104 (2025) 105236

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj

Full-Length Article

Environmental contamination and horizontal transmission of *Salmonella* Enteritidis among experimentally infected layer pullets in indoor cage-free housing☆

Richard K. Gast [a,*], Javier S. Garcia [a], Rupa Guraya [a], Deana R. Jones [a], Darrin M. Karcher [b]

[a] U. S. National Poultry Research Center, USDA Agricultural Research Service, Athens, 30605, Georgia
[b] Department of Animal Sciences, Purdue University, West Lafayette, IN, 47907, USA

ARTICLE INFO

Key words:
Salmonella Enteritidis
Cage-free housing
Environmental contamination
Horizontal transmission

ABSTRACT

The persistence and transmission of the egg-associated pathogen *Salmonella* Enteritidis in laying flocks are significantly influenced by the poultry housing environment. The present study assessed environmental contamination and horizontal transmission of *S*. Enteritidis within groups of layer pullets in cage-free housing after infection just before the age of sexual maturity. In each of 3 trials, 144 pullets were transferred from a rearing facility at 15 wk of age and randomly distributed between 2 isolation rooms simulating commercial cage-free barns with perches and nest boxes (72 birds/room). One wk after placement in the containment facility, a proportion of the 72 pullets in each room were orally inoculated with approximately 6×10^7 cfu of *S*. Enteritidis: 1/3 in trial 1, 1/6 in trial 2, and 1/12 in trial 3. At 2 wk post-inoculation in each trial, samples of liver, spleen, and intestinal tract were collected from 40 uninoculated (contact-exposed) birds in each room for bacteriologic culturing to detect horizontal transmission of *S*. Enteritidis. At 6 intervals between inoculation and necropsy, 5 types of environmental samples (wall dust swab, nest box swab, perch swab, flooring substrate drag swab, and flooring substrate composite) were collected and cultured for *S*. Enteritidis. The overall frequencies of *S*. Enteritidis recovery from both environmental samples and internal organs from contact-exposed pullets after initial oral inoculation of 1/3 of the birds in each room (97 % and 75 %, respectively) were significantly greater than after initial infection of 1/6 of the birds (78 % and 58 %), and *S*. Enteritidis recovery from birds inoculated at a 1/12 proportion (10 % of environmental samples and 18 % of organs) was significantly lower than from the 1/6 inoculation group. Flooring substrate composites were the most efficient environmental sample type for all 3 trials combined (72 % positive), providing significantly better *S*. Enteritidis recovery than the least efficient samples (flooring substrate drag swabs; 53 % positive). These data suggest that a high frequency of environmental contamination may be an important contributor to horizontal transmission of *S*. Enteritidis infections among pullets in cage-free housing.

Introduction

The sustained international implementation of comprehensive risk reduction programs for food safety in commercial egg production has led to considerable progress toward public health goals, but *Salmonella enterica* serovar Enteritidis infections among consumers of eggs continue to challenge producers and regulators (Chousalkar et al., 2018; Chanamé Pinedo et al., 2022). More human *S.* Enteritidis infections have been attributed to eggs and egg products than to any other food vehicle of transmission (Sher et al., 2021), and the prevalence of this pathogen in the housing environment of commercial egg-laying poultry has been identified as a useful indicator of the risk for human exposure to contaminated eggs (Arnold et al., 2014). Strains of *S*. Enteritidis responsible for human disease outbreaks are often genetically identical to isolates found in layer flocks (Denagamage et al., 2017). The edible interior portions of eggs become contaminated with *S*. Enteritidis before oviposition as the result of a sequence of events that typically begins with bacterial colonization of the intestinal tract of laying hens, followed within hours by invasion to internal tissues such as the liver and spleen (Zeng et al., 2018). Further dissemination to the ovary or oviduct can

☆ Primary Audience: Researchers, Veterinarians, Laying Flock Managers

* Corresponding author.
E-mail address: Richard.Gast@usda.gov (R.K. Gast).

https://doi.org/10.1016/j.psj.2025.105236
Received 23 February 2025; Accepted 29 April 2025

lead to deposition inside the yolk or albumen as the forming egg is released and descends the reproductive tract before enclosure within the shell (Gast et al., 2024a). Experimental infection studies have attributed a substantially higher propensity for egg contents contamination to *S.* Enteritidis than to other serovars (Gast et al., 2007, 2021), putatively associated with better survival in chicken macrophages (He et al., 2012), stronger adherence to reproductive tract mucosa (Wales and Davies, 2011), greater invasion of ovarian granulosa cells (Babu et al., 2016), or reduced induction of inflammatory responses (Kilroy et al., 2017). However, substantially lower frequencies of *S.* Enteritidis contamination are usually found in commercially produced eggs because naturally occurring infections of laying hens most often involve relatively low initial doses of the pathogen ingested orally from environmental reservoirs (DeWinter et al., 2011; Esaki et al., 2013).

