

Research Note: Internal organ colonization by *Salmonella* Enteritidis in experimentally infected layer pullets reared at different stocking densities in indoor cage-free housing

Richard K. Gast ^{*,1} Deana R. Jones ^{*} Rupa Guraya^{*} Javier S. Garcia^{*} and Darrin M. Karcher [†]

^{*}*U. S. National Poultry Research Center, USDA Agricultural Research Service, Athens, GA 30605, USA; and*
[†]*Department of Animal Sciences, Purdue University, West Lafayette, IN 47907, USA*

ABSTRACT Contamination of eggs by *Salmonella* has often been identified as a source of food-borne human illness. *S. Enteritidis* is deposited inside developing eggs when invasive infections of laying hens reach the reproductive organs. The susceptibility of hens in cage-based housing systems to *S. Enteritidis* has been associated with their stocking density, but the applicability of this information to extensive (cage-free) systems is uncertain. The present study assessed internal organ colonization by *S. Enteritidis* in egg-type pullets reared at 2 different stocking densities in cage-free housing. Pullets were reared at either 374 cm² or 929 cm² of floor space per bird. At 16 wk of age, 4 groups of 72 pullets were moved into isolation rooms simulating commercial cage-free barns; 1/3 of the pullets in 2 rooms were orally inoculated with *S. Enteritidis* immediately after transfer and pullets in 2

rooms were similarly infected at 19 wk. At 6 and 12 d postinoculation, the pullets were euthanized and samples of liver, spleen, and intestinal tract were removed for bacteriologic culturing. No significant differences ($P > 0.05$) in *S. Enteritidis* isolation frequencies from any tissue were observed between high and low density rearing groups following infection at either age. However, *S. Enteritidis* was found significantly ($P < 0.05$) more frequently among pullets infected orally at 19 wk than at 16 wk in spleens and intestines. Likewise, the frequency of *S. Enteritidis* isolation from all birds (inoculated plus contact-exposed) at 19 wk was significantly higher than at 16 wk in livers and spleens. This increased susceptibility to invasive *S. Enteritidis* infection at reproductive maturity emphasizes the importance of risk reduction at a critical stage in the egg production cycle.

Key words: *Salmonella*, pullet rearing, stocking density, cage-free housing, internal organs

2022 Poultry Science 101:102104

<https://doi.org/10.1016/j.psj.2022.102104>

INTRODUCTION

Eggs contaminated by *Salmonella* have been implicated as prominent sources of human illness for many years, a problem which poses a continuing challenge to public health agencies and poultry industries on several continents (Chousalkar et al., 2018). The incidence of human *Salmonella enterica* serovar Enteritidis infections is directly related to the prevalence of this organism in commercial laying flocks, and isolates from poultry sources are often genetically identical to those responsible for human disease outbreaks. Epidemiologists have identified eggs and egg products as the most common food vehicles for transmitting human salmonellosis in Europe and as the animal product most often

linked to human *S. Enteritidis* infections in the United States. The reported incidence of human *S. Enteritidis* infections in the United States in 2018 had not declined for more than a decade, despite the implementation of a comprehensive national risk reduction and flock testing program for commercial egg production in 2010.

Oral ingestion of *S. Enteritidis* by chickens can lead to colonization of the gastrointestinal tract and invasion to reach the liver and spleen within hours of exposure. In mature laying hens, further dissemination of the pathogen to the ovaries and oviducts sometimes results in bacterial deposition inside the edible portions (yolk and albumen) of forming eggs prior to enclosure within the shell and oviposition (Gantois et al., 2009). This process has been variously ascribed to intracellular survival in chicken macrophages, adherence to reproductive tract mucosa, and invasion of ovarian granulosa cells. Although *S. Enteritidis* is typically cleared from the internal organs of hens within a few weeks after exposure, continuing fecal shedding into the laying house environment by a few persistently infected individuals

Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received June 3, 2022.

Accepted July 23, 2022.

