Development of a wild-type *Escherichia coli* environmental bloom model to evaluate alternatives to formaldehyde fumigation in broiler chicken hatch cabinets

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ABSTRACT Horizontal transmission of opportunistic *Escherichia coli* during hatch can have detrimental effects on early performance, particularly as pioneer colonizers. Commercially, formaldehyde is often applied in the United States to combat the bacterial bloom that occurs inside of the hatching environment. The purpose of these experiments was to develop a replicable *E. coli* horizontal challenge model to evaluate alternatives to formaldehyde sanitation applied to the hatching environment. In experiment 1, two trials were conducted for 2 wild-type (**WT**) *E. coli* isolates (isolate 1 [**I1**] or isolate 2 [**I2**]) to determine the appropriate in ovo challenge dose and day of embryogenesis (**DOE**) for challenge administration. In experiment 1 trial 1, the most appropriate inoculation dose and time point were determined to be 10^2 cfu/embryo on DOE 19. Experiment 1 trial 2 evaluated whether placement of seeder (direct-challenged) embryos with contact (indirect-challenged) embryos during hatch affected contact hatchability. Trial 2 showed no differences in hatchability between groups. A 7-day experiment (experiment 1 trial 2) was conducted to evaluate the effects of I1 or I2 on horizontal transmission, gram-negative bacterial (**GNB**) recovery from the gastrointestinal tract (**GIT**), and impact on BW gain (**BWG**). Compared with the negative control, seeder, and contact chicks challenged with I1 or I2, we observed increased ($P < 0.05$) GNB recovered from GIT on the day of hatch. There was a marked ($P < 0.05$) reduction in 7-day BWG between the I1 indirect-challenged group and the negative control group. To further validate the model, 2 7-day trials (experiment 2, experiment 3) were conducted to evaluate the effects of formaldehyde fumigation on coliform recovery from the hatching environment and on early performance using I1 for the challenge. Isolate 1 positive control hatchers had increased levels of circulating coliforms compared with the negative control and formaldehyde-treated hatchers, although there was no significant impact on performance induced by challenge or formaldehyde treatment in experiment 2 or experiment 3. These data provide a potential model for investigations related to horizontal transmission of WT *E. coli* at a low dose on DOE 19 to promote simulated commercially relevant bacterial blooms under laboratory conditions.

Key words: *Escherichia coli*, *in ovo*, broiler, hatcher, model

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INTRODUCTION

Commercial hatcheries serve as microbial reservoirs and promote the proliferation of opportunistic pathogens, such as antimicrobial-resistant *Escherichia coli* (Osman et al., 2018). Vertical transmission between the flock (breeder) and progeny (broiler) has been correlated for avian pathogenic *E. coli* strains (Giovanardi et al., 2005). Exposure to apathogenic microorganisms may have little risk; however, avian pathogenic *E. coli* strains cause extraintestinal lesions resulting in systemic disease associated with elevated 7-day morality (Kemmett et al., 2014).

Previously, *in ovo* inoculation into the chorioallantois sac with a nalidixic-resistant *E. coli* (36-55 cfu/100uL/embryo) at 12 d of embryogenesis negatively impacted hatchability and BW gain (**BWG**) and increased mortality compared with the negative control group (Montgomery et al., 1999). Furthermore, Montgomery et al. (1999) indicated that the placement of seeder (direct-challenged) eggs had little effect on contact (indirect-challenged) chick hatchability and BW over the 21-day experimental period. This suggests that chicks exposed to microbes during oviposition or the hatching period can serve as reservoirs for opportunistic pathogens during the grow out period. Recently, it was shown that the highest prevalence of select antimicrobial

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resistance *E. coli* was associated with the neonatal period suggesting the opportunistic pathogens could be transmitted vertically or horizontally (Apostolakos et al., 2019). In addition, nonviable embryos have also been implicated as potential reservoirs for antimicrobial resistant *E. coli* (Karunarathna et al., 2020).

