Investigation of the potential of aerosolized *Salmonella* Enteritidis on colonization and persistence in broilers from day 3 to 21

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ABSTRACT The presence of *Salmonella* in air of poultry houses has been previously confirmed. Therefore, it is important to investigate the entry of *Salmonella* into broilers through air. The present study aimed to evaluate different levels of *Salmonella* Enteritidis aerosol inoculations in broiler chicks for colonization of ceca, trachea, and liver/spleen and persistence over time. In 3 independent trials, 112 one-day-old birds were randomly divided into 4 groups (n = 28/group). On d 1 of age, one group was exposed to an aerosol of sterile saline and the remaining three groups were exposed to an aerosol generated from one of 3 doses (10^3, 10^6, or 10^9 CFU/mL) of *S.* Enteritidis inoculum. Aerosol exposure time was 30 min/group and was performed using a nebulizer. On d 3, 7, 14, and 21 of age, ceca, trachea, and liver/spleen were aseptically removed. Ceca were cultured for *Salmonella* counts ($\log_{10}$ CFU/g) and all tissues were cultured for *Salmonella* prevalence. All tissues from the control group were *Salmonella* negative for all sampling days. On sampling d 3 and 7, ceca *Salmonella* counts were highest (5.14 and 5.11, respectively) when challenged with 10^9 *Salmonella* ($P \leq 0.0281$). Ceca *Salmonella* counts increased from d 3 (2.43) to d 7 (4.43), then remained constant when challenged at 10^3 *Salmonella*, and counts decreased over time for all other groups. Tissue *Salmonella* prevalence increased with increasing challenge levels at all sampling timepoints ($P \leq 0.0213$). *Salmonella* prevalence was low (0/18 to 4/18) and did not change over time following 10^3 *Salmonella* challenge ($P \geq 0.2394$). Prevalence decreased over time in ceca and trachea following 10^6 and 10^9 *Salmonella* challenge ($P \leq 0.0483$). Liver/spleen *Salmonella* prevalence increased from d 3 (13/18) to d 14 (18/18) and then decreased at d 21 (10/18) in birds exposed to an aerosol of 10^9 *Salmonella* but remained constant over time for rest of the *Salmonella* inoculated groups. Overall, this study demonstrated the *Salmonella* colonization and persistence in different tissues in broilers following exposure to aerosolized *Salmonella*.

Key words: *Salmonella*, broiler, aerosol, tissue, poultry

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INTRODUCTION

More than 2,500 *Salmonella* serotypes have been characterized, and >100 serotypes have been linked to human infections (CDC, 2020). *Salmonella* causes human salmonellosis which is a major foodborne illness encountered in the United States. Poultry products have been frequently found to be linked to *Salmonella* outbreaks (CDC, 2018).

Presence of *Salmonella* in live poultry populations is one of the major factors for *Salmonella* contamination of poultry meat and eggs (Hugas and Beloeil, 2014). During poultry production, *Salmonella* spread can be possible by both horizontal and vertical pathways through several possible sources including breeders, hatcheries, feed, production house environment, rodents, and insects (Liljebjelke et al., 2005). In poultry production houses, *Salmonella* colonization in birds can be possible through several routes. Previously, Cox et al. (1996) found that *Salmonella* administration in broiler chicks through mouth, cloaca, eyes, and nasal passages readily results in seeder birds which may then spread *Salmonella* throughout the poultry production pen or house. The entry of bacteria from air through the respiratory route in poultry has not been deeply explored, although some

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studies have examined and confirmed this possibility by performing inoculation of poultry (broilers, turkeys, and layers) with bacterial contaminated aerosol (Cox et al., 1996; Knab et al., 2018; Cheng et al., 2020).

