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Variable Effects of the In Ovo Administration of an *Escherichia coli* Vaccine in the Amnion or Air Cell on Commercial Layer Embryo and Hatchling Development †

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Abstract: The effects of injecting the Poulvac *E. coli* vaccine (PECV) into either the air cell (AC) or amnion (AM) at different dosages at 18 days of incubation (DOI) on Hy-Line W-36-layer embryo and hatchling development were investigated. Serial dilutions of the PECV in diluent provided either 6.5×10^4, 6.5×10^3, 6.5×10^2, or 6.5×10^1 CFU dosages of *E. coli*. A diluent only injection treatment was included as a control. A total of 19 live embryonated eggs in each of 10 treatment groups were represented on each of 16 replicate levels (3040 total) in the hatcher unit. At 19 DOI, swabs of the AM indicated that the 6.5×10^1 and 6.5×10^2 CFU dosages provided a 50% level of PECV presence, whereas the 6.5×10^3 and 6.5×10^4 CFU dosages provided a 100% level of PECV presence. Conversely, only the 6.5×10^3 and 6.5×10^4 CFU dosages provided a 50% level of PECV presence in the AC. At all *E. coli* dosage levels, injection in the AM led to higher percentages of live or dead embryos that failed to pip (PEIS) ($p = 0.001$) or complete hatch (PEPE) ($p \leq 0.001$) and a lower percentage of live fully hatched chicks (HI) ($p \leq 0.001$), when compared to those injected in the AC. Like HI, significantly lower percentages of female hatchlings were also observed at 22 DOI for the AM compared to the AC injection, for all dosages except for the 6.5×10^2 CFU dosage. However, at all the dosages above the 6.5×10^1 CFU dosage, the AM injection resulted in a lower mean hatchling body weight ($p = 0.010$) at 22 DOI. In conclusion, *E. coli* populations were more prevalent in the AM than in the AC after the injection of the PECV in those sites. Furthermore, the injection of the PECV in the AM at all *E. coli* dosages generally increased late embryo mortality and decreased hatchability and hatchling body weight in comparison to an AC injection. It is concluded that the negative impact of the in ovo administration of the PECV in the AM at 18 DOI on the hatch process is dose dependent. However, effects of an increase in AC dosages and a decrease in AM dosages should be further investigated.

Keywords: *E. coli* vaccine; hatchability; in ovo injection; layers; site of injection

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1. Introduction

Avian pathogenic *E. coli* (**APEC**) is a strain of *E. coli* that can cause significant economic loss within the poultry industry [1]. Contact with APEC can begin in the hatchery [2] or even as early as in the hen's oviduct, before shell formation [3]. Chickens can acquire an APEC infection through contact with feces, especially if they are housed in poor conditions [1]. Contamination can also occur through the inhalation of dust in the chicken house [2]. An

APEC infection can be manifested in multiple ways, including swollen-head syndrome, cellulitis, salpingitis, peritonitis, yolk sac infection, and acute fatal septicemia [1]. Bacterial infections, such as those of APEC, are one of the leading causes of mortality in the first wk of a chicken's life [4]. Birds that have an APEC infection will normally show clinical signs, such as problems with gait and balance, and the inability to eat or drink [1].

Due to the economic losses associated with APEC, the development of a vaccine for the protection of commercial poultry became imperative. Consequently, live attenuated and non-attenuated, and inactivated vaccines have been tested [1]. The vaccine commonly used today is the Poulvac *E. coli* vaccine (**PECV**; Zoetis, Parsippany, NJ, USA) [5,6]. The vaccine offers protection for both broilers and layers. In layers, it is usually administered at one day of age and again at 14 to 16 weeks of age [7–9]. The current application method is to apply the vaccine via drinking water or by coarse spray [7]. However, the in ovo administration of the PECV has not been previously tested.