During hatch, RH increases markedly, promoting the proliferation of the microbial bloom within hatching environment. Thus, hatchery sanitation is crucial. To evaluate circulating bacteria (such as total aerobic bacteria, presumptive lactic acid bacteria, and gram-negative bacteria) within the commercial hatching environment, the open agar plate method was previously used to compare environmental treatments with spray application of a probiotic with conventional formaldehyde fumigation (Graham et al., 2018). Formaldehyde application inhibits proliferation of opportunistic pathogens within the hatching environment (Whistler et al., 1989), but it is a potential carcinogen (Swenberg et al., 2013) and has been shown to have negative effects on the tracheal epithelium of poultry (Sander et al., 1995). As a result, development of both virulent (Graham et al., 2019) and wild-type *E. coli* challenge models are of importance for the investigation of mitigative strategies, other than formaldehyde, to control the microbial bloom. The purpose of the present study was to develop a consistent *E. coli* horizontal challenge model to evaluate formaldehyde fumigation alternatives under laboratory conditions. A commercially relevant laboratory model for simulating *E. coli* horizontal transmission is needed.

MATERIALS AND METHODS

E. Coli *Culture and Challenge*

Two wild-type *E. coli* isolates were evaluated in these experiments: isolate 1 (**I1**) and isolate 2 (**I2**). Both isolates were recovered postmortem from diseased chicks, and identification was confirmed using the API 20E kit (cat. no. 95060-674; VWR, Suwanee, GA). Aliquots of each isolate, consisting of 30% glycerol, were stored at −80°C for long-term preservation. For the challenge culture, 100 μL of *E. coli* was removed from a frozen aliquot and added to 10 mL of tryptic soy broth (Tryptic soy broth, cat. no. 90000-378; VWR, Suwanee, GA). The culture was incubated at 37°C for 18 h. After incubation, bacterial cells were washed 3 times with sterile 0.9% saline by centrifugation at 1,800 × *g* for 15 min and reconstituted in saline. *E. coli* cfu enumeration was determined by serial dilution and plating on MacConkey agar (MacConkey Agar, cat. no. 89429–342; VWR, Suwanee, GA) to determine the stock concentration, and then, cells were held overnight, approximately 16 h, at 4°C. The culture was then serially diluted to desired cfu concentration for in ovo administration. Actual *E. coli* challenge dose (cfu/mL) was confirmed as described previously and reported in Table 1.

Enumeration of Bacteria

For experiment 1, trial 2, experiment 2, and experiment 3, the gastrointestinal tract (from the proventriculus to the ileocecal junction including the ceca) was aseptically removed postmortem and collected into sterile bags. Samples were weighed and homogenized, and 1:4 wt/vol dilutions were made using sterile 0.9% saline. Ten-fold dilutions of each sample, from each group, were made in sterile 96-well Bacti flat-bottom plates, and the diluted samples were plated on culture media to evaluate presumptive gram-negative bacteria on MacConkey agar (MacConkey Agar, cat. no. 89429–342; VWR, Suwanee, GA). Plates were incubated at 37°C for 18 h, and bacterial counts were expressed as Log_{10} cfu/g of sample. In addition, the open agar plate method (Berrang et al., 1995; Kim et al., 2010; Graham et al., 2018) was used for enumeration of circulating presumptive gram-negative bacteria within the hatching environment. Up to 3 agar plates (with the lids removed) were placed open side up on the top tray of the hatchers (GQF 1550 Digital Cabinet Egg Incubator) for 1 min or 5 min. The plates were incubated at 37°C for 18 h to enumerate presumptive gram-negative bacteria present in the hatching cabinets; data were expressed as cfu/plate.

Animal Source

For all experiments, eighteen-day-old Ross 308 embryos were candled, randomly allocated, and placed in separate hatchers based on the treatment group. Mortality was recorded for the duration of each trial (7-day trial period). Chicks were provided ad libitum access to water and a balanced, unmedicated corn and soybean diet meeting the nutritional requirements for broilers

Table 1. Confirmed group in ovo challenge doses by trial.

	Isolate 1		Isolate 2	
Experiment	Group	Confirmed cfu/200uL/embryo	Group	Confirmed cfu/200uL/embryo
Experiment 1 trial 1	d18 10^2	6×10^1	d18 10^2	3×10^1
	d18 10^3	6×10^2	d18 10^3	3×10^2
	d19, d19.5 10^2	7×10^1	d19, d19.5 10^2	3×10^1
	d19, d19.5 10^3	7×10^2	d19, d19.5 10^3	3×10^2
Experiment 1 trial 2	d19 10^2	1.5×10^2	d19 10^2	2.1×10^2
Experiment 2	d19 10^2	1.00×10^2	-	-
Experiment 3	d19 10^2	1.12×10^2	-	-

Table 2. Effect of *in ovo* administration of select wild-type *Escherichia coli* during late embryogenesis on hatchability (%) – experiment 1 trial 1.

