samples using a commercial qPCR assay in the third experiment, and statistical comparisons were made using one-sided F-tests ($p = 0.8061$) for bootsock (red) and bootsock-roller (grey) samples. The average variance and Ct value with standard error for each methodology is provided in the bottom left and the variances with *p*-values derived from one-sided F-tests for each house along the bottom. No significant differences were established at $p < 0.05$ between sampling methodologies in each house.

limited surface area and number of grabs that are taken, which is an inherit limitation of these particular sampling methods. For instance, one study took four fecal grabs from the central area of the house, and individually assessed each fecal grab (Berghaus et al. 2013). Subsequently, they reported that fecal grabs showed the lowest prevalence in their study with only 13.6 % *Salmonella* positive samples, which was substantially lower than bootsocks (68.2 %). In the present study, this challenge was addressed by increasing the number of fecal grabs per house (20 per house, pooled into two composites of 10 grabs per side of each house), and by evenly distributing the grab sites along the side of the house. Despite these efforts, fecal grabs in this study underreported *Salmonella* prevalence when screened by qPCR, reporting 20 % less *Salmonella* positive samples than the prevalence observed in bootsock samples (87.5 %).

For a sampling method to generate useful data for integrators, it must generate reproducible and consistent prevalence results by both molecular and traditional culture-based detection. In the first experiment for litter grab, fecal grab, and cloacal swab samples, this was not the case, which led to the discontinuation of these sampling methodologies in subsequent experiments. While we expected to find discordance between culture and qPCR detection, we expected that we would observe higher prevalence by qPCR given the increased sensitivity of this method. However, for these three sampling methods, qPCR underperformed compared to culture. We suspect that this was due to high levels of background microflora in these sample types and we note that fecal, litter, and cloacal samples are not a validated matrix for the commercial qPCR assay used here. There is the potential for adapting sample preparation or culture conditions in the future that may make these sample types more amenable to qPCR prevalence testing. Cloacal swabs were found by our study and others (Velasquez et al. 2018) to outperform other sample types, including litter and feces, at detecting *Salmonella* from broilers but were poor indicators of overall flock prevalence. This is likely due to the small number of cloacal swabs being collected (12) relative to the number of broilers within each house (20,000-44,500) in this study. Further, because collecting cloacal swabs is more labor intensive and necessitates broiler handling, which could induce unnecessary distress on the broilers (Kannan and Mench 1996, 1997). ESP-rollers had the greatest concordance between the two *Salmonella* detection methods but had the lowest proportion of positive samples by culture in this experiment (i.e., larger number of false negative samples). In a recent study where a pre-moistened paint roller cover was used to sample experimental floor pens for *Salmonella*, roller samples were found to be less sensitive than bootsocks but more sensitive than litter grabs (Talorico et al. 2021). This finding and our results led us to modify the sampling methodology in the subsequent experiment from using an ESP secured around the roller to utilizing a pre-moistened bootsock. These bootsock rollers performed better than ESP-rollers, which we suspect is because moist bootsocks improve *Salmonella* recovery compared to the dry ESPs that quickly lose their electrostatic property following contact with the poultry house litter. Bootsocks and feather swabs were the best performing methods for determining prevalence in the first experiment. Feather swab samples represent an external environmental sample that more closely resembles the *Salmonella* profile that ultimately enters the processing facility following harvest than other sampling methodologies as the contamination on the feathers and external broiler surfaces is a leading cause of early processing contamination with *Salmonella* (Slader et al. 2002; Zeng et al. 2021). To increase the recovery with feather swabs by qPCR, in the second experiment we decided to sample fewer broilers with each sample and to collect twice as many samples from each house. As in the first experiment, there were no significant differences across sampling methods by culture-based prevalence in the second experiment. However, by qPCR, feather swab prevalence was significantly lower than prevalence in bootsock rollers. Further, feather swabs also demonstrated

discordance between qPCR and culture prevalence in this experiment. By increasing sampling frequency and decreasing the composited nature of the sample (i.e., reducing the number of broilers per swab) quantity of broilers sampled with each swab in the second experiment, we were unable to reduce the difference in prevalence between detection means. This suggested that feather swabs were not an appropriate sample type for indicating *Salmonella* prevalence. There were no significant differences observed between bootsock and bootsock roller prevalence when comparing culture and qPCR, and it is notable that qPCR prevalence by bootsock rollers was 100 % in this experiment.

In the final experiment, bootsocks and bootsock rollers were sampled sequentially in triplicate from each side of 20 houses across 10 farms. This approach was designed to evaluate the number of repeated sampling events required to capture the best representation of *Salmonella* prevalence and load within each broiler house. No significant differences were observed between the culture or qPCR results across all three replicates and both sampling methods. In both bootsock-rollers and bootsocks, however, there were differences between culture and qPCR prevalence, with prevalence by qPCR being higher for both methods, which contrasts with the results found in both previous experiments. This may reflect high false positives, but as we did not attempt to isolate *Salmonella* from the MP cultures that were used for qPCR, we cannot confirm this. A study using the same qPCR assay in broiler cecal samples noted a number of false negatives, but zero false positives (Chaney et al. 2022). We did consider whether it was a sensitivity issue and that the qPCR was able to detect very low levels of *Salmonella* that could not be detected by culture. However, the Ct values of qPCR-positive and culture-negative samples compared to those of qPCR-positive and culture-positive samples did not differ (two-tailed *t*-test, $p = 0.39$; data not shown).