In livestock houses, there are several sources of airborne microorganisms including litter, feed, animal respiratory tracts, animal skin, feces, and farm workers (Zhao et al., 2014). Diverse kinds of bacteria (including *Salmonella* spp.) have been confirmed from the air in broiler houses (Chinivasagam et al., 2009; Fallschissel et al., 2009). *Salmonella* can travel in air by either being carried on dust particulate or in aerosol (Gast et al., 1998). Some studies have reported the airborne transmission of *Salmonella* in poultry facilities. Specifically, Gast et al. (1998) reported transmission of *S.* Enteritidis through air from challenged to nonchallenged groups of layers when both bird groups were physically separated from each other but sharing the same air circulation in a controlled environmental isolation cabinet. They found *Salmonella* positive results both from circulating air and in nonchallenged birds. Similarly, the observations of *Salmonella* infection in turkeys after exposure to aerosol, containing *Salmonella* contaminated fecal dust particles, confirmed the airborne transmission of *Salmonella* (Harbaugh et al., 2006). Additionally, Kallapura et al. (2014) recovered *Salmonella* from ceca-cecal tonsil, trachea, and liver/spleen after intratracheal administration of *Salmonella* in broiler chicks, and thereby they confirmed the possibility of respiratory route to serve as an entry point for *Salmonella* in poultry birds. Moreover, when 2 different *Salmonella* serotypes (*S.* Enteritidis and *S.* Heidelberg) were administered in day-of-hatch broiler chicks via one of several different inoculation routes (oral, intratracheal, subcutaneous, ocular, and cloacal), then the overall highest recovery from the samples (trachea, crop, liver/spleen, cecum, and cloacal swab) of market-age broilers was observed following intratracheal inoculation compared to the other inoculation routes for both *Salmonella* serotypes (Chadwick et al., 2020).

Therefore, published research implicate airborne *Salmonella* as a risk factor for *Salmonella* infections or colonization in chickens by detecting the existence of *Salmonella* in air of poultry houses, airborne transmission of *Salmonella*, and the possibility of respiratory route to serve as an entry portal for *Salmonella*. However, this phenomenon can be explained by inoculation of chickens through *Salmonella* contaminated aerosol, that mimics the natural route of bacterial infection through air, in a more concise way. In this regard, our objective was to evaluate the potential of different levels of *Salmonella* Enteritidis aerosol inoculation in day-old broiler chicks for colonization of their ceca, trachea, and liver/spleen (pooled) over time.

MATERIALS AND METHODS

All the procedures conducted in this study were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) (PRN #2021-3841).

Experimental Design

For each of the 3 independent trials, a total of 112 one-d old broilers (trial 1: Ross708, trials 2 and 3: YPMxRoss708) were randomly divided into 4 groups (n = 28/group). On d 1 of bird age, one group was exposed to an aerosol of sterile saline and the remaining 3 groups were exposed to an aerosol generated from one of 3 doses (10^3 CFU/mL, 10^6 CFU/mL, or 10^9 CFU/mL) of *S.* Enteritidis inoculum. Aerosol exposure time of 30 min/group was selected based on the nebulizing rate and inoculum or saline holding capacity of the nebulizer cup used for aerosol exposure. Following aerosol exposures, all the birds were placed in battery cages, having litter-free environment, at the Auburn University Poultry Research Farm Battery House (2 cages/group, total cages = 8). The amount of allotted space per bird (d 1: 51 in^2/bird, d 3: 66 in^2/bird, d 7: 91 in^2/bird, d 14: 145 in^2/bird, d 21: 364 in^2/bird) exceeded the minimum allowed space for broilers up to 21 d of age. Cages were separated from each other by one empty cage (66 cm). Birds were provided ad-libitum feed and water (in external troughs) during growout. On d 3, 7, 14, and 21 of age, ceca, trachea, and liver/spleen of 6 birds/group/trial (or 3 birds/cage/trial) were aseptically removed after euthanizing the birds by CO_2 asphyxiation and placed separately into sterile sampling bags (Nasco whirl-pak sample bag, Madison, WI). The sampling time intervals were based on allowing time for transient *Salmonella* to pass by d 3, then repeated measures at d 7, 14, and 21 were performed to evaluate *Salmonella* over time. Sampling after d 21 was not performed due to bird and battery cage size. After collecting samples, bags were placed on ice and then transported to the laboratory for microbiological examination. Collected tissues were cultured for *Salmonella* prevalence and ceca for *Salmonella* enumeration ($\log_{10}$ CFU/g).