In ovo injection is the process of injecting a substance into an egg to confer a benefit to the embryo before the incubation period is completed [10]. There are multiple advantages to this method, including lowering the cost of labor, reducing bird stress, and having better assurance that each chick is receiving an equal and correct dosage of the injected material. This is of particular importance for the injection of vaccines [11]. Different sites within the egg have been tested for efficacy. Wakenell et al. [12] administered the Marek's disease vaccine by in ovo injection into various sites including the amnion (**AM**) and air cell (**AC**) of specific-pathogen-free eggs at 18 days of incubation (**DOI**). The AM has been described as a primary injection site for optimal vaccine efficacy [13,14]. It has been confirmed that injection into the AM or embryo proper yields the most posthatch protection against Marek's disease [14,15]. Embryos imbibe AM fluid before hatch [16], whereas, when injected in the AC, the integument and mucosa of the hatching chick may make contact with the vaccine during the pipping process. It has been shown that the in ovo injection of the 6/85 strain of *Mycoplasma galisepticum* (**6/85** MG) in the AC of layer hatching eggs resulted in a lower embryonic mortality but insufficient humoral immune response when compared to an injection in the AM [17]. The in ovo administration of the PECV into the AM or AC on hatchling development has not been previously investigated. It is hypothesized that in ovo administration of some of the dosages of the PECV could negatively affect the hatch process as well as hatchling quality. Because the efficacy of an in ovo-injected vaccine can be influenced by its injection into the AM or AC at various incremental dosages, the primary objective of this study was to identify the effects of site of injection (AM or AC) within various dosage levels of the PECV on commercial layer embryo and hatchling development when the vaccine is administered in ovo at 18 DOI. An additional objective was to test for the possible occurrences of *E. coli* populations in the AM and AC at the dosages administered.

2. Materials and Methods

2.1. Incubational Conditions and Monitoring

All handling and care of broiler hatchlings was conducted under the approval of the Mississippi State University Institutional Animal Care and Use Committee (Protocol # IACUC-20-248). An Avida ChickMaster incubator (ChickMaster Incubator Company, Medina, OH, USA) was used as both a setter and hatcher unit in this study, and the incubational regimens in both phases followed the procedure described by Fatemi et al. [18]. The air temperature and relative humidity within the incubator were also recorded in accordance with the method described by Fatemi et al. [19].

2.2. Vaccine Preparation

2.2.1. Preliminary Plating

Prior to d of injection, the titer of the PECV (Serial # 438524) was determined via plating on LB plates. Upon testing 2 different vials of vaccine, it was determined that a

single dosage of the undiluted vaccine contained between 4.30×10^7 and 5.43×10^7 *E. coli* colony forming units (**CFU**).

2.2.2. Pre-Injection Plating at 18 DOI

At 18 DOI, the PECV was diluted using ten-fold serial dilutions to create 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, and 1×10^{-8} dilutions. Plating of 100 µL volumes of the 1×10^{-4}, 1×10^{-5}, and 1×10^{-6} dilutions resulted in colonies that were too numerous to count. In a 100 µL volume, an average of 130 and 20 CFU were counted in the 1×10^{-7} and 1×10^{-8} dilutions, respectively. Based on calculations derived from the 1×10^{-7} dilution, the amount of PECV *E. coli* delivered in a 50 µL volume to each egg from the 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} dilutions corresponded to 6.5×10^4, 6.5×10^3, 6.5×10^2, and 6.5×10^1 CFU, respectively (Table 1). Because the PECV *E. coli* dosage in the 1×10^{-8} dilution was less than 1 CFU, it was excluded as a treatment in the study.

Table 1. The Poulvac *E. coli* vaccine (PECV) dilutions that corresponded with the 6.5×10^1, 6.5×10^2, 6.5×10^3, and 6.5×10^4 *E. coli* dosages (CFU) in each 50 µL solution volume administered in the amnion and air cell.

***E. coli* Dosage (CFU)**	**PECV Dilution**
Marek's disease commercial diluent	---------
6.5×10^1	1×10^{-7}
6.5×10^2	1×10^{-6}
6.5×10^3	1×10^{-5}
6.5×10^4	1×10^{-4}

2.2.3. Post-Injection Plating at 18 DOI

The vaccine dilutions were also plated post-vaccination. In a 100 µL volume, the CFU of the 1×10^{-4} and 1×10^{-5} dilutions were likewise too numerous to count. However, for the 1×10^{-6} dilution, 179 CFU were observed, which exceeded the extremely low/undetectable CFU levels of the 1×10^{-7} and 1×10^{-8} dilutions. The 130 CFU level in the pre-injection plating of the 1×10^{-7} dilution and the 179 CFU level in the post-injection plating of the 1×10^{-6} dilution would indicate that a slight amount of bacterial death occurred in the diluent.