¹Corresponding author: Richard.Gast@usda.gov

can perpetuate horizontal transmission of *Salmonella* within a flock.

The prevalence of food-borne pathogens such as *Salmonella* in commercial laying flocks depends on both the physical design characteristics of poultry housing facilities and the management practices employed to operate them. Commercial egg production in much of the world is currently undergoing a transition from conventional cage-based hen housing systems toward less space-intensive alternatives such as indoor cage-free aviaries or outdoor free-range systems. This transformation is intended to address animal welfare concerns regarding traditional caging but its food safety consequences are not yet entirely clear. Understanding how housing and management decisions affect the presence and levels of *Salmonella* in laying hens and their environment (and ultimately the production of contaminated eggs) is vital for ensuring a safe transition of the industry into cage-free systems. Stocking density (floor space per bird) is an important parameter with relevance across all housing systems. Although prior research has established a relationship between the stocking density at which laying hens are held and the observed outcomes of *Salmonella* infection (Gast et al., 2016), the available data do not indicate whether stocking densities utilized during pullet rearing could subsequently affect susceptibility to *Salmonella* infection when exposure to the pathogen occurs after transfer to laying facilities and at or near the age of sexual maturity. The objective of the present study was to assess horizontal transmission and invasion of internal organs by *S. Enteritidis* in pullets reared at 2 different stocking densities in cage-free housing and experimentally infected at either 16 or 19 wk of age.

MATERIALS AND METHODS

Experimental Housing of Layer Pullets

In each of 2 similar trials, 288 female Tetra 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. Pullets in one trial were reared at a stocking density of 374 cm² of floor space per bird (high density rearing) and pullets in the other trial were reared at a stocking density of 929 cm² of floor space per bird (low density rearing). At 16 wk of age, the birds in both trials were transferred to the U.S. National Poultry Research Center (Athens, GA), distributed evenly (72 per room) between four separate isolation rooms in a disease-containment facility, and housed on wood shavings at a density of 1,710 cm² of floor space per bird with a lighting schedule typical for commercial egg production. 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-2019-001) was approved by the Institutional Animal Care and Use Committee of the U.S. National Poultry Research Laboratory.

Preinoculation 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. 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 broth (Acumedia) and incubated for 24 h at 41.5°C. A 10- μ L portion from each of these broth cultures was then streaked onto brilliant green (BG) agar (Acumedia) supplemented with 0.02 mg/mL of novobiocin (Sigma Chemical Co., St. Louis, MO). 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

After transfer to the containment facility in each trial, 24 of the 72 pullets in 2 replicate isolation rooms were orally inoculated with a measured dose of a 2-strain mixture (phage types 4 and 13a) of *S. Enteritidis* at 1 d after placement into the containment facility at 16 wk of age. An identical proportion of pullets in the other two replicate rooms were similarly orally inoculated at 21 d post-placement (19 wk of age). Both *Salmonella* strains were originally isolated from internal organs of naturally infected chickens in commercial settings. Each inoculum strain was resuscitated by transfer into tryptic soy broth (Acumedia) for two 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 two 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 BG agar confirmed that the final cell concentration in each 1.0-ml oral inoculum dose was 5.3×10^7 cfu (equalized for both trials). Orally infected pullets were identified by colored leg bands.

Internal Organ Samples

At 6 d postinoculation in each trial, 36 pullets per room were randomly selected (12 orally infected birds and 24 uninoculated birds) and humanely 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 s. 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- μ L aliquot of each culture was then streaked onto BG agar plus novobiocin. Following incubation of these plates for 24 h at 37°C, typical *S. Enteritidis* colonies were subjected to biochemical and serological confirmation (Gast et al., 2019). This sampling procedure was repeated for the remaining 36 pullets in each isolation room at 12 d postinoculation. The two sampling dates were chosen to represent peak and post-peak intervals for the frequency of systemic dissemination of infection following oral exposure (Gast et al., 2020).