Salmonella Inoculum Preparation

Salmonella inoculums were prepared as previously described (Pal et al., 2021). *Salmonella enterica* serotype Enteritidis, resistant to 100 μg/mL nalidixic acid, was used for aerosol inoculations of birds. The marker strain, stored in glycerol at −80°C, was first plated onto plate count agar (Hardy Diagnostics, Santa Maria, CA). The colonies were collected from plate count agar plates after the incubation period of 24 h at 37°C and then suspended in sterile saline to achieve an optical density approaching 10^9 CFU/mL. The actual counts were confirmed by plating the appropriate inoculum dilutions onto 100 μg/mL nalidixic acid containing Xylose Lysine Tergitol-4 **(XLT4)** (Criterion, Hardy Diagnostics) agar plates in duplicate. *Salmonella* counts from XLT4 agar plates were reported after an incubation period of 24 h at 37°C. The actual obtained *Salmonella* counts ($\log_{10}$

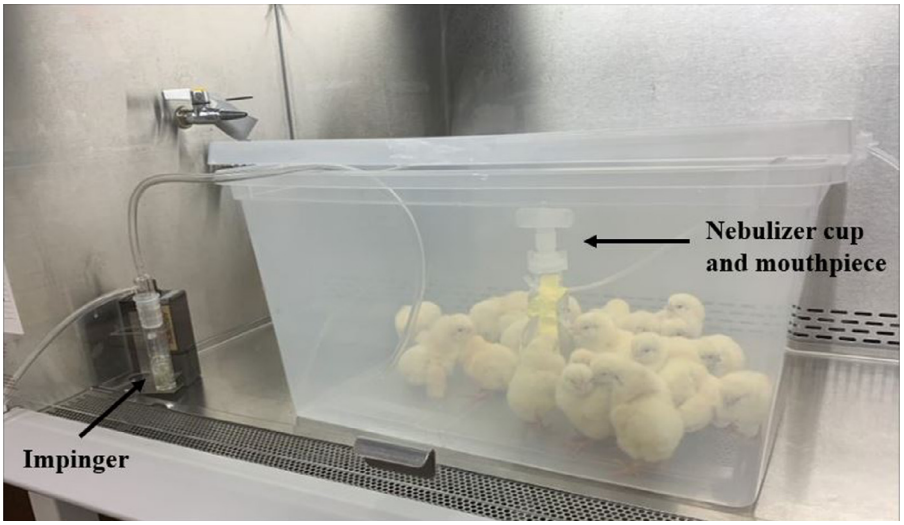

Figure 1. Experiment setup for exposure of broiler chicks to different aerosol treatments.

CFU/mL) were 8.70, 8.54, and 8.48 for trials 1, 2, and 3, respectively. Each prepared inoculum was further serially diluted in sterile saline to obtain the desired levels of *Salmonella* required for aerosol inoculations.

Procedure of Aerosol Exposure

Aerosol exposure protocols were developed for the purpose of this study. Within each trial, for aerosol exposure of each group, 28 birds were first placed into a cleaned and sanitized plastic tub (58.4 cm × 41.3 cm × 31.4 cm, LWH, Sterilite, Townsend, MA) within a biosafety cabinet (Figure 1). The plastic tub was equipped with a disposable nebulizer cup and mouthpiece (Aeromist Compact, Medline Industries, Inc., Northfield, IL) in the middle. The nebulizer cup contained 8 mL of *Salmonella* inoculum dose or sterile saline depending on the assigned group treatment. *Salmonella* or saline was nebulized for 30 min from the nebulizer cup to the birds within the tub through a mouthpiece which had 2 open ends. The tub was closed with a lid on top during aerosol exposure treatments. The nebulizer compressor (Aeromist Compact, Medline Industries, Inc.) was attached to the nebulizer cup to generate the *Salmonella* or sterile saline aerosol through the mouthpiece and itself was placed outside the biosafety cabinet. The average rate of *Salmonella* inoculum and sterile saline distribution in air was 0.20 mL/min. Based on the manufacturer's specifications, the nebulized particles size was less than 5 μm. After nebulization for 30 min, the plastic tub remained untouched in the biosafety cabinet for 5 min to allow suspended aerosol to settle.