2.3. Setter Phase Egg Incubation and Subsequent In Ovo Injection at 18 Days of Incubation

Hy-Line W-36 [20] fertile layer hatching eggs were obtained commercially from a 36-week-old parent flock, and stored under commercial conditions as described by Fatemi et al. [19]. The eggs were incubated at 37.5 °C (dry bulb) and 28.9 °C (wet bulb) temperatures form 0 to 18 DOI, and at 36.9 °C (dry bulb) and 29.9 °C (wet bulb) temperatures from 18 to 22 DOI. Hens had been previously vaccinated for *E. coli* in accordance with the vaccine recommendations of Hy-Line. Eggs were not set if they were dented, cracked, or were excessively large or small. The eggs were stored for 3 days under commercial conditions prior to set. A total of 125 eggs were randomly set in each of 2 trays (one in each of the 2 incubator columns) belonging to each of the 16 levels of the incubator (4000 total eggs among 32 total trays). At 11 and 18 DOI, eggs were candled to verify live embryonation, with eggs that were non-embryonated or that contained dead embryos being discarded.

Prior to injection at 18 DOI, eggs were randomly selected from each of the 32 trays in the incubator to constitute each dosage-site injection treatment group. At 18 DOI, a 50 µL volume of Marek's disease commercial diluent (Merial, Inc. Athens, GA, USA) was injected into the AM or AC of live embryonated eggs alone or in combination with PECV containing 6.5×10^1, 6.5×10^2, 6.5×10^3, or 6.5×10^4 CFU of *E. coli*. Dosage descriptions and the corresponding dilutions of the PECV are listed in Table 1. Eggs belonging to different dosage groups were kept separate and remained in the hatcher unit until they were injected.

Eggs were injected according to dosage using an Embrex Inovoject M machine (Zoetis, Durham, NC, USA). Due to needle depth adjustment requirements, AM injections were administered prior to AC injections. For each site of injection, injections of diluent alone were first administered, followed by the lowest to highest *E. coli* dosages. To prevent treatment cross-contamination, sterile water was flushed through the machine between treatment applications. A machine cleaning cycle was further included between AM and AC injections.

After egg injection at 18 DOI, 19 randomly selected eggs from each of the 10 treatment combination groups (2 sites × 5 dosages) were transferred to divided hatching basket sections (5 sections in each of 2 baskets) assigned to each treatment group. Each treatment group was represented on each of the 16 replicate levels (units or blocks) of the incubator so that a total of 304 eggs belonged to each treatment combination group. The assignment of the various treatment groups to the hatching basket sections are displayed in Table 2. To avoid bacterial cross-contamination from feces produced by hatchlings from the different treatments, hatching basket sections containing eggs belonging to a common treatment combination occupied the same column in the incubator. As specified by Elliott et al. [21], injection sites were confirmed and embryo developmental stage determinations were performed on 21 AC-injected and 21 AM-injected eggs using Coomassie brilliant blue R-250 dye (MP Biomedicals, LLC., Solon, OH, USA) at 18 DOI. Injection site evaluations were conducted according to methods described by Williams and Hopkins [15]. Embryo developmental staging scores were recorded as described by Sokale et al. [22].

Table 2. Treatment arrangement in the incubator at 18 d of incubation. Each hatch basket had 5 divided segments, with a single treatment replicate placed in a single divided segment. Site and dosage treatments (1 to 10) were spread throughout the hatch baskets, with a replicate of each treatment represented on every level. S = swab and represents the area where the swabbed eggs were kept for an additional day, until 19 d of incubation. E = an empty basket section. IL = incubator level. LS = left side of the incubator. RS = right side of the incubator. AM = amnion. AC = air cell. 1 = Marek's disease commercial diluent in AM, 2 = 6.5×10^1 CFU of *E. coli* in AM, 3 = 6.5×10^2 CFU of *E. coli* in AM, 4 = 6.5×10^3 CFU of *E. coli* in AM, 5 = 6.5×10^4 CFU of *E. coli* in AM, 6 = Marek's disease commercial diluent in AC, 7 = 6.5×10^1 CFU of *E. coli* in AC, 8 = 6.5×10^2 CFU of *E. coli* in AC, 9 = 6.5×10^3 CFU of *E. coli* in AC, and 10 = 6.5×10^4 CFU of *E. coli* in AC.