S. Enteritidis is usually cleared from the internal organs of most infected hens within a few weeks after initial exposure (Gast et al., 2007, 2011), but some individual birds may remain persistently colonized until the onset of egg production (Gast et al., 2015). Fecal shedding of the pathogen into the laying house environment can also continue to spread infection horizontally within flocks and thereby increase the likelihood of eventual egg contamination (Crabb et al., 2019; Gast et al., 2020). Pullets reaching the age of reproductive maturity were found to exhibit heightened susceptibility to *S.* Enteritidis infection and a high likelihood of remaining infected until the age when egg production commences (Groves et al., 2021; Gast et al., 2024b). The opportunities for *Salmonella* to be introduced into and disseminated throughout laying flocks (ultimately also affecting egg safety outcomes) are strongly influenced by environmental conditions, associated with both facility design characteristics and management practices (Trampel et al., 2014). Although the environmental complexity of commercial egg production (and the diversity of conditions in different facilities) challenges the risk assessment process, the factors most often linked with a higher prevalence of *S.* Enteritidis in egg-laying poultry include larger flock size, greater flock age, housing in older facilities, and multiple-age stocking (Pitesky et al., 2013; Denagamage et al., 2015). Persistent environmental contamination in poultry houses can serve as a reservoir for the infection of successive laying flocks with *S.* Enteritidis (Dewaele et al., 2012; Trampel et al., 2014).

Another challenge to assessing food safety risks in commercial egg layers is posed by the ongoing transition within the industry from conventional multiple-bird cages (housing small groups of hens at relatively high stocking densities) toward alternative systems such as indoor aviaries, featuring lower stocking densities for larger hen groups plus environmental enhancements including perches, nesting areas, open floors with scratching pads, and freedom to move between different levels. Driven principally by animal welfare considerations, this shift has generated many new questions about flock management, poultry health, and food safety. Each type of poultry housing system has unique inherent characteristics which present correspondingly unique inherent risk reduction hurdles to overcome (Jones et al., 2015). Different microbial communities have been found in both birds and environmental sources from different housing systems (Wiersema et al., 2021; Wilson et al., 2021). Meaningful comparisons between systems can be confounded by differences in hen genetics, bird stocking densities, facility construction, levels of exposure to contaminated dust and feces, and populations of biological vectors (Holt et al., 2011). Prior research has shown that the outcomes of *S.* Enteritidis infections, including organ invasion and horizontal transmission, can vary with different rearing conditions for egg-type pullets and these effects are especially pronounced at or near the age of reproductive maturity (Gast et al., 2022). One of the pivotal considerations for understanding the role of housing system design and management in the progress and perpetuation of *S.* Enteritidis infections in laying flocks is the relationship between contamination of specific environmental reservoirs and bird-to-bird dissemination of the pathogen. The present study addressed this issue by oral introduction of *S.* Enteritidis into groups of layer pullets in cage-free housing just before the age of sexual maturity and the subsequent determination of both the frequency of detecting the pathogen in several types of environmental samples and the frequency of horizontal transmission of infection among co-housed birds.

Materials and methods

Experimental housing of layer pullets

In each of 3 similar trials, 144 female Lohmann Brown chicks (a strain used by the commercial egg industry) were obtained from a breeding company and reared on floors covered with wood shavings in cage-free barns at Purdue University (West Lafayette, IN) without vaccination against *Salmonella* infection. At 15 wk of age, the birds in each trial were transferred to the U.S. National Poultry Research Center (Athens, GA), distributed evenly (72 per room) between 2 separate isolation rooms in an ABSL-2 containment facility with central heating and air conditioning, and housed on wood shavings at a density of 1,710 cm^2 of floor space per bird with a lighting schedule specified for this line of birds and typical for commercial egg production (Lohmann Tierzucht, 2025). Each isolation room simulated a commercial cage-free barn with community kick-out nest boxes and perches. Water was supplied *ad libitum* via automatic nipple-type drinkers and feed (antibiotic-free, ground-mash layer ration) was provided according to standards for commercial egg production. The experimental protocol (USNPRC-2025-05) was approved by the Institutional Animal Care and Use Committee of the U.S. National Poultry Research Laboratory.

Pre-inoculation cloacal swab samples

Immediately before pullets in each containment room were inoculated, sterile cotton swabs were used to collect cloacal swab samples from 60 randomly selected birds per room to ensure the absence of *Salmonella* infection within the group. Each sample was transferred into 10 ml of buffered peptone water (BPW; Acumedia, Neogen Corp., Lansing, MI) and incubated for 24 h at 37°C. A 0.1 ml portion of each culture was then transferred into 10 ml of Rappaport-Vassiliadis (RV) broth (Acumedia) and incubated for 24 h at 42°C. A 10-µl portion from each of these broth cultures was then streaked onto brilliant green sulfa (BGS) agar (Acumedia). These plates were incubated for 24 h at 37°C and then examined for the presence of typical *Salmonella* colonies.