For each treatment group, simultaneously during aerosol exposure, the counts of *Salmonella* in tub air were assessed similarly to a previously described method (Pal et al., 2021). Air was collected from the tub for 30 min into 10 mL of buffered peptone water **(BPW)** (BBL, Becton Dickinson and Company, Sparks, MD) using an impinger system that had an air collection rate of 0.75 L/min (ACE Glass Incorporated, 7531 − 10 Midget Impinger Comp., Vineland, NJ). After that, direct or an appropriate serial dilution in BPW was duplicate plated onto XLT4 agar plates, containing 100 μg/mL of nalidixic acid, and then presumptive *Salmonella* counts were recorded after the incubation period of 24 h at 37°C. The remaining volume of the BPW air sample (8.8 mL) was further incubated for 24 h at 37°C for enrichment. After 24 h, the enriched BPW air sample was streaked onto XLT4 agar plates (containing 100 μg/mL of nalidixic acid) and then presumptive colonies of *Salmonella* were reported after the incubation period of 24 h at 37°C. The levels of *Salmonella* in tub air, to which the chicks were exposed, with respect to trial and assigned group treatment, are given in Table 1. The biosafety cabinet and plastic tubs were sanitized with ethanol each time before and after every aerosol exposure. After completing one group aerosol exposure cycle

Table 1. *Salmonella* counts or presence in air (within the tub), during aerosol exposures of broilers, with respect to trial number and assigned group treatment.

	Salmonella counts ($\log_{10}$ CFU/m^3) or presence (positive or negative)		
Aerosol exposure treatments	Trial 1	Trial 2	Trial 3
Sterile saline	ND[1] (negative)	ND (negative)	ND (negative)
10^3 CFU/mL SE[2]	ND (positive)	3.35	ND (positive)
10^6 CFU/mL SE	5.25	6.04	6.05
10^9 CFU/mL SE	8.32	8.25	8.05

[1]ND = Not detected by direct plating for *Salmonella* counts. Minimum detection limit was 3.35 $\log_{10}$ CFU/m^3.
[2]SE,*Salmonella* Enteritidis.

(30 + 5 min), birds were transferred individually by hand to a cleaned and sanitized plastic tub and then transported to the battery house. During transport, birds remained in the plastic tub and were not handled until present in the room in which they were housed. Aerosol nebulization was performed in the order of control (Group 1), 10^3 CFU/mL (Group 2), 10^6 CFU/mL (Group 3), and then 10^9 CFU/mL (Group 4).

Microbial Analyses of Collected Tissues

Collected tissues were analyzed using previously described protocols (Cox et al., 1996; Chadwick et al., 2020). Collected ceca, trachea, and liver/spleen were first macerated within their respective sampling bag and then the average weight of each type of tissue was calculated using 5 random samples. Ceca were collected as an indicator of intestinal colonization, trachea as an indicator of respiratory colonization, and liver/spleen as an indicator of systemic infection. Liver and spleen samples were pooled to maximize *Salmonella* detection potential. Next, BPW (10 mL when the tissue weight was <3.3 g or 3 times the weight of tissue when tissue weight was >3.3g) was added into each sampling bag of collected tissues. Following this, tissues were stomached for 1 min. For *Salmonella* enumeration from ceca, an aliquot from direct BPW homogenates or their appropriate dilutions, in sterile saline, were duplicate plated onto XLT4 agar plates that contained 100 μg/mL of nalidixic acid. The *Salmonella* counts were recorded after the incubation period of 24 h at 37°C. For *Salmonella* prevalence detection from each type of tissues, the original BPW homogenates were incubated for 24 h at 37°C for enrichment. After 24 h, each sample was streaked onto 100 μg/mL nalidixic acid containing XLT4 agar plates and the confirmation of *Salmonella* was completed after incubation of 24 h at 37°C.

Statistical Analyses

Salmonella counts were transformed into $\log_{10}$ CFU/g before data analysis. Ceca *Salmonella* count data were analyzed using two-way ANOVA. Means value of *Salmonella* counts were compared among the inoculated groups using Tukey's HSD test and level of significance was set at $P \leq 0.05$. *Salmonella* prevalence data was analyzed using Fisher's exact test. *Salmonella* prevalence data comparisons were performed between all the possible combinations and level of significance was set at $P \leq 0.05$. All data of this study was analyzed using SAS Studio, release 3.8 Enterprise Edition.