IL	LS	RS										
1	E	1	2	3	4	5	6	7	8	9	10	S
2	E	1	2	3	4	5	6	7	8	9	10	E
3	E	1	2	3	4	5	6	7	8	9	10	E
4	E	1	2	3	4	5	6	7	8	9	10	E
5	E	1	2	3	4	5	6	7	8	9	10	E
6	E	1	2	3	4	5	6	7	8	9	10	E
7	E	1	2	3	4	5	6	7	8	9	10	E
8	E	1	2	3	4	5	6	7	8	9	10	E
9	E	1	2	3	4	5	6	7	8	9	10	E
10	E	1	2	3	4	5	6	7	8	9	10	E
11	E	1	2	3	4	5	6	7	8	9	10	E
12	E	1	2	3	4	5	6	7	8	9	10	E
13	E	1	2	3	4	5	6	7	8	9	10	E
14	E	1	2	3	4	5	6	7	8	9	10	E
15	E	1	2	3	4	5	6	7	8	9	10	E
16	E	1	2	3	4	5	6	7	8	9	10	E

2.4. Air Cell and Amnion Membrane Swabs and Yolk Sac and Yolk-Free Embryo Weights at 19 Days of Incubation

At 19 DOI (24 h post-vaccination), 2 extra eggs from each treatment (20 total), which previously had been randomly designated for swabbing, were removed from the incubator and the AC or AM membrane of each egg was aseptically swabbed to test for the specific presence of Poulvac *E. coli* via real time PCR. Sterile cotton-tipped swabs (Puritan® Medical Products Co LLC., Guilford, ME, USA) were used for all internal egg and subsequent hatchling swabs. The eggs were sequentially swabbed in order of increasing injection dosage. For eggs that had been injected in the AC, a pre-wetted swab was gently wiped across the surface of the AC membrane. For eggs that had been injected in the AM, the AC membrane was gently punctured, and without making contact with the AC membrane, a pre-wetted swab was inserted through the opening that was created. Upon penetration of the amniotic sac, the inner surface of the AM membrane overlaying the embryo was gently wiped. Each swab was swirled in PBS in separate microcentrifuge tubes that were then sealed. After swabbing, total embryo body weight (**BW**), including the yolk sac, was determined and yolk sacs were separated from the embryo at their point of attachment to the intestine to determine yolk sac weight. Yolk sacs were weighed and embryos were weighed without their yolk sacs to determine yolk sac weight as a percentage of embryo BW and yolk-free embryo BW.

2.5. Hatchery Residue Analysis and Hatchling Evaluation at 22 Days of Incubation

All handling and care of layer hatchlings was conducted under the approval of the Mississippi State University Institutional Animal Care and Use Committee (Protocol #: IACUC-20-351). All hatchlings were removed from the incubator at 22 DOI by the sequential removal of the PECV-injected treatments from the least to greatest dosage. There was a 6 h period between the removal of the diluent-injected groups and the removal of the highest dosage groups from the hatcher unit. However, because the site treatment groups (AM and AC) within PECV dosage were pulled from the incubator at a similar time, it was possible to statistically test for the effects of site treatment within dosage for each variable examined beginning on day of hatch.