Experimental infection of layer pullets with S. enteritidis

In each of 3 trials, a proportion of the 72 pullets in each of 2 replicate containment rooms were orally inoculated with a measured dose of a 2-strain mixture (phage types 4 and 13a) of *S.* Enteritidis at 16 wk of age (1 wk after placement into the containment facility). Both *Salmonella* strains were originally isolated from internal organs of naturally infected chickens in commercial settings. One-third of the pullets in each room (24/72) were orally infected in trial 1, 1/6 of the pullets in each room (12/72) were inoculated in trial 2, and 1/12 (6/72) of the pullets in each room were inoculated in trial 3. Each inoculum strain was resuscitated by transfer into tryptic soy broth (Acumedia) for 2 successive cycles of 24-h incubation at 37°C. After cell numbers in each incubated culture were estimated by determining its optical density at 600 nm, equal numbers of the 2 inoculum component strains were combined, and further serial dilutions in 0.85 % saline were performed to achieve the desired final cell concentration. Plate counts on BGS agar indicated that the average final *S.* Enteritidis cell concentration in each 1.0-ml oral inoculum dose was approximately 6×10^7 cfu for the 3 trials. All inoculations and necropsies were performed at the same time of day, 24 h after the birds last received feed. Orally infected pullets were identified by colored leg bands.

Pullet housing environmental samples

1 d prior to the oral inoculation of pullets with *S*. Enteritidis and again at 1, 3, 6, 8, 10, and 13 d post-inoculation in each trial, 5 types of environmental samples were collected from each room as described by Garcia et al. (2023) and cultured to detect *Salmonella* contamination. Each environmental test set consisted of 2 samples each of flooring substrate composite (1 gloved handful each from the drinker and feeder areas), drag swabs of flooring substrate, wall dust swabs, perch swabs, and nest box swabs. Samples were transported to the laboratory and 10 ml of BPW was added to each swab sample and 250 ml of BPW was added to a 25 g portion of each flooring substrate composite sample. All samples were then mixed by stomaching for 1 min. After incubation for 24 h at 37°C, 0.1 ml portions from each sample were transferred into 10 ml of RV broth and incubated for 24 h at 42°C. A 10-µl aliquot of each RV broth culture was then streaked onto BGS agar and incubated for 24 h at 37°C. Typical *Salmonella* colonies on these plates were subjected to biochemical and serological confirmation (Waltman and Gast, 2016).

Internal organ samples

At 14 d post-inoculation in each trial, 46-48 pullets from 1 containment room were randomly selected (8 orally infected birds and 40 uninoculated birds in trials 1 and 2; 6 inoculated and 40 uninoculated birds in trial 3) and euthanized for bacteriologic culture of internal tissues. Portions (approximately 5-10 g) of the liver, spleen, and intestinal tract (including the ileocecal junction and adjacent regions of both ceca) from each pullet were aseptically removed, transferred to 20 ml of BPW, and mixed by stomaching for 30 sec. After incubation for 24 h at 37°C, a 1-ml portion of each culture was transferred to 9 ml of tetrathionate broth (Acumedia) and incubated for 24 h at 37°C. A 10-µl aliquot of each culture was then streaked onto BGS agar. Following incubation of these plates for 24 h at 37°C, typical *S*. Enteritidis colonies were subjected to biochemical and serological confirmation. This sampling procedure was repeated for pullets from the other containment room in each trial at 15 d post-inoculation.

Statistical analysis

Significant differences ($P < 0.05$) in the frequency of *S*. Enteritidis isolation between the 3 groups of pullets orally inoculated with *S*. Enteritidis at different proportions, the 5 types of environmental samples, or the 6 post-inoculation sampling intervals were determined by Fisher's exact test. Because the 2 replicate rooms within each trial never differed significantly in *Salmonella* recovery from any type of samples in this analysis, their results were combined for presentation in the text and tables. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA).

Results

No pre-infection samples (from either the birds or their environment) were *Salmonella*-positive in any of the 3 trials. After 1/3 of the pullets were orally inoculated with *S*. Enteritdis in trial 1, 97 % of all housing environmental samples collected between 1 and 13 d post-inoculation were positive for the pathogen (Table 1). Significantly fewer (78 %; $P < 0.0001$) of these samples were positive after oral infection of 1/6 of the hens in trial 2, and *S*. Enteritidis environmental positivity associated with inoculation of 1/12 of the pullets in trial 3 (10 %) was significantly ($P < 0.0001$) lower than for the 1/6 inoculation group. Following oral infection of 1/3 of the pullets, the frequencies of *S*. Enteritidis isolation from environmental samples collected during the 1st and 2nd wk post-inoculation were similar (Table 1). However, when 1/6 of the birds were initially infected, *S*. Enteritidis was isolated from environmental samples significantly ($P = 0.0018$) more often during the 2nd wk than during the 1st wk post-inoculation (88 % vs. 65 %). Similarly, although not statistically significant, results from inoculating 1/12 of the pullets followed the same pattern as the 1/6 group (13 % positive for *S*. Enteritidis in wk 2 vs. 7 % in wk 1).

Table 1
Isolation of *Salmonella* Enteritidis from environmental samples collected in cage-free rooms during the first 2 wk after oral inoculation of different proportions of pullets in co-housed groups.[1].