Statistical Analysis

Significant differences ($P < 0.05$) between rearing densities, postplacement infection intervals, or postinfection sampling intervals in the mean frequencies of *S. Enteritidis* isolation from internal organs were determined by Fisher's exact test. Because the 2 replicate rooms within each trial never differed significantly in *Salmonella* recovery from any of the 3 sampled tissues, their results were combined for analysis and presentation. Data were analyzed with InStat biostatistics software (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

No preinfection cloacal swab samples were *Salmonella*-positive in either trial. After pullets were infected with *S. Enteritidis* at either 16 or 19 wk of age (1 d and 3 wk, respectively, following transfer from the rearing facility) and sampled for internal organ colonization 6 d later, the pathogen was recovered from at least 54.2% of livers, 58.3% of spleens, and 83.3% of intestines from groups of orally inoculated birds and from at least 14.6% of livers, 16.7% of spleens, and 64.6% of intestines from groups of contact-exposed birds (Table 1). When samples were collected 12 d postinfection, *S. Enteritidis* was isolated from at least 29.2% of livers, 25.0% of spleens, and 75.0% of intestines from groups of orally inoculated pullets as well as from at least 14.6% of livers, 14.6% of spleens, and 54.2% of intestines from groups of contact-exposed pullets (Table 1). There were no significant differences ($P > 0.05$) in *S. Enteritidis* isolation frequencies between high and low density rearing groups associated with either of the 2 postplacement infection intervals or with either of the 2 postinfection sampling intervals. For both sampling dates (6 plus 12 d postinfection) and both rearing densities (high and low) combined, *S. Enteritidis* was found at significantly higher frequencies among pullets infected orally at 19 wk than at 16 wk in spleen (68.8% vs. 49.0%; $P = 0.0081$) and intestinal (96.9% vs. 84.4%; $P = 0.0051$) samples. Similarly, for both routes of pathogen introduction (orally inoculated plus

contact-exposed) combined, the frequency of *S. Enteritidis* isolation after infection at 19 wk was significantly higher than at 16 wk from livers (38.9% vs. 29.2%; $P = 0.0175$) and spleens (39.9% vs. 28.1%; $P = 0.0037$).

The introduction, transmission, and persistence of *Salmonella* and other food-borne pathogens in poultry are influenced by numerous characteristics of their housing environment, including levels of dust and moisture and the presence of vectors such as rodents and insects. These environmental risk factors can vary substantially according to flock biosecurity and sanitation practices, but design elements inherent to poultry housing systems can also play important roles. A considerable body of published work has extensively described the progression of *S. Enteritidis* infections in caged laying hens, but the corresponding documentation for more extensive systems is not as thorough. The intestinal microbiota of hens in cage-free systems has been reported as less diverse than that of caged birds (Wiersema et al., 2021), implying a possibility for different responses to pathogen exposure in different systems.

Studies comparing housing systems have generated diverse and often contradictory results about their associated frequencies of *Salmonella* environmental prevalence, hen infection, and egg contamination (Whiley and Ross, 2015). Variations within and between housing systems in bird stocking densities, levels of exposure to contaminated dust and feces, and populations of biological vectors often confound the dependability of comparative conclusions. In a large field study conducted under commercial management conditions, the prevalence of *Salmonella* in egg shell and environmental samples was similar for hens in conventional cages, enriched colonies, and cage-free aviaries (Jones et al., 2015), although unique pathogen risk factors were identified in each type of housing. One specific observation from prior research with broad potential significance is that high hen stocking densities were linked to more frequent invasion of internal organs by *S. Enteritidis* in experimentally infected hens housed in either conventional cages or enriched colonies (Gast et al., 2016, 2019), perhaps attributable to immunologically suppressive stress. However, the possibility that the stocking densities at which egg-type pullets are reared might affect their susceptibility to *S. Enteritidis* infection in a manner that persists until exposure at or near sexual maturity has not been previously explored.