Hatch residue analysis, feather sexing of individual birds, and determination of mean hatchling BW in each dosage-site group were conducted when hatch was pulled. A total of 532 residue eggs across all dosages and sites were evaluated. Embryo residue analysis in each dosage-site group at 22 DOI included determination of the percentages of middle incubation (8 to 13 DOI) embryonic deaths. Additionally, as percentages of injected eggs that contained live embryos as of 18 DOI, values were determined for embryos that failed to pip externally and remained in the shell (live and dead; **PEIS**), embryos that pipped externally but did not complete hatch (live and dead; **PEPE**), and live chicks that fully hatched (**HI**). An analysis of the chicks at 22 DOI also included percentage determinations of dead chicks (free from the shell) and culled chicks (chicks with dry, rough navels, or externalized intestines). In addition, the number of live fully hatched females was counted to calculate the percentage of female chicks hatched (**PFCH**). Females from each replicate group within each dosage-site treatment were pooled, and 3 from each pool were randomly selected for measurement of their body length. In an elongated position, body length was measured from the tip of the beak to the end of the extended third toe of the left leg [23,24]. The chicks were then humanely euthanized and placed in sealed bags and refrigerated for later swabbing and sampling. All remaining female chicks, as well as all male chicks, were also humanely euthanized.

2.6. Somatic and Yolk Content Determinations of Hatchlings at 22 Days of Incubation

The female chicks that were stored in the sealed bags were weighed for determination of total BW, including the yolk sac. The yolk sacs of the chicks were then removed and weighed, and the dry matter and moisture contents of the yolks were determined according to the procedures of Peebles et al. [25]. Yolk sac weight was expressed as a percentage of

total BW, and dry yolk matter and moisture were expressed as percentages of total yolk sac weight. Using the total BW and yolk sac weight data of the chicks, their yolk-free BW was calculated. Furthermore, BW-to-length ratio and body mass were also calculated for each sampled female chick. The BW-to-length ratio of the chicks was calculated by dividing the BW of the chick by its length, and percent chick body mass was calculated by dividing the yolk-free BW of the chick by its whole BW and multiplying the result by 100.

2.7. PCR Procedures

DNA extractions were performed using a BioSprint 96 One-For-All-Vet Kit (Qiagen, Valencia, CA, USA). Primers and probes were designed for differentiation of PECV (Table 3). The forward and reverse primers were designed according to the procedure described by Fatemi [26]. Real Time PCR assays were performed using a QuantStudio^{TM} 6 Flex Fast 96-Well Real Time PCR System (Applied Biosystems, Foster City, CA, USA). All reactions (25 µL) were performed in duplicate in 96-well format and contained 2.5 µL of the extracted DNA and primers and probe at 0.5 µM and 0.1 µM, respectively. Reaction cycle included 50 °C for 2 min, 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s, 58.5 °C for 30 s, and 72 °C for 30 s. Each plate contained non-template controls and 6 10-fold dilutions (1:10–1:1,000,000) of a PECV standard in duplicate. The specificity of the Real-time PCR results were determined on a qualitative basis (positive or negative for PECV) according to the method described by Elliott et al. [27] and Poudel et al. [28].

Table 3. Base sequence of primers and probe used in PCR analysis to specifically identify the DNA of the *E. coli* in the Poulvac *E. coli* vaccine apart from sample associated *E. coli*.

Item Identification	5′→3′ Base Sequence
Primer 1	TTAATGACTGCGCCTCTTGC
Primer 2	ACGTCCAATACCGGTCACTT
Probe	[6FAM]TTAAGAATTCCAGTCTCCGGGT[BHQ1]

2.8. Statistical Analysis

Due to the extended time (6 h) between the sequential pull of the in ovo PECV dosage treatments from the incubator, there was a potential confounding influence of time on the effects of in ovo dosage treatment on the hatch variables examined. Therefore, only the effects of site of injection within the various dosages administered were analyzed. LSMeans tests of effect slices were utilized to examine differences between the 2 sites at each dosage level. In the analyses of hatch residue, mean hatchling BW, HI, and PFCH at 22 DOI, hatch basket section served as the experimental unit in a complete block experimental design, with tray level as the blocking factor. Using the chicks taken from a pool of the replicate groups within each dosage-site treatment combination, the following variables were analyzed with individual sample representing the experimental unit: embryo and chick BW, absolute and percent yolk sac weight, and yolk-free BW; and chick body length, BW-to-length ratio, percent body mass, and percent yolk moisture and dry matter contents. Least squares means were compared using Fisher's Protected LSD test in the event of significant global effects. Global effects and LS means differences were considered significant at $p \leq 0.05$. All data were analyzed by ANOVA using the MIXED procedure of SAS software 9.4 [29–31]. Swab data were not subjected to statistical analysis, and LS means comparisons for variables that were not significantly affected by treatment are not shown.