	Salmonella-positive/total (%)			
Post-inoculation sampling dates	1/3 inoculated	1/6 inoculated	1/12 inoculated	All pullets
1-6 d	60/60 (100)[a]	39/60 (65)[a]	4/60 (7)[a]	103/180 (57)[a]
8-13 d	56/60 (93)[a]	54/60 (88)[b]	8/60 (13)[a]	118/180 (66)[a]

[1] In 3 separate trials, 3 different proportions of 144 pullets housed in cage-free disease containment rooms were orally inoculated with 6.1×10^7 cfu of a 2-strain mixture of *S*. Enteritidis at 16 wk of age. Samples from the housing environment were collected at 6 intervals between 1 and 13 d post-inoculation and cultured for *S*. Enteritidis.

[a,b] Values in columns that share no common superscripts are significantly ($P < 0.05$) different.

Within the 3 individual trials, which were initiated by the oral infection of 3 different proportions of pullets, there were no significant differences in the frequency of *S*. Enteritidis recovery between the 5 types of environmental samples that were collected and tested (Table 2). For all 3 trials combined, the frequency of *S*. Enteritidis isolation using the most efficient sample type (flooring substrate composites; 72 %) was significantly ($P = 0.0248$) higher than was obtained via the least efficient samples (flooring substrate drag swabs (53 %) . However, when only data from the 2 higher proportions of oral inoculation (1/3 and 1/6 of the pullets) were combined, the most efficient environmental samples were wall dust swabs (96 % positive), which provided significantly ($P = 0.0273$) better recovery of *S*. Enteritidis than the least efficient type of samples (flooring substrate drag swabs; 79 %).

When tissues were harvested from orally inoculated pullets at 2 wk post-challenge, *S*. Enteritidis was recovered from 91 % of ileocecal samples, 84 % of livers, and 66 % of spleens, with no significant differences observed between the 3 different proportions of initial oral exposure (Table 3). Among contact-exposed (uninoculated) pullets, the

Table 2
Isolation of *Salmonella* Enteritidis from 5 types of environmental samples collected in cage-free rooms after oral inoculation of different proportions of pullets within co-housed groups.[1].

	Salmonella-positive/total (%)			
Environmental Sample Type	1/3 inoculated	1/6 inoculated	1/12 inoculated	All pullets
Flooring substrate drag swab	23/24 (96)[a]	15/24 (63)[a]	0/24 (0)[a]	38/72 (53)[a]
Flooring substrate composite	23/24 (96)[a]	20/24 (83)[a]	9/24 (38)[a]	52/72 (72)[b]
Nest box swab	23/24 (96)[a]	16/24 (67)[a]	3/24 (13)[a]	42/72 (58)[ab]
Perch swab	23/24 (96)[a]	20/24 (83)[a]	0/24 (0)[a]	43/72 (60)[ab]
Wall dust swab	24/24 (100)[a]	22/24 (92)[a]	0/24 (0)[a]	46/72 (64)[ab]

[1] In 3 separate trials, 3 different proportions of 144 pullets housed in a cage-free disease containment facility were orally inoculated with 6.1×10^7 cfu of a 2-strain mixture of *S*. Enteritidis at 16 wk of age. 5 types of samples from the housing environment were collected at 6 intervals between 1 and 13 d post-inoculation and cultured for *S*. Enteritidis.

[a,b] Values in columns that share no common superscripts are significantly ($P < 0.05$) different.

Table 3
Isolation of *Salmonella* Enteritidis from internal organs of orally inoculated and contact-exposed pullets in cage-free rooms after initial infection of different proportions within co-housed groups.[1].

	Orally Inoculated			Contact-Exposed		
	Liver	Spleen	Ileum/Ceca	Liver	Spleen	Ileum/Ceca
	Salmonella-positive/total (%)					
1/3 inoculated	11/16 (69)[a]	12/16 (75)[a]	14/16 (88)[a]	56/80 (70)[b]	60/80 (75)[c]	65/80 (81)[c]
1/6 inoculated	10/16 (63)[a]	12/16 (75)[a]	15/16 (94)[a]	43/80 (54)[b]	45/80 (56)[b]	52/80 (65)[b]
1/12 inoculated	8/12 (67)[a]	9/12 (75)[a]	11/12 (92)[a]	8/80 (10)[a]	12/80 (15)[a]	23/80 (29)[a]

[1] In 3 separate trials, 3 different proportions of 144 pullets housed in a cage-free disease containment facility were orally inoculated with 6.1×10^7 cfu of a 2-strain mixture of *S.* Enteritidis at 16 wk of age. The remaining pullets in each room were exposed to infection by horizontal contact. 6-8 inoculated pullets and 40 contact-exposed pullets per room were sampled for the presence of *S.* Enteritidis in internal organs at 2 wk post-infection.