Challenge	Hatchability (%)			
	DOE 18 inoculation	DOE 19 inoculation	DOE 19.5 inoculation	Total hatchability by challenge
I1 – 10^2	20/37 (54.05)	36/40 (90)	39/39 (100)	95/116 (81.89)
I1 – 10^3	16/39 (41.03)	37/40 (92.50)	39/40 (97.50)	92/119 (77.31)
I2 – 10^2	8/37 (21.62)	36/40 (90)	33/38 (86.84)	69/115 (60)
I2 – 10^3	4/37 (10.81)	28/40 (70)	34/40 (85)	66/117 (56.41)

n = one hatcher per isolate with each inoculation day receiving its own tray level in its respective hatch cabinet (n = 240 eggs for negative control hatcher; n = 37–40 eggs/challenge, n = 3 tray/cabinet).
Negative control hatchability; 238 of 240 (99.17%).
Abbreviation: DOE, d of embryogenesis; I1, isolate 1; I2, isolate 2.

recommended by Aviagen (Aviagen, 2018). All experiments and animal handling procedures complied with the Institutional Animal Care and Use Committee at the University of Arkansas, protocol #17073.

Experiment 1 Design

Experiment 1 consisted of 2 trials. In experiment 1 trial 1, I1 or I2 (10^2 or 10^3 cfu/200uL/embryo) was administered into the amnion via in ovo inoculation at day 18, 19, or 19.5 of embryogenesis (n = 37–40/treatment). The impact of late embryogenesis challenge was compared with a negative control group (no treatment, n = 240) to determine optimal administration time point and appropriate challenge dose for future studies. Each hatcher contained 3 trays capable of holding 80 eggs per tray. For experiment 1 trial 1, the negative control eggs were housed in 1 hatcher (n = 80/tray) and both doses for each day of challenge were allocated as follows: tray 1, day 18 challenge; tray 22, day 19 challenge; tray 33, day 19.5 challenge. The I1 and I2 challenge groups were placed in separate hatchers. In experiment 1 trial 2, seeder embryos (n = 15 seeders/hatcher or n = 50 seeders/hatcher) were inoculated with I1 or I2 at 10^2 cfu/200uL/embryo via in ovo injection into the amnion and segregated into mesh hatching bags (reusable mesh nylon netting, IDS, Amazon) to evaluate the impact of horizontal transmission of *E. coli* on contact chick hatchability. In experiment 1 trial 2 and experiment 2, the open agar plate method (Berrang et al., 1995; Kim et al., 2010; Graham et al., 2018) was used to evaluate circulating coliforms within the hatching environment at selected time points during the hatch. A MacConkey's agar plate, a selective media for gram-negative bacteria, was placed on the top tray of the hatcher for 1 min (experiment 1 trial 2 only) or 5 min at 80% hatch day 20 of embryogenesis) or immediately before hatch pull. Experiment 1 trial 2 was the initial evaluation of the open agar plate method under these specific challenge conditions, and 1 plate was placed per hatcher (n = 1 hatcher/treatment) per time point. On day 21 of embryogenesis (day of hatch [**DOH**]), dry chicks were removed from the hatching environment. In addition, gram-negative enteric colonization (n = 12/treatment) was evaluated on DOH, day 3, and day 7, and BW was recorded on DOH, day 3, and day 7. Chicks were neck-tagged and allocated into separate pens with fresh pine shavings on DOH. BW of I1 and I2 seeder treatment groups (n = 15 seeders/cabinet) were not evaluated because of low animal numbers (i.e., 15 total chicks for placement). Six replicate pens per treatment were placed (n = 15 chicks/pen), except seeders of the I2 50% group, which had 5 replicate pens (n = 15 chicks/pen).

Experiment 2 and Experiment 3 Design

Based on experiment 1 results, I1 was selected as the challenge strain for experiment 2 and experiment 3. There were 2 hatchers/treatment (n = 210 eggs/hatcher;

Table 3. Effect of horizontal transmission of wild-type *Escherichia coli* on hatchability and gram-negative enteric colonization at DOH, d3, and d7 – experiment 1 trial 2.