3. Results

3.1. Percent Egg Weight Loss, Site of Injection and Embryo Staging Confirmations, and Embryonic Somatic Variables

For the eggs injected with Coomassie blue dye, all 21 AC injections were successfully administered without penetration of the inner AC membrane, and 19 of the 21 AM injections

were successfully administered in the AM after penetration through the inner AC membrane. The mean embryo stage score at 18 DOI was 1.1, which was prior to a pipping response and the positioning of the head of the embryo under its left wing. Based on individual embryo samples at 19 DOI, BW, absolute yolk weight, yolk-free BW, and yolk sac weight as a percentage of BW were not significantly affected by site of injection treatment.

3.2. PCR at 19 Days of Incubation

The PCR data for the eggs swabbed at 19 DOI are provided in Table 4. All diluent-injected eggs, regardless of site of injection, were negative for the presence of Poulvac *E. coli*. Eggs injected with either 6.5×10^1 or 6.5×10^2 CFU of *E. coli* into the AM were 50% positive, with all AC eggs at those dosages having negative results. The eggs injected at 6.5×10^3 or 6.5×10^4 CFU showed 100% positive results in the AM and 50% positive results in the AC.

Table 4. Percentage of positive PCR tests for the presence of Poulvac *E. coli* on swabs of the amnion (AM) and air cell (AC) at 19 d of incubation after in ovo administration of 6.5×10^1, 6.5×10^2, 6.5×10^3, and 6.5×10^4 CFU dosages of *E. coli* in the Poulvac *E. coli* vaccine.

***E. coli* Dosage (CFU)**	**AM**	**AC**
	----------Positives (%)----------	
Marek's disease commercial diluent	0	0
6.5×10^1	50	0
6.5×10^2	50	0
6.5×10^3	100	50
6.5×10^4	100	50

3.3. Residue Egg Breakout and Hatchability

As expected, because eggs were not injected until 18 DOI, there were no significant ($p \geq 0.135$) differences between the injection sites at any dosage for mid-incubation dead embryos. However, for both PEIS and PEPE, there were significant differences between the sites of injection at all dosages except for the diluent injection treatment (Table 5). The values of PEIS and PEPE were significantly higher in eggs injected in the AM compared to eggs injected in the AC at the dosages of 6.5×10^1, 6.5×10^2, 6.5×10^3, and 6.5×10^4 CFU (Table 5). Except for the diluent injection treatment, there were also significant differences between the sites of injection at all dosages for HI (Table 5). Mean HI was significantly higher in eggs injected in the AC compared to eggs injected in the AM within the 6.5×10^1, 6.5×10^2, 6.5×10^3, and 6.5×10^4 CFU dosages (Table 5). Furthermore, except for the diluent injection and the 6.5×10^2 CFU injection treatments, there were significant differences between the sites of injection at all dosages for PFCH, and mean PFCH was significantly higher in eggs injected in the AC compared to eggs injected in the AM within the 6.5×10^1, 6.5×10^3, and 6.5×10^4 CFU dosages (Table 5).

Except for the diluent and 6.5×10^1 CFU injection treatments, mean hatchling BW was significantly affected by site of injection at all dosage levels (Table 5). Mean BW was significantly higher in eggs injected in the AC compared to eggs injected in the AM within the 6.5×10^2, 6.5×10^3, and 6.5×10^4 CFU dosages (Table 5). There were no significant ($p = 0.116$) site differences at any dosage for chicks found dead (free from the shell) in the hatch baskets. However, compared to the AC site treatment, the percentage of culled chicks was significantly ($p = 0.009$) higher in the AM site treatment only at the 6.5×10^1 CFU dosage level.

Table 5. Comparisons between in ovo injection sites (amnion and air cell) within the 5 separate Poulvac *E. coli* dosages that were administered for the following variables at 22 days of incubation (DOI): percent of embryos that failed to pip externally and remained in the shell (live and dead; PEIS), percent of embryos that pipped externally but did not complete hatch (live and dead; PEPE), percent hatch of injected eggs that contained live embryos as of 18 DOI (HI), percent of females hatched (PFCH), and mean chick body weight (BW) at hatch.