[a,b,c] Values in columns that share no common superscripts are significantly ($P < 0.05$) different.

frequency of *S.* Enteritidis isolation from ileocecal samples in the group with 1/3 of the pullets orally infected (81 %) was significantly ($P = 0.0317$) greater than for the 1/6 inoculated group (65 %), and a further significant difference ($P < 0.0001$) separated the 1/6 inoculated group from the 1/12 inoculated group for these samples (29 % positive). Likewise, S. Enteritidis was recovered at a significantly ($P = 0.0194$) higher frequency from spleens of contact-exposed pullets in the 1/3 inoculation group (75 %) than from the 1/6 inoculation group (56 %), and a further significant reduction ($P < 0.0001$) differentiated the 1/12 inoculation group (15 %). Liver samples from contact-exposed birds in the 1/3 and 1/6 inoculation groups did not differ significantly in the frequency of *S.* Enteritidis recovery (70 % and 54 %, respectively), but both were significantly ($P < 0.0001$) greater than the 1/12 inoculation group (10 %).

Discussion

Both the interior and exterior environments of poultry facilities can be reservoirs of *Salmonella* contamination (Soria et al., 2017). Chicken feces, dust and aerosols, and biological vectors all contribute to *Salmonella* dissemination and persistence in poultry houses (Guard et al., 2018; McWhorter and Chousalkar, 2020) and create extended opportunities for infection to spread horizontally within and between flocks (Dewaele et al., 2012; Gast et al., 2014). Feces and dust are widely distributed throughout laying houses and can remain contaminated with *Salmonella* for many months (Im et al., 2015; McWhorter and Chousalkar, 2020). High moisture levels in poultry house flooring substrates can support bacterial survival and increase the transfer of contamination to circulating dust (McWhorter and Chousalkar, 2020; Pal et al., 2021a). Henzler et al. (1998) found that laying flocks with high levels of *S.* Enteritidis in their manure were 10 times more likely to produce contaminated eggs than flocks with low levels. Because laying flocks infected with *S.* Enteritidis typically produce contaminated eggs at very low frequencies, most *S.* Enteritidis control plans rely on testing environmental samples to identify flocks which pose a potential public health threat and trigger additional actions such as egg testing or egg marketing restrictions (Gast et al., 2024a).

Environmental samples can provide highly effective detection of infections with salmonellae, even at a low prevalence within flocks (Apentag et al., 2020), although the common practice of pooling swabs for testing has been shown to significantly reduce the sensitivity of detecting low bacterial levels (Jones et al., 2020). Diverse environmental sampling methods have been successfully utilized to find *Salmonella* in poultry facilities, including drag swabs, boot swabs, and the collection of litter material or dust from locations such as egg belts, fan blades, or nest boxes. Feces voided by infected hens are principal sources for introducing *Salmonella* contamination into laying house environments, but testing dust or other environmental matrices often provides equivalent or better *Salmonella* detection than sampling feces from individual birds (Sodagari et al., 2020; Pacholewicz et al., 2023). Electrostatic collection of airborne dust provided highly sensitive detection of *S.* Enteritidis infection in groups of experimentally infected laying hens (Gast *et al.*, 2004). The frequency of *Salmonella* contamination in poultry house dust has been observed to increase from bird placement to depopulation (Kim et al., 2024). At the 2 higher oral inoculation frequencies (33 % and 17 %) in the present study, wall dust was the most efficient environmental sample for detecting *S.* Enteritidis. However, when the lowest (8 %) oral inoculation frequency was also included in the analysis, flooring substrate composite sampling was most effective. These most efficient samples provided significantly better *S.* Enteritidis recovery than the least efficient samples (drag swabs of flooring substrate). In a prior investigation, boot swabs were found to be the preferred samples for non-cage housing, whereas dust was more reliable for cage systems (Pacholewicz et al., 2023).

Direct contact between hens, ingestion of contaminated feed or feces, circulation of contaminated dust and aerosols, and the movement of personnel and equipment within poultry facilities can all mediate rapid and extensive horizontal transmission of *S.* Enteritidis infection throughout laying flocks after initial exposure from environmental sources (Gast et al., 2014). Airborne dust or aerosols have been shown to disseminate both environmental contamination and invasive infection (Gast et al., 1998; Pal et al., 2021b; Khan et al., 2024). Although intestinal colonization by salmonellae typically declines steadily during the initial weeks after infection of laying hens (Gast et al., 2005, 2011), instances of persistent colonization with prolonged fecal shedding into the housing environment could perpetuate opportunities for infection to continue spreading (Li et al., 2007; Gast et al., 2011). Cage-free housing systems have been reported to allow greater horizontal transmission of *Salmonella* infection within flocks, perhaps because cage-free conditions give birds greater access to each other and to feces-contaminated flooring substrate (Devylder et al., 2011). A relatively less diverse intestinal microbiota in cage-free birds may result in reduced protection against pathogen colonization (Wiersema et al., 2021),. Floor rearing of egg-type pullets has been associated with a subsequently increased prevalence of *S.* Enteritidis in commercial laying houses (Garber et al., 2003). In the present study, oral inoculation of relatively higher proportions of pullets (17-33 %) in cage-free housing resulted in rapid environmental contamination and extensive horizontal transmission of invasive infection during the first 2 weeks after exposure. Oral inoculation of a smaller initial proportion (8 %) of pullets led to less (and more slowly developing) environmental contamination and less horizontal transmission of infection. The contrast between the downward trend in environmental positivity from 1 to 2 wk post-inoculation in the 33 % oral infection group and the upward trend over time for the corresponding data in the other 2 groups illustrates a more slowly progressing transmission of infection (and correlated seeding of the environment via contaminated feces) at the lower initial incidences of oral infection. The results of this study suggest that a high frequency of environmental contamination can be an important contributor to horizontal transmission of *S.* Enteritidis infections among pullets in cage-free housing, potentially leading to the persistence of infection until reproductive maturity and the associated production of contaminated eggs.