Treatment - % seeder embryos	Contact or seeder	Hatchability (%)	DOH $\log_{10}$ CFU/g	D 3 $\log_{10}$ CFU/g	D 7 $\log_{10}$ CFU/g
Negative control	-	209/210 (99.52)	2.80 ± 0.94^{b}	8.28 ± 0.16	6.65 ± 0.17
I1 – 7.14	Contact	195/195 (100)	7.60 ± 0.77^{a}	8.04 ± 0.16	7.02 ± 0.24
	Seeder	15/15 (100)	8.38 ± 0.13^{a}	-	-
I1 – 50	Contact	104/105 (99.05)	8.19 ± 0.39^{a}	7.88 ± 0.12	6.95 ± 0.26
	Seeder	104/105 (99.05)	8.54 ± 0.12^{a}	7.64 ± 0.12	6.94 ± 0.18
I2 – 7.14	Contact	195/195 (100)	6.65 ± 0.65^{a}	8.09 ± 0.14	6.54 ± 0.14
	Seeder	15/15 (100)	8.18 ± 0.41^{a}	-	-
I2 – 50	Contact	105/105 (100)	8.02 ± 0.33^{a}	7.78 ± 0.18	6.65 ± 0.22
	Seeder	103/105 (98.10)	8.29 ± 0.39^{a}	8.07 ± 0.21	6.62 ± 0.17

[a,b]Significant ($P < 0.05$) difference between treatments.
Data expressed as mean ± SE.
n = 210 total eggs placed/hatcher (7.14%: n = 15/hatcher, 50%: n = 105/hatcher).
For gram-negative bacterial recovery, n = 12/treatment.
Abbreviations: DOE, d of embryogenesis; DOH, d of hatch; I1, isolate 1; I2, isolate 2.

Table 4. Effect of horizontal transmission of wild-type *Escherichia coli* on average BW, BWG, and 7-d mortality of contact and seeder chicks – experiment 1 trial 2.

Treatment – % seeder embryos	Contact or seeder	BW (g) DOH	BW (g) day 3	BW (g) day 7	BWG (g) day 0–day 7	Mortality (%)
Negative control	-	42.78 ± 0.35	81.31 ± 0.77[a]	171.84 ± 2.72[a]	129.13 ± 2.70[a]	0/90 (0)
I1 – 7.14	Contact	42.76 ± 0.28	77.86 ± 0.87[a,b]	158.87 ± 2.83[b]	116.60 ± 2.76[b]	0/90 (0)
I1 – 50	Contact	42.64 ± 0.31	80.97 ± 0.74[a]	164.59 ± 2.59[a,b]	121.71 ± 2.59[a,b]	0/90 (0)
	Seeder	41.92 ± 0.33	78.02 ± 0.85[a,b]	160.40 ± 3.12[a,b]	118.52 ± 3.07[a,b]	3/90 (3.33)
I2 – 7.14	Contact	42.49 ± 0.38	79.24 ± 0.82[a,b]	167.73 ± 3.07[a,b]	125.46 ± 3.01[a,b]	0/90 (0)
I2 – 50	Contact	42.02 ± 0.38	78.22 ± 0.85[a,b]	159.66 ± 2.80[a,b]	117.97 ± 2.74[a,b]	0/90 (0)
	Seeder	42.27 ± 0.36	76.94 ± 1.31[b]	155.16 ± 3.91[b]	113.16 ± 3.75[b]	8/75 (10.75)

[a,b]Indicates significant differences between treatments groups within columns ($P < 0.05$).
Data expressed as mean ± SE.
n = 6 pens/treatment, n = 15 chicks/pen all groups except for I2 – 50 seeder group, n = 5 pens/treatment, n = 15 chicks/pen.
Low n for I1 and I2 – 7.14 seeder chicks–chicks were not placed.
Abbreviations: BWG, BW gain; DOH, d of hatch; I1, isolate 1; I2, isolate 2.

n = 15 seeders/hatcher or 7.14%), and 3 replicate MacConkey plates were placed in the respective hatcher for 5 min for gram-negative bacterial recovery. The hatchers were sampled at 4 time points during the hatching phase: 20% hatch, 50% hatch, 80% hatch, and before hatch pull on the DOH. For the formaldehyde-treated hatch cabinets, fumigation was performed via a drip application of 6 mL of formalin every 3 h after transfer from the incubator to the hatching cabinet and ceased 12 h before hatch pull. On the DOH, dry chicks were removed from the hatching environment. Gastrointestinal tract samples were collected postmortem for presumptive gram-negative bacterial recovery, as described previously on the DOH and day 7 (n = 12/treatment). For both trials, weight allocation on the DOH was performed to normalize BW and prevent the initial treatment effect on BW. Pen BW was determined at placement and on day 7 to determine BWG with 12 replicate pens per treatment (n = 20 chicks/pen). Hatchability and 7-day mortality were not impacted as a result of the challenge (data not shown).