RESULTS

Data of *Salmonella* counts or presence in the air, that was circulating within the tub during exposure of broilers to different aerosol treatment levels, are given in Table 1. All the air samples were *Salmonella* negative when birds were exposed to an aerosol of sterile saline. When birds were exposed to an aerosol of *Salmonella* inoculum of 10^3 levels, 100% *Salmonella* prevalence in air samples was observed and *Salmonella* counts in air were $\leq$3.35 $\log_{10}$ CFU/m^3. *Salmonella* counts in air ($\log_{10}$ CFU/m^3) ranged between 5.25 to 6.05 and 8.05 to 8.32 when air samples were obtained from the tub simultaneously during bird exposure to an aerosol of *Salmonella* inoculum of 10^6 and 10^9 levels, respectively.

Salmonella counts ($\log_{10}$ CFU/g) in ceca obtained at different ages (d 3, d 7, d 14, d 21) from broilers after exposure to different aerosol treatments are presented in Table 2. No *Salmonella* counts were observed in ceca for control group birds exposed to an aerosol of sterile saline. Ceca *Salmonella* counts increased ($P = 0.0188$) from d 3 (2.43) to d 7 (4.43) and then remained constant for birds exposed to an aerosol generated from lowest dosed inoculum of *Salmonella* (10^3). For bird groups exposed to an aerosol of *Salmonella* inoculum of 10^6 and 10^9 levels, ceca *Salmonella* counts decreased with broiler age ($P = 0.005$ and $P < 0.0001$, respectively) from 4.56 at d 3 to 2.59 at d 21 and 5.14 at d 3 to 2.81 at d 21, respectively. Differences in ceca *Salmonella* counts among *Salmonella* aerosol-inoculated bird groups were observed at d 3 ($P < 0.0001$) and d 7 ($P = 0.0281$). On d 3 and 7, the highest *Salmonella* counts in ceca (5.14 and 5.11, respectively) were observed for the birds challenged with an aerosol of *Salmonella* inoculum of 10^9 levels. The lowest ceca *Salmonella* counts on d 3 (2.43) and 7 (3.85) was observed in the bird groups challenged with

Table 2. *Salmonella* counts in ceca obtained at different ages (d 3, d 7, d 14, d 21) from broilers following exposure to aerosol of different levels of *S.* Enteritidis inoculum or sterile saline for 30 min at d 1 of age. (n = 18/group/sampling day).

	Salmonella counts ($\log_{10}$ CFU/g ± Standard error)[5]				
Aerosol exposure treatments	d 3	d 7	d 14	d 21	*P* value
Sterile saline[1]	ND[4]	ND	ND	ND	-
10^3 CFU/mL SE[2]	$2.43 \pm 0.33^{b,y}$	$4.43 \pm 0.42^{a,xy}$	3.68 ± 0.04^{ab}	3.10 ± 0.67^{ab}	0.0188
10^6 CFU/mL SE[3]	$4.56 \pm 0.22^{a,x}$	$3.85 \pm 0.40^{ab,y}$	2.84 ± 0.26^{b}	2.59 ± 0.32^{b}	0.0005
10^9 CFU/mL SE	$5.14 \pm 0.21^{a,x}$	$5.11 \pm 0.22^{a,x}$	2.94 ± 0.36^{b}	2.81 ± 0.29^{b}	<0.0001
P value	<0.0001	0.0281	0.5666	0.7781	-

[1]Sterile saline = The data of this group were not used for statistical analysis.
[2]SE, *Salmonella* Enteritidis.
[3]10^6 CFU/mL (SE) = One of the ceca samples of this treatment group was lost at d 3.
[4]ND, Not detected by either direct plating or enrichment for *Salmonella*.
[5]*Salmonella* counts ($\log_{10}$ CFU/g ± Standard error) = Only *Salmonella* positive samples were included for statistical analysis.
[a-b]Values within a row with different superscripts are significant different ($P \leq 0.05$).
[x-y]Values within a column with different superscripts are significant different ($P \leq 0.05$).

Table 3. *Salmonella* prevalence in ceca, trachea, and liver/spleen detected at different ages (d 3, d 7, d 14, d 21) from broilers following exposure to aerosol of different levels of *S.* Enteritidis inoculum or sterile saline for 30 min at d 1 of age. (n = 18/group/ sampling day).