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.

Acknowledgments

We gratefully express appreciation for excellent technical assistance from Stephen Norris, and Robin Woodroof (U. S. National Poultry Research Center, Athens, GA). This study was supported by appropriated funds of the USDA Agricultural Research Service.

References

Apenteng, O.O., Arnold, M..E., Vigre, H., 2020. Using stochastic dynamic modelling to estimate the sensitivity of current and alternative surveillance program of *Salmonella* in conventional broiler production. Sci. Rep. 10, 19441.

Arnold, M.E., Martelli, F.., McLaren, I., Davies, R.H., 2014. Estimation of the rate of egg contamination from *Salmonella*-infected chickens. Zoonoses Pub. Health 61, 18–27.

Babu, U.S., Harrison, L..M., Patel, I.R., Ramirez, G.A., Williams, K.M., Pereira, M., Balan, K.V., 2016. Differential antibacterial response of chicken granulosa cells to invasion by *Salmonella* serovars. Poult. Sci. 95, 1370–1379.

Chanamé Pinedo, L., Mughini-Gras, L., Franz, E., Hald, T., Pires, S.M., 2022. Sources and trends of human salmonellosis in Europe, 2015-2019: an analysis of outbreak data. Int. J. Food Microbiol. 379, 109850.

Chousalkar, K., Gast, R., Martelli, F., Pande, V., 2018. Review of egg-related salmonellosis and reduction strategies in United States, Australia, United Kingdom and New Zealand. Crit. Rev. Microbiol. 44, 290–303.

Crabb, H.K., Gilkerson, J..R., Browning, G.F., 2019. Does only the age of the hen matter in *Salmonella enterica* contamination of eggs? Food Microbiol. 77, 1–9.

De Vylder, J., Dewulf, J., Van Hoorebeke, S., Pasmans, F., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2011. Horizontal transmission of *Salmonella* Enteritidis in groups of experimentally infected hens housed in different housing systems. Poult. Sci. 90, 1391–1396.

Denagamage, T., Jayarao, B., Patterson, P., Wallner-Pendleton, E., Kariyawasam, S., 2015. Risk factors associated with *Salmonella* in laying hen farms: systematic review of observational studies. Avian Dis. 59, 291–302.

Denagamage, T., Jayarao, B., Wallner-Pendleton, E., Patterson, P., Kariyawasam, S., 2017. A retrospective study of *Salmonella* Enteritidis isolated from commercial layer flocks. Avian Dis. 61, 330–334.

Dewaele, I., Van Meirhaeghe, H., Rasschaert, G., Vanrobaeys, M., De Graef, E., Herman, L., Ducatelle, R., Heyndrickx, M., De Reu, K., 2012. Persistent *Salmonella* Enteritidis environmental contamination on layer farms in the context of an implemented national control program with obligatory vaccination. Poult. Sci. 91, 282–291.

DeWinter, L.M., Ross, W..H., Couture, H., Farber, J.F., 2011. Risk assessment of shell eggs internally contaminated with *Salmonella* Enteritidis. Int. Food Risk Anal. J. 1, 40–81.

Esaki, H., Shimura, K., Yamazaki, Y., Eguchi, M., Nakamura, M., 2013. National surveillance of *Salmonella* Enteritidis in commercial eggs in Japan. Epidemiol. Infect. 141, 941–943.

Garber, L., Smeltzer, M., Fedorka-Cray, P., Ladely, S., Ferris, K., 2003. Salmonella enterica serotype enteritidis in table egg layer house environments and in mice in U. S. layer houses and associated risk factors. Avian Dis. 47, 134–142.

Garcia, J.S., Jones, D..R., Gast, R.K., Karcher, D.M., Erasmus, M.A., 2023. Environmental sampling methods' influence on detection of pathogens in cage-free aviary housing. Poult. Sci. 102, 102381.

Gast, R.K., Dittoe, D..K., Ricke, S.C., 2024a. *Salmonella* in eggs and egg laying chickens: pathways to effective control. Crit. Rev. Microbiol. 50, 39–63.

Gast, R.K., Guard-Bouldin, J.., Holt, P.S., 2005. The relationship between the duration of fecal shedding and the production of contaminated eggs by laying hens infected with strains of *Salmonella* Enteritidis and *Salmonella* Heidelberg. Avian Dis. 49, 382–386.

Gast, R.K., Guraya, R.., Guard, J., Holt, P.S., 2011. The relationship between the numbers of *Salmonella* Enteritidis, *Salmonella* Heidelberg, or *Salmonella* Hadar colonizing reproductive tissues of experimentally infected laying hens and deposition inside eggs. Avian Dis. 55, 243–247.