Statistical Analysis

All data were subjected to 1-way ANOVA using JMP Pro 13 (SAS, 2016). Data are expressed as mean ± SE. Significant differences ($P < 0.05$) among the means were further separated using Tukey's multiple range test for gram-negative bacterial recovery with individual bird (experiment 1 trial 2) or pen (experiment 2 or experiment 3) as the experimental unit for BW data. Mortality was compared using the chi-square test of independence to determine the significance ($P < 0.05$) for these studies (Zar, 1984).

RESULTS

Challenge dose(s) for each experiment are reported in Table 1. In ovo administration of I1 or I2 at 10^2 or 10^3 cfu/embryo on day 18 of embryogenesis negatively impacted hatchability, with I2 10^3 cfu/embryo being the most lethal and I1 10^2 cfu/embryo having less of an impact compared with the I2 challenge (Table 2). Experiment 1 trial 1 data suggest that in ovo challenge with I1 or I2 at 10^2 cfu/embryo on day 19 of embryogenesis did not negatively affect development since hatchability was 90%, although the I1 10^2 cfu/embryo, when administered at day 19.5 of embryogenesis, had no effect on hatchability.

Horizontal transmission of I1 or I2 between the seeder chicks (direct-challenged) at a level of 7.14% or 50% of the population did not affect the hatchability of the contact (indirect-challenged) chicks (Table 3). However, gram-negative bacteria recovered from gastrointestinal tract samples on the DOH, of both seeder and contact chicks, were higher ($P < 0.05$) than that from the negative control group as a result of in ovo challenge (Table 3). There was no statistical difference in gram-negative bacterial recovery between all groups at day 3 or day 7 (Table 3). BW on the DOH was not impacted

Table 5. Gram-negative bacterial recovery from the hatching environment at 80% hatch and DOH – experiment 1 trial 2.

Treatment – % seeder embryos	Sampling duration	80% hatch cfu/plate	DOH cfu/plate	Total (80% hatch + DOH cfu/plate)
Negative control	1m	19	0	19
	5m	46	8	54
I1 – 7.14	1m	35	24	59
	5m	126	76	202
I1 – 50	1m	140	71	211
	5m	632	224	856
I2 – 7.14	1m	4	8	12
	5m	30	27	57
I2 – 50	1m	50	16	66
	5m	760	92	852

n = 1 hatcher/treatment.
n = 1 MacConkey agar plate/sample time point.
Abbreviations: DOH, d of hatch; I1, isolate 1; I2, isolate 2.

Table 6. Effect of horizontal transmission of *Escherichia coli* and formaldehyde fumigation on gram-negative enteric colonization at DOH, d 3, and d 7 – experiment 2 and experiment 3.

		DOH log_{10} cfu/g		D 3 log_{10} cfu/g		D 7 log_{10} cfu/g	
Treatment	Contact or seeder	Experiment 2	Experiment 3	Experiment 2	Experiment 3	Experiment 2	Experiment 3
Negative control	-	1.13 ± 0.71^{b}	0.56 ± 0.56^{b}	7.18 ± 0.19^{b}	7.98 ± 0.12	6.40 ± 0.18	6.75 ± 0.22
I1	Contact	6.32 ± 0.94^{a}	5.96 ± 0.38^{a}	8.18 ± 0.19^{a}	8.34 ± 0.11	6.48 ± 0.31	7.07 ± 0.23
	Seeder	7.56 ± 0.39^{a}	7.89 ± 0.09^{a}	-	-	-	-
I1 + formaldehyde	Contact	1.70 ± 0.92^{b}	1.65 ± 0.93^{b}	$7.67 \pm 0.21^{a,b}$	8.09 ± 0.20	6.72 ± 0.21	6.58 ± 0.25
	Seeder	7.22 ± 0.62^{a}	7.15 ± 0.41^{a}	-	-	-	-