		Salmonella prevalence (Positive samples/Total samples)				
Sampled tissues	Aerosol exposure treatments	d 3	d 7	d 14	d 21	*P* value
Ceca	Sterile saline[1]	0/18	0/18	0/18	0/18	-
	10^3 CFU/mL (SE[2])	4/18^{y}	4/18^{y}	4/18^{y}	2/18^{y}	0.8125
	10^6 CFU/mL (SE)[3]	17/17$^{a, x}$	18/18$^{a, x}$	15/18$^{a, x}$	8/18$^{b, xy}$	<0.0001
	10^9 CFU/mL (SE)	18/18$^{a, x}$	18/18$^{a, x}$	18/18$^{a, x}$	12/18$^{b, x}$	0.0005
	P value	<0.0001	<0.0001	<0.0001	0.0020	-
Trachea	Sterile saline[1]	0/18	0/18	0/18	0/18	-
	10^3 CFU/mL (SE)	0/18^{y}	2/18^{z}	0/18^{y}	0/18^{y}	0.2394
	10^6 CFU/mL (SE)	17/18$^{a, x}$	10/18$^{b, y}$	11/18$^{b, x}$	5/18$^{b, x}$	0.0004
	10^9 CFU/mL (SE)	18/18^{x}	18/18^{x}	16/18^{x}	14/18^{x}	0.0483[4]
	P value	<0.0001	<0.0001	<0.0001	<0.0001	-
Liver/spleen	Sterile saline[1]	0/18	0/18	0/18	0/18	-
	10^3 CFU/mL (SE)	1/18^{y}	2/18^{y}	3/18^{z}	2/18^{y}	0.9542
	10^6 CFU/mL (SE)	12/18^{x}	12/18^{x}	10/18^{y}	6/18^{xy}	0.1703
	10^9 CFU/mL (SE)	13/18$^{bc, x}$	17/18$^{ab, x}$	18/18$^{a, x}$	10/18$^{c\ x}$	0.0015
	P value	<0.0001	<0.0001	<0.0001	0.0213	-

[1]Sterile saline = The data of this group were not used for statistical analysis.
[2]SE, *Salmonella* Enteritidis.
[3]10^6 CFU/mL (SE) = One of the ceca samples of this treatment group was lost at d 3.
[4]0.0483 = Although the overall *P* value was significant, but direct comparisons between days were not significant.
[a-c]Values within a respective tissue type and within a row with different superscripts are significant different ($P \leq 0.05$).
[x-z]Values within a respective tissue type and within a column with different superscripts are significant different ($P \leq 0.05$).

an aerosol of *Salmonella* inoculum of 10^3 and 10^6 levels, respectively.

Salmonella prevalence in the tissues obtained at different ages (d 3, d 7, d 14, d 21) from broilers after exposure to the aerosol treatments are presented in Table 3. All the tissues from control group birds were *Salmonella* negative. *Salmonella* prevalence did not change over time in any of the sampled tissues (ceca, trachea, and liver/spleen) for the bird group exposed to an aerosol generated from lowest dosed inoculum of *Salmonella* (10^3, $P \geq 0.2394$). For this group of birds, *Salmonella* prevalence ranged between 2/18 to 4/18, 0/18 to 2/18, and 1/18 to 3/18 in ceca, trachea, and liver/spleen, respectively. For birds exposed to an aerosol of *Salmonella* inoculum of 10^6 and 10^9 levels, *Salmonella* prevalence decreased over time in ceca from 17/17 to 8/18 and 18/18 to 12/18, respectively, and in trachea from 17/18 to 5/18 and 18/18 to 14/18, respectively. *Salmonella* prevalence in liver/spleen did not change with increasing broiler age ($P \geq 0.1703$) for bird groups exposed to an aerosol of *Salmonella* inoculum of 10^3 or 10^6 levels. However, *Salmonella* prevalence in liver/spleen increased from d 3 (13/18) to d 14 (18/18) and then decreased at d 21 (10/18) for birds exposed to an aerosol generated from highest dosed inoculum of *Salmonella* (10^9, $P = 0.0015$). In each kind of sampled tissue, *Salmonella* prevalence increased with increasing *Salmonella* inoculum levels, at all sampling timepoints ($P \leq 0.0213$). Overall, *Salmonella* persisted in both ceca and liver/spleen at all inoculum levels. However, in the trachea, *Salmonella* only persisted through 21 d of age at the higher 10^6 and 10^9 inoculum levels.