In the present study, rearing pullets at 2 different stocking densities did not affect the susceptibility of birds to internal organ colonization by *S. Enteritidis*, either when they were infected immediately after transfer into cage-free containment rooms or 3 wk later. As all pullets were reared in cage-free facilities, one possible explanation for the absence of a significant rearing density effect on infection outcomes is that any stress associated with higher stocking densities in conventional cage or enriched colony systems in prior experiments was not caused at similarly high stocking densities in a cage-free environment. Alternatively, any stress-induced increase in infection susceptibility that occurred during rearing

Table 1. Recovery of *Salmonella* Enteritidis from internal organs of orally infected and contact-exposed pullets in cage-free housing after rearing at different stocking densities.¹

	Orally inoculated			Contact-exposed		
	Liver	Spleen	Ileum/Ceca	Liver	Spleen	Ileum/Ceca
	----- <i>Salmonella</i> -positive/total (%)-----					
Infected at 16 wk of age						
Sampled 6 d postinfection						
High density	17/24 (70.8) ^a	16/24 (66.7) ^{ab}	24/24 (100) ^a	11/48 (22.9) ^{ab}	8/48 (16.7) ^a	35/48 (72.9) ^{ab}
Low density	13/24 (54.2) ^{ab}	14/24 (58.3) ^{ab}	20/24 (83.3) ^{ab}	7/48 (14.6) ^a	9/48 (18.8) ^a	31/48 (64.6) ^{ab}
Sampled 12 d postinfection						
High density	9/24 (37.5) ^b	11/24 (45.8) ^{ac}	19/24 (79.2) ^b	11/48 (22.9) ^{ab}	7/48 (14.6) ^a	35/48 (72.9) ^{ab}
Low density	7/24 (29.2) ^b	6/24 (25.0) ^c	18/24 (75.0) ^b	9/48 (18.8) ^{ab}	10/48 (20.8) ^a	32/48 (66.7) ^{ab}
Infected at 19 wk of age						
Sampled 6 d postinfection						
High density	23/24 (95.8) ^c	22/24 (91.7) ^d	24/24 (100) ^a	18/48 (37.5) ^b	15/48 (31.3) ^a	39/48 (81.3) ^b
Low density	19/24 (79.2) ^{ac}	17/24 (70.8) ^{bd}	24/24 (100) ^a	18/48 (37.5) ^b	15/48 (31.3) ^a	35/48 (72.9) ^{ab}
Sampled 12 d postinfection						
High density	9/24 (37.5) ^b	14/24 (58.3) ^{ab}	23/24 (95.8) ^{ab}	7/48 (14.6) ^a	8/48 (16.7) ^a	28/48 (58.3) ^a
Low density	9/24 (37.5) ^b	13/24 (54.2) ^{abc}	22/24 (91.7) ^{ab}	9/48 (18.8) ^{ab}	11/48 (22.9) ^a	26/48 (54.2) ^a

^{a,b,c,d}Values in columns that share no common superscripts are significantly ($P < 0.05$) different.

¹Pullets were reared in cage-free housing at stocking densities of either 374 cm² (high density) or 929 cm² (low density) of floor space per bird and placed into 4 rooms of a cage-free disease containment facility at 16 wk of age. 24 of 72 pullets in each of 2 rooms were orally inoculated with approximately 5.3×10^7 cfu of a two-strain mixture of *S. Enteritidis* at 1 d post-placement. Pullets in the other 2 rooms were similarly inoculated at 21 d postplacement. The remaining pullets in each room were exposed to infection by horizontal contact. $\frac{1}{2}$ of the orally inoculated birds and $\frac{1}{2}$ of the contact-exposed birds in each room were sampled for the presence of *S. Enteritidis* in internal organs at 6 d postinfection and the other $\frac{1}{2}$ at 12 d.

at a higher stocking density may not have persisted after the pullets were transferred into cage-free containment housing at a lower density.