Gast, R.K., Guraya, R.., Guard-Bouldin, J., Holt, P.S., Moore, R.W., 2007. Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with *Salmonella enteritidis* or *Salmonella heidelberg*. Avian Dis. 51, 40–44.

Gast, R.K., Guraya, R.., Jones, D.R., Anderson, K.E., 2014. Horizontal transmission of *Salmonella* Enteritidis in experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 93, 3145–3151.

Gast, R.K., Guraya, R.., Jones, D.R., Anderson, K.E., 2015. Persistence of fecal shedding of *Salmonella* Enteritidis by experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 94, 1650–1656.

Gast, R.K., Jones, D..R., Guraya, R., Anderson, K.E., Karcher, D.M., 2020. Research note: horizontal transmission and internal organ colonization by *Salmonella* Enteritidis and *Salmonella* Kentucky in experimentally infected laying hens in cage-free housing. Poult. Sci. 99, 6071–6074.

Gast, R.K., Jones, D..R., Guraya, R., Anderson, K.E., Karcher, D.M., 2021. Research note: contamination of eggs by *Salmonella* Enteritidis and *Salmonella* Typhimurium in experimentally infected laying hens in indoor cage-free housing. Poult. Sci. 100, 101438.

Gast, R.K., Jones, D..R., Guraya, R., Garcia, J.S., Karcher, D.M., 2022. Internal organ colonization by *Salmonella* Enteritidis in experimentally infected layer pullets reared at different stocking densities in indoor cage-free housing. Poult. Sci. 101, 102104.

Gast, R.K., Jones, D..R., Guraya, R., Garcia, J.S., Karcher, D.M., 2024b. Internal organ colonization by *Salmonella* Enteritidis in layer pullets infected at two different ages during rearing in cage-free housing. Avian Dis. 68, 141–144.

Gast, R.K., Mitchell, B..W., Holt, P.S., 1998. Airborne transmission of Salmonella enteritidis infection between groups of chicks in controlled-environment isolation cabinets. Avian Dis. 42, 315–320.

Gast, R.K., Mitchell, B..W., Holt, P.S., 2004. Detection of airborne Salmonella enteritidis in the environment of experimentally infected laying hens by an electrostatic sampling device. Avian Dis. 48, 148–154.

Groves, P.J., Williamson, S..L., Ahaduzzaman, M.d., Diamond, M., Ngo, M., Han, A., Sharpe, S.M., 2021. Can a combination of vaccination, probiotic and organic acid treatment in layer hens protect against early life exposure to *Salmonella* Typhimurium and challenge at sexual maturity. Vaccine 39, 815–824.

Guard, J., Henzler, D.J., Ramadan, H., Jones, D.R., Gast, R.K., Davison, S., Allard, M.W., 2018. Serotyping of *Salmonella Enterica* isolated from mice caught on US poultry farms 1995 through 1998. Food Safety 6, 44–50.

He, H., Genovese, K.J., Swaggerty, C.L., Nisbet, D.J., Kogut, M.H., 2012. A comparative study on invasion, survival, modulation of oxidative burst, and nitric oxide responses of macrophages (HD11), and systemic infection in chickens by prevalent poultry *Salmonella* serovars. Foodborne Pathogens Dis. 9, 1104–1110.

Henzler, D.J., Kradel, D..C., Sischo, W.M., 1998. Management and environmental risk factors for *Salmonella enteritidis* contamination of eggs. Am. J. Vet. Res. 59, 824–829.

Holt, P.S., Davies, R..H., Dewulf, J., Gast, R.K., Huwe, J.K., Jones, D.R., Waltman, D., Willian, K.R., 2011. The impact of different housing systems on egg safety and quality. Poult. Sci. 90, 251–262.

Im, M.C., Jeong, S..J., Kwon, Y.-K., Jeong, O.-M., Kang, M.-S., Lee, Y.J., 2015. Prevalence and characteristics of *Salmonella* spp. Isolated from commercial layer farms in Korea. Poult. Sci. 94, 1691–1698.

Jones, D.R., Cox, N..A., Guard, J., Fedorka-Cray, P.J., Buhr, R.J., Gast, R.K., Abdo, Z., Rigsby, L.L., Plumblee, J.R., Karcher, D.M., Robison, C.I., Blatchford, R.A., Makagon, M.M., 2015. Microbiological impact of three commercial laying hen housing systems. Poult. Sci. 94, 544–551.

Jones, D.R., Gast, R..K., Regmi, P., Ward, G.E., Anderson, K.E., Karcher, D.M., 2020. Pooling of laying hen environmental swabs and efficacy of *Salmonella* detection. J. Food Prot. 83, 943–950.

Khan, S., McWhorter, A.R., Andrews, D.M., Underwood, G.J., Moore, R.J., Van, T.T.H., Gast, R.K., Chousalkar, K.K., 2024. Dust sprinkling as an effective method for infecting layer chickens with wild-type *Salmonella* typhimurium and changes in host gut microbiota. Environ. Microbiol. Rep. 16, e13265.