DISCUSSION

Salmonella colonization of internal tissues of broilers through *Salmonella* contaminated aerosol within commercial poultry houses is still an undefined phenomenon. However, there are a few experimentally conducted studies indicating the possibility of spread of *Salmonella* infection among poultry birds via air and *Salmonella* entry in broilers through the respiratory tract (Gast et al., 1998; Kallapura et al., 2014). Moreover, Cheng et al. (2020) observed tissue colonization and significant inflammatory cytokine expressions in leghorn chickens after exposure to *S.* Pullorum contaminated aerosol. Therefore, the objective of this experiment was to explore the colonization of different tissues (ceca, trachea, and liver/spleen) of broilers over time following exposure to an aerosol of *S.* Enteritidis.

In this study, the colonization of each of the sampled tissues of birds occurred following bird exposure at d 1 to an aerosol generated from *Salmonella* inoculum at each of the different levels (10^3, 10^6, and 10^9). The actual counts of *Salmonella* in air during aerosol inoculations of birds ranged between <3.35 and 8.32 $\log_{10}$ CFU/m^3 (Table 1). The lowest infectious dose of airborne *S.* Enteritidis for broilers colonization in this study was less than 3.35 $\log_{10}$ CFU/m^3 for 30 min. Previously, the minimum levels of airborne *S.* Pullorum responsible for colonization of lungs and livers of poultry were reported to be 2.10 $\log_{10}$ CFU/m^3 for 30 min (Cheng et al., 2020). In commercial poultry production houses, the airborne *Salmonella* levels were reported to range between 1.82 and 2.52 $\log_{10}$ CFU/m^3 (Venter et al., 2004; Fallschissel et al., 2009). Therefore, there is potential for *Salmonella* colonization in broilers at commercial poultry houses through *Salmonella* contaminated aerosol. Moreover, it has also been experimentally examined that the inhalation of 2.46 $\log_{10}$ CFU (or 290 cells) of *S.* Enteritidis by laying hens can result in colonization (Chart et al., 1992). However, the ability of chickens to inhale at least this many cells of *Salmonella* through air and the existence of continuous airborne exposure of chickens to aerosolized *Salmonella* in poultry houses still

requires investigation. These findings may help to elucidate the threat of airborne *Salmonella* to poultry animals at commercial poultry farms.

In the present study, the *Salmonella* counts ($\log_{10}$ CFU/g) in ceca ranged between 2.43 to 4.43, 2.59 to 4.56, and 2.81 to 5.14 for bird groups exposed to an aerosol of *Salmonella* inoculum of 10^3, 10^6, and 10^9 levels, respectively. Overall, the decreasing trend of ceca *Salmonella* counts was observed with broilers growth/age. Also, *Salmonella* prevalence in ceca and trachea was diminishing over time during growout. However, *Salmonella* prevalence in liver/spleen changed over time only in one of the bird groups that was exposed to an aerosol generated from *Salmonella* inoculum of 10^9 levels, where *Salmonella* prevalence was increased first up to d 14 and then decreased on d 21. Previously, when broiler chicks were inoculated directly into the crop at 1 d after hatching with 10^7 and 10^6 CFU of *S.* Typhimurium per chick, the ceca *Salmonella* counts ($\log_{10}$ CFU/g) and prevalence decreased with broiler age (Gast and Beard, 1989). Specifically, they observed that ceca *Salmonella* counts (ceca *Salmonella* prevalence) were 8.0 (100%) and 7.4 (100%) at 1 wk, and 3.6 (87.5%) and 3.4 (75.0%) at 7 wk, after inoculation of 10^7 and 10^6 CFU of *S.* Typhimurium per chick, respectively. In the same study, when chicks were inoculated with 10^8 CFU of *S.* Typhimurium directly into the crops of birds at 1 d after hatching, a decreasing trend of *Salmonella* prevalence in liver and spleen, 100% to 16.7% and 100% to 0.00%, respectively, with broiler age was observed. The reason for initial rise of *Salmonella* prevalence in liver/spleen for bird group, exposed to the highest inoculum level, in this present study is not clear. It may have been due to slow invasion or translocation of *S.* Enteritidis from the aerosol exposure to the liver/spleen or due to an increase in systemic infection over time at this high inoculum dose. It is also important to note that *Salmonella* persisted in all types of sampled tissue at d 21 following 30 min *Salmonella* aerosol exposure of day of hatch chicks. Continued persistence will need to be assessed through to market age of broilers. Recently, when day-of-hatch broiler chicks were administered with *S.* Enteritidis and *S.* Heidelberg via different inoculation routes such as oral, intratracheal, cloacal, ocular, and subcutaneous, the recovery of both *Salmonella* serotypes from trachea, crop, liver/spleen, ceca, and cloacal swab occurred when broilers reached market weight (Chadwick et al., 2020).