In a study conducted using the same cage-free housing system as the present investigation, widespread horizontal dissemination of *S. Enteritidis* was reported during the first 2 wk after the pathogen was introduced into groups of laying hens, and high frequencies of invasion to internal organs were observed in both orally infected and contact-exposed hens (Gast et al., 2020). The extensive access of birds to each other and to feces-contaminated flooring substrate in cage-free systems may facilitate horizontal transmission of salmonellae and thereby prolong the production of contaminated eggs by newly infected hens. In the present study, the frequencies of *S. Enteritidis* isolation from internal organs of both orally inoculated and contact-exposed pullets infected at 19 wk of age were similar to previously noted values for laying hens infected at 24 wk (Gast et al., 2020). However, the overall frequencies of *S. Enteritidis* recovery from all 3 sampled tissues of pullets infected at 16 wk in the current study were significantly lower than for the birds infected 3 wk later (even though the younger group potentially experienced transport stress from being moved between facilities just before *Salmonella* inoculation). Prior data have shown in several instances that the *Salmonella* load in the tissues of infected birds can increase at the point of sexual maturity, perhaps due to temporarily decreased immunological responsiveness. For example, Wigley et al. (2005) determined that the loss of T-cell activity at the point of lay was associated with *S. Pullorum* infection of the reproductive tract and deposition in eggs. Moreover, the frequency of egg contamination by *Salmonella* has been found to be greater at the onset of laying than at any later time during egg production (Crabb et al., 2019). Additional accumulation of dust and feces over time might also facilitate

horizontal transmission of infection in older birds. The increased susceptibility to invasive *S. Enteritidis* infection at sexual maturity observed in the present study may represent a significant risk factor for this food safety pathogen in young hens and thus calls for attentive risk reduction efforts at this critical stage in the life cycle of laying flocks.

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.

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.

REFERENCES

- Chousalkar, K., R. Gast, F. Martelli, and V. Pande. 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., J. R. Gilkerson, and G. F. Browning. 2019. Does only the age of the hen matter in *Salmonella enterica* contamination of eggs? *Food Microbiol.* 77:1–9.
- Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, R. Gast, T. J. Humphrey, and F. Van Immerseel. 2009. Mechanisms of egg contamination by *Salmonella* Enteritidis. *FEMS Microbiol. Rev.* 33:718–738.
- Gast, R. K., R. Guraya, D. R. Jones, K. E. Anderson, and D. M. Karcher. 2016. Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens housed in

- enriched colony cages at different stocking densities. *Poult. Sci.* 95:1363–1369.
- Gast, R. K., D. R. Jones, R. Guraya, K. E. Anderson, and D. M. Karcher. 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., P. Regmi, R. Guraya, D. R. Jones, K. E. Anderson, and D. M. Karcher. 2019. Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens of four commercial genetic lines in conventional cages and enriched colony housing. *Poult. Sci.* 98:1785–1790.
- Jones, D. R., N. A. Cox, J. Guard, P. J. Fedorka-Cray, R. J. Buhr, R. K. Gast, Z. Abdo, L. L. Rigsby, J. R. Plumblee, D. M. Karcher, C. I. Robison, R. A. Blatchford, and M. M. Makagon. 2015. Microbiological impact of three commercial laying hen housing systems. *Poult. Sci.* 94:544–551.
- Whiley, H., and K. Ross. 2015. *Salmonella* and eggs: from production to plate. *Int. J. Environ. Res. Public Health* 12:2543–2556.
- Wiersema, M. L., L. R. Koester, S. Schmitz-Esser, and D. A. Koltes. 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.
- Wigley, P., S. D. Hulme, C. Powers, R. K. Beal, A. Berchieri Jr., A. Smith, and P. A. Barrow. 2005. Infection of the reproductive tract and eggs with *Salmonella enterica* serovar Pullorum in the chicken is associated with suppression of cellular immunity at sexual maturity. *Infect. Immun.* 73:2986–2990.