Kilroy, S., Raspoet, R., Martel, A., Bosseler, L., Appia-Ayme, C., Thompson, A., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2017. *Salmonella* Enteritidis flagellar mutants have a colonization benefit in the chicken oviduct. Comp. Immunol. Microb. 50, 23–28.

Kim, M.B., Jung, H.-R., Lee, Y.J., 2024. Emergence of *Salmonella* Infantis carrying the pESI megaplasmid in commercial of five major integrated broiler operations in Korea. Poult. Sci. 103, 103516.

Li, X., Payne, J.B., Santos, F.B., Levine, J.F., Anderson, K.E., Sheldon, B.W., 2007. *Salmonella* populations and prevalence in layer feces from commercial high-rise houses and characterization of the *Salmonella* isolates by serotyping, antibiotic resistance analysis, and pulsed field gel electrophoresis. Poult. Sci. 86, 591–597.

Lohmann Tierzucht. 2025. Management guide: alternative systems. Accessed Apr. 2025. https://lohmann-breeders.com/e-guide/alternative-housing/.

McWhorter, A.R., Chousalkar, K.R., 2020. *Salmonella* on Australian cage egg farms: observations from hatching to end of lay. Food Microbiol. 87, 103384.

Pacholewicz, E., WIsselink, H.J., Koene, M.G.J., van der Most, M., Gonzales, J.L., 2023. Environmental sampling methods for detection of *Salmonella* infections in laying hens: a systematic review and meta-analysis. Microorganisms. 11, 2100.

Pal, A., Bailey, M.A., Talorico, A.A., Krehling, J.T., Macklin, K.S., Price, S.B., Buhr, R.J., Bourassa, D.V., 2021a. Impact of poultry litter Salmonella levels and moisture on transfer of *Salmonella* through associated in vitro generated dust. Poult. Sci. 100, 101236.

Pal, A., Riggs, M.R., Urrutia, A., Osborne, R.C., Jackson, A.P., Bailey, M.A., Macklin, K.S., Price, S.B., Buhr, R.J., Bourassa, D.V., 2021b. Investigation of the potential of aerosolized *Salmonella* Enteritidis on colonization and persistence in broilers from day 3 to 21. Poult. Sci. 100, 101504.

Pitesky, M., Charlton, B., Bland, M., Rolfe, D., 2013. Surveillance of *Salmonella* Enteritidis in layer houses: a retrospective comparison of the Food and Drug Administration's Egg Safety Rule (2010-0211) and the California Egg Quality Assurance Program (2007-2011). Avian Dis. 57, 51–56.

Sher, A.A., Mustafa, B..E., Grady, S.C., Gardiner, J.C., Saeed, A.M., 2021. Outbreaks of foodborne *Salmonella enteritidis* in the United States between 1990 and 2015: an analysis of epidemiological and spatial-temporal trends. Int. J. Infect. Dis. 105, 54–61.

Sodagari, H.R., Habib, I.., Whiddon, S., Wang, P., Mohammed, A.B., Robertson, I., Goodchild, S., 2020. Occurrence and characterization of *Salmonella* isolated from table egg layer farming environments in western Australia and insights into biosecurity and egg handling practices. Pathogens. 9, 56.

Soria, M.C., Soria, M..A., Bueno, D.J., Godano, E.K., Gómez, S.C., ViaButron, I.A., Padin, V.M., Rogé, A.D., 2017. *Salmonella* spp. Contamination in commercial layer hen farms using different types of samples and detection methods. Poult. Sci. 96, 2820–2830.

Trampel, D.W., Holder, T..G., Gast, R.K., 2014. Integrated farm management to prevent *Salmonella* Enteritidis contamination of eggs. J. Appl. Poult. Res. 23, 353–365.

Wales, A.D., Davies, R.H., 2011. A critical review of *Salmonella* typhimurium infection in laying hens. Avian Pathol. 40, 429–436.

Waltman, W.D., Gast, R.K., 2016. Salmonellosis. In: Williams, S.M., Dufour-Zavala, L., Jackwood, M.W., Lee, M.D., Lupiani, B., Reed, W.M., Spackman, E., Woolcock, P.R. (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 6th ed. American Association of Avian Pathologists, Jacksonville, FL, pp. 013–112.

Wiersema, M.L., Koester, L..R., Schmitz-Esser, S., Koltes, D.A., 2021. Comparison of intestinal permeability, morphology, and ileal microbial communities of commercial hens housed in conventional cages and cage-free housing systems. Poult. Sci. 100, 1178–1191.

Wilson, A., Chandry, P.S., Turner, M.S., Courtice, J.M., Fegan, N., 2021. Comparison between cage and free-range egg production on microbial composition, diversity and the presence of *Salmonella enterica*. Food Microbiol. 97, 103754.

Zeng, J., Lei, C., Wang, Y., Chen, Y., Zhang, X., Kang, Z., Zhai, X., Ye, X., Wang, H., 2018. Distribution of *Salmonella Enteritidis* in internal organs and variation of cecum microbiota in chicken after oral challenge. Microb. Pathog. 122, 174–179.