Overall, the order of *Salmonella* prevalence in sampled tissues was ceca (138/287) > trachea (111/288) > liver/spleen (106/288) in this study. High *Salmonella* prevalence in the trachea indicates that broiler chicks did inhale airborne *Salmonella*. Among 111 birds, which had *Salmonella* in their trachea, 102 and 84 of the birds had *Salmonella* presence in their ceca and liver/spleen, respectively (data not shown). This indicates that *Salmonella* might follow a systemic route of infection after entering the respiratory tract of birds through aerosol. Likewise, when *Salmonella* was administered intratracheally in broiler chicks in a previous study, the recovery of *Salmonella* from liver/spleens and ceca-cecal tonsils along with trachea was observed (Kallapura et al., 2014). The authors suggested that the recovery of *Salmonella* from ceca-cecal tonsils and liver/spleens indicates the systemic pathway of infection of *Salmonella* following intratracheal challenge in birds. Gast et al. (1998) also pointed out the possibility of the transfer of inhaled bacteria into the gastrointestinal tract within the oropharynx. Moreover, we observed that the overall prevalence in ceca (138/287) was greater compared to trachea (111/288), and *Salmonella* was recovered from ceca and liver/spleen but not from trachea in some instances, 36/138 and 22/106, respectively. This finding suggested that *Salmonella* might enter broilers from other body openings (mouth, eyes, cloaca etc.) along with the nasal passage during aerosol inoculation. This could explain why a higher ceca *Salmonella* prevalence was observed. Previously, Cox et al. (1996) observed a higher level of *Salmonella* colonization in ceca compared to lungs when broiler chicks were inoculated with *Salmonella* through aerosol. Moreover, the same study also demonstrated that, using different methods of *Salmonella* inoculations in chicks, the entry of *Salmonella* in chickens can be possible through multiple body openings such as mouth, nasal passage, cloaca, navel, and eyes, and passage of *Salmonella* through all these different pathways resulted in ceca colonization. Additionally, the possibility of mouth breathing in 1-day-old broilers was speculated when they were being exposed to microsphere aerosols of different sizes (Corbanie et al., 2006). Furthermore, during aerosol exposure of birds, *Salmonella* could be deposited on external surfaces of birds from where it later entered in birds during growout via oral ingestion during instances like preening or picking, and thereby increased intestinal colonization.

We observed the absence of *Salmonella* in each sampled tissue, on all sampling days, from the control group of birds that were exposed to an aerosol of sterile saline. This indicates that there was an absence of airborne spread of *Salmonella* among bird groups, which were housed in the same room within separated battery cages having litter-free environment. However, the previously conducted experimental studies observed the airborne transmission of *S.* Enteritidis from infected to uninfected chicks (control) when both sets of chicks were reared in the same house (Lever and Williams, 1996; Gast et al., 1998). This contrast in findings might be because of very low levels of *Salmonella* in air during growout that were not enough to colonize the control groups of chicks or might be due to failure of aerosolization of *Salmonella* during growout, in the present study. However, the counts or presence of *Salmonella* in air were not assessed in this study during growout.

Overall, the findings of this study indicate that airborne *Salmonella* may enter broiler chicks through aerosol, acquire systematic route of infection, and colonized multiple tissues. We observed the persistence of *Salmonella* colonization of tissues up to 21 d of birds age after *Salmonella* aerosol exposures of chicks at d 1, and the persistence of *Salmonella* colonization needs to be further assessed

through market age of birds. Further investigation regarding the likelihood of inhalation of airborne *Salmonella* by chickens in commercial poultry houses is still needed and would provide more knowledge about the aerosol route of *Salmonella* colonization in poultry.

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DISCLOSURES

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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