Quantitative PCR is used to indicate relative increase or decrease in template between samples. Where standard curves are available, this method can be used to calculate a value that indicates the amount of starting template. In Experiment 3, where our goal was to compare different sample types, a standard curve was only available for bootsock samples, but not for rollers. Therefore, we only reported the Ct values and we inferred relative differences in Ct value using comparison of variance. A recent study showed variability across technical replicates using SalQuant on poultry rinsates when Ct values were used with a standard curve to generate logCFU/ml values (Schmidt et al. 2024). We did not use technical replicates in this study, however, we had a total of six biological replicates per method per house (three replicates per side of each house). These replicates generated variance values of 12.26 and 11.65 for bootsocks and rollers, respectively, which did not differ significantly, suggesting no difference between the two methods for indicating the Salmonella load across the dataset.

By evaluating the Ct value data with a linear mixed-effect model, the choice of sample type influenced the amount of *Salmonella* recovered. Across the random effects, farm was the greatest contributor to overall variance followed by house and then side of house, followed by replicate. This highlights that there are more differences in *Salmonella* quantity between farms than between houses on a single farm. The model also showed that houses contributed 24.6 % of the total variance. Further, removal of house as a factor from the model resulted in reduced model fitness (data not shown). This means that sampling a single house may not be indicative of *Salmonella* loads in other houses on the same farm, implying a need to sample multiple houses on a farm. Replicates contributed a small but meaningful variance to the model (3.85 %) as model performance was reduced when replicates were removed as a factor (data not shown), suggesting that a single replicate may not be sufficient to capture *Salmonella* levels. Similarly to replicate, side of house also contributed a small proportion of the variance and its removal decreased model fitness (data not shown); therefore both sides of a house should be sampled. Congruent with our findings, a previous study used a nestled intercept-only multilevel mixed-effects logistic regression model for variance in *Salmonella* prevalence and found that house and farm were important contributors of variance (combined 96.3 % of total variance by intercluster correlations)(Obe et al. 2023).

Our findings contrast with a past study suggesting that as many as five pairs of bootsocks may be required to reliably detect the *Salmonella* present in a broiler house, but the differences may be attributed to differences in detection methodologies applied (Skov et al. 1999). In the present study, it was found that while only one sampling replicate was required and that side did not influence prevalence, sampling both left and right sides was necessary to capture all *Salmonella* present in the houses. As in this study, Skov et al. found that there were no spatial differences in *Salmonella* distribution between sides of broiler houses in terms of *Salmonella* prevalence (Skov et al. 1999). Other studies, however, that have utilized drag swabs and litter grabs suggest that there are spatial differences in *Salmonella* distribution within broiler houses (Hayes et al. 2000; Roberts et al. 2013), supporting our finding that both sides of a broiler house should be sampled to best capture any *Salmonella* present. The differences observed between our results and these past studies may be explained by the greater *Salmonella* detection capabilities of the applied sampling and detection methodologies in the third experiment and serve as support for the decision to discontinue utilizing litter grab samples following the first experiment. When directly comparing the quantification values between bootsocks and bootsock-rollers, there was no difference in the Ct values generated. However, in the linear mixed-effects model that considered other effects, there was a difference between the two methods. While bootsocks had lower Ct values than bootsock-rollers, suggesting better *Salmonella* recovery, this may not be meaningful as it is not possible to compare *Salmonella* load differences between these sample types without validated quantification curves. The data presented here demonstrates that there were no differences between bootsock and bootsock-roller performance and that individual farm variation is responsible for the bulk of the variability in *Salmonella* observed in the third experiment. Collectively, this data demonstrates the challenge faced by integrators concerning not only *Salmonella* detection but also regarding the reliability and applicability of *Salmonella* quantification in broiler production and the challenges associated with surveillance sample collection. The large variation in the quantification values from both sampling methods may demonstrate that *Salmonella* prevalence and load are not equally distributed throughout a broiler house. Alternatively, there may be challenges in quantification methodology. Nonetheless, high variation in *Salmonella* load presents a challenge that could influence the viability of using quantification as a risk assessment metric or for assessing the efficacy of on-farm *Salmonella* controls. It is important to measure *Salmonella* load and the high throughput nature of the assay used is helpful for industry. Perhaps a more useful application of the quantification data would be to separate *Salmonella* data into high, medium, and low/negative categories.

The data presented here indicates that sampling method significantly influences *Salmonella* prevalence (and therefore any downstream tests such as quantification, serotyping, etc.). Bootsocks and bootsock-rollers were identified as highly reproducible, user friendly, and the most reliable sampling methods for determining *Salmonella* prevalence in broiler houses. However, this data only provides a snapshot (in this case, in flocks aged 27-29 days) of the *Salmonella* within the surveyed houses. An effective pre-harvest sampling method sample type must also be able to reflect the *Salmonella* profile detected at the processing facility, ideally the *Salmonella* that are ultimately found in final product. In this study, the recovery of *Salmonella* was variable and influenced by sampling method, again highlighting the importance of utilizing an appropriate sampling method. This body of work portrays the current challenges that poultry integrators face in generating meaningful pre-harvest *Salmonella* data which can be used to improve *Salmonella* control in broiler production. Future studies should evaluate the ability of these sampling methods to correlate *Salmonella* in production to *Salmonella* at processing and establish when is the optimal timepoint for sampling pre-harvest broilers.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nikki Shariat reports financial support was provided by US Poultry and Egg Association. Emily Cason reports financial support was provided by Foundation for Food and Agriculture Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104963.

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