[a,b]Indicates significant differences between treatments groups within columns ($P < 0.05$).
Data expressed as mean ± SE.
Abbreviations: DOH, d of hatch; I1, isolate 1.

by treatment, although seeder chicks of the I2–50 treatment group – were markedly ($P < 0.05$) lighter at day 3 and day 7 (Table 4). The day 7 BW and 7-day BWG were significantly ($P < 0.05$) reduced for the contact chicks of the I1–7.14% treatment group compared with the negative control but was not different from the other treatment groups (Table 4). In addition, there was 10.75% mortality over the 7-day trial period in the I2–50 seeder group – yet not statistically different than the negative control (Table 4).

As expected, exposing a MacConkey's agar plate to the hatching environment for 5 min resulted in higher recovery of gram-negative bacteria compared with sampling the air within the hatch cabinet for 1 min (Table 5). Moreover, inoculating 50% of the embryos at day 19 of embryogenesis as compared with 7.14% of the embryo increased the total number of colonies recovered (80% hatch cfu/plate + DOH cfu/plate) on MacConkey's agar compared with the negative control (Table 5). In experiment 2 and experiment 3, there was a significant difference ($P < 0.05$) in gram-negative bacterial recovery between the negative control and contact chicks of the I1 + formaldehyde treatment group compared with the positive control contact and seeder chicks and the seeders of the I1 + formaldehyde treatment group on DOH (Table 6). There was a significant ($P < 0.05$) difference in day 3 gram-negative bacterial recovery between the negative control and contact chicks of the positive control group only in experiment 2 (Table 6). No differences were observed for gram-negative bacterial recovery between treatment groups by day 7 (Table 6). Seeder challenge increased coliform recovery from the hatching environment, and formaldehyde fumigation effectively controlled the artificial microbial bloom (Table 7). No differences were observed in DOH or day 7 BW or 7-day BWG between all treatments in experiment 2 and experiment 3 (Table 8). In experiment 2 and experiment 3, 7-day mortality was not impacted as a result of challenge (data not shown).

DISCUSSION

Colibacillosis is one of the leading causes of morbidity and mortality in poultry and is of significant economic importance to the industry (Kabir, 2010). Commercial hatch cabinet temperatures and humidity levels promote the proliferation of opportunistic pathogens, such as *E. coli* during hatch (Thermote, 2006). Hatchery sanitation methods, including formaldehyde fumigation, are used to prevent the spread of pathogens. Formaldehyde fumigation is commonly implemented as a precautionary measure in commercial hatcheries owing to its biocidal efficacy regardless of the research that has been conducted for decades to evaluate potential carcinogenicity (Swenberg et al., 2013). Fumigation can affect the tracheal epithelial integrity of chicks exposed to formaldehyde during the late hatching phase, such as a reduction in cilia and extensive mucus accumulation (Fauziah et al., 1996; Zulkifli et al., 1999). In addition to chemical treatments, a probiotic application to control the microbial loads during hatch has been investigated as a formaldehyde fumigation alternative (Graham et al., 2018). Probiotic application vs. chemical application would expose the chicks to presumptive beneficial pioneer colonizers and reduce formaldehyde exposure for the hatching chicks and hatchery employees. Thus, there is a need for an in ovo seeder challenge model to investigate formaldehyde fumigation methods imitating commercial horizontal transmission and the microbial bloom within the hatcher.

Table 7. Gram-negative bacterial recovery from the hatching environment (DOE 20 and DOH) – experiment 2 and experiment 3.

	20% hatch cfu/plate		50% hatch cfu/plate		80% hatch cfu/plate		DOH cfu/plate	
Treatment	Experiment 2	Experiment 3	Experiment 2	Experiment 3	Experiment 2	Experiment 3	Experiment 2	Experiment 3
Negative Control	2.5	0	1.5	0	0.5	2	0.5	0
I1	1.0	7.0	22.5	20	18	112	120	7
I1 + formaldehyde	0	0	0	3	2	1	0	0

n = 2 hatchers/treatment.
n = 3 MacConkey plates/sample time point.
Abbreviations: DOE, d of embryogenesis; DOH, d of hatch; I1, isolate 1.

Table 8. Average BW and BWG – experiment 2, trial 1 and trial 2.

Treatment	BW (g) DOH		BW (g) day 7		BWG (g) day 0–day 7	
	Experiment 2	Experiment 3	Experiment 2	Experiment 3	Experiment 2	Experiment 3
Negative control	43.01 ± 0.03	42.59 ± 0.03	142.52 ± 1.77	138.97 ± 1.71	99.51 ± 1.76	96.39 ± 1.73
I1	42.93 ± 0.03	42.74 ± 0.02	144.86 ± 1.56	140.68 ± 1.98	101.94 ± 1.56	97.96 ± 1.98
I1 + formaldehyde	42.91 ± 0.04	42.69 ± 0.03	141.04 ± 1.80	140.08 ± 2.05	98.12 ± 1.81	97.37 ± 2.05

Data expressed as mean ± SE.
n = 12 pens/treatment, n = 20 chicks/pen.
Abbreviations: BWG, BW gain DOH, d of hatch; I1, isolate 1.

In the present study, *in ovo* inoculation with ~10^2 or 10^3 cfu/embryo at day 18 of embryogenesis with wild-type *E. coli* negatively impacted hatchability, but hatchability improved when challenge administration was delayed to day 19 or 19.5 of embryogenesis. This suggests that the in vivo replication of the bacteria, when 1,000 cfu/embryo or less was administered on day 19 of embryogenesis, reduced lethality to the chick. As such, previous research indicates that the doubling time of *E. coli* in vitro is between 22 and 40 min (Helmstetter, 1968). Thus, a later in ovo challenge during embryogenesis reduced the time for the *E. coli* to replicate within the developing embryo.

Horizontal challenge models, which consist of comingling seeder (challenged) and contact (nonchallenged) chicks, have been developed to mimic natural challenge conditions (Weinack et al., 1981; Montgomery et al., 1999; Jarquin et al., 2007; Graham et al., 2019). Previously, a low-dose (<100 cfu/embryo) *in ovo* inoculation with a nalidixic acid–resistant *E. coli* at day 12 of embryogenesis negatively affected the hatchability of directly challenged chicks, although there was no significant effect on contact chick hatchability (Montgomery et al., 1999). Experiment 1 trial 1 results suggested that day 19 administration of ~100 cfu of *E. coli* was not damaging to the developing embryo, and contact chick hatchability was also not impacted as a result of seeder challenge. In addition, gram-negative bacterial recovery was increased in the contact chicks compared with the negative control on DOH, but no differences were observed at day 3 or day 7 after hatch. The lack of difference in gram-negative bacterial recovery between the negative control and the treatment groups on day 3 and day 7 can be attributed to the presence of commensal *E. coli* within the gastrointestinal tract. Because wild-type *E. coli* strains were used for the challenge, differentiation between lactose-fermenting colonies (commensal and challenge strain) on MacConkey agar was not possible using the employed culture methods.

Although transmission via the fecal–oral route has been considered the primary route of infection for *Salmonella*, respiratory transmission has also been noted as a portal of entry (Kallapura et al., 2014) and fluff circulating in the cabinet during hatch can harbor pathogenic organisms with respiratory tropisms. Presently, formaldehyde fumigation effectively reduced the number of gram-negative bacteria in the hatching environment and in the gastrointestinal tract of contact chicks on the DOH compared with the nontreated challenged control group. However, horizontal transmission of *E. coli* or formaldehyde treatment did not alter 7-day performance compared with controls. These results are similar to those reported by Zulkifli et al. (1999), where no significant effects on overall (41 d) performance as a result of formaldehyde fumigation were observed. While not directly compared or evaluated in these studies, the common coliform blooms and effects on horizontal transmission during commercial hatch primarily cause relatively subtle effects on overt 7-day mortality and performance, similar to the findings of these experiments. The primary purpose of the model was to be able to compare alternative solutions to formaldehyde during hatch. *E. coli* are predominantly involved in secondary infections and perhaps why there was not a consistent impact on early performance. However, this model could be used to evaluate formaldehyde fumigation alternatives to control wild-type *E. coli* bloom within the hatching environment. Further research is being conducted to determine the effects of a multipathogen horizontal transmission model on the microbial load within the hatching cabinet, hatchability, and posthatch morbidity/mortality.

DISCLOSURES

The authors declare no conflicts of interest.

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