	Diluent (0 CFU *E. coli*)	**6.5×10^1 CFU *E. coli***	**6.5×10^2 CFU *E. coli***	**6.5×10^3 CFU *E. coli***	**6.5×10^4 CFU *E. coli***
			PEIS (%)		
Amnion	9.54	13.91 [a]	11.88 [a]	13.82 [a]	15.13 [a]
Air Cell	6.91	5.59 [b]	6.91 [b]	9.21 [b]	6.91 [b]
p-value	0.2578	0.001	0.034	0.049	0.001
			Pooled SEM = 1.6376		
N = 16					
			PEPE (%)		
Amnion	2.63	10.58 [a]	9.61 [a]	7.90 [a]	12.83 [a]
Air Cell	5.26	3.62 [b]	5.26 [b]	3.29 [b]	1.32 [b]
p-value	0.140	<0.001	0.016	0.011	<0.001
			Pooled SEM = 1.2801		
N = 16					
			HI (%)		
Amnion	87.48	75.19 [b]	78.17 [b]	77.94 [b]	71.70 [b]
Air Cell	87.17	89.73 [a]	87.84 [a]	87.16 [a]	91.12 [a]
p-value	0.918	<0.001	0.002	0.003	<0.001
			Pooled SEM = 2.1396		
N = 16					
			PFCH (%)		
Amnion	56.53	43.60 [b]	52.09	46.35 [b]	42.85 [b]
Air Cell	51.71	52.53 [a]	48.88	54.92 [a]	54.24 [a]
p-value	0.270	0.042	0.461	0.051	0.010
			Pooled SEM = 3.0723		
N = 16					
Chick BW (g)					
Amnion	39.75	39.35	38.65 [b]	38.69 [b]	38.23 [b]
Air Cell	39.99	39.48	39.48 [a]	39.55 [a]	39.34 [a]
p-value	0.410	0.650	0.005	0.004	0.001
Pooled SEM for PEIS, PEPE, HI, and PFCH = 0.2124. SEM for chick BW = 0.2191. N = 16					

[a,b] Means among site of injection within injection dosage for each variable with no common superscript differ significantly ($p \leq 0.05$).

3.4. Hatchling Somatic and Yolk Content Variables

There was a significant ($p = 0.044$) difference between injection sites in the 6.5×10^3 CFU dosage for absolute yolk sac weight, and between injection sites in the 6.5×10^4 CFU dosage for percent yolk sac weight ($p = 0.012$) and percent yolk dry matter ($p = 0.031$), with the AM having a significantly higher value than the AC. Conversely, there were significant differences between injection sites in the 6.5×10^4 CFU dosage for yolk-free BW ($p = 0.031$), body length ($p = 0.001$), and percent body mass ($p = 0.012$), with the AC having a significantly higher value than the AM. However, there were no significant differences between the injection sites at any dosage for percent yolk moisture ($p = 0.364$), hatchling BW ($p = 0.082$), or for BW-to-length ratio ($p = 0.104$).

4. Discussion

The 18 DOI pre-injection plating results showed that the CFU of the PECV in a 100 µL volume of the 1×10^{-6} dilution were too numerous to count and were 130 in the 1×10^{-7} dilution. However, in the 18 DOI post-injection plating of the same volume of the PECV, there were 179 CFU in the 1×10^{-6} dilution. This would indicate a 10-fold loss of bacteria between the pre- and post-injection periods at 18 DOI. The loss of bacteria may be because in ovo injection of the treatments took approximately 5 h to complete. It is well documented that the in ovo injection of caffeine and electrolyte solutions in broiler hatching eggs altered yolk utilization as well as hatchling quality [32–34]. These results indicate that changes in the composition of in ovo-injected solutions can influence nutrient absorption rate and subsequent chick quality. It is also possible that the contents of the diluent used, which was formulated specifically for the Marek's disease vaccine, might not be optimal for long-term survival of *E. coli* species. It is suggested that there may be the need for a more immediate direct dispensing of the PECV after its fresh preparation. Although there was a notable reduction in the bacteria numbers between the pre- and post-injection periods, an appreciable number of bacteria would have been administered in the 1×10^{-6}, 1×10^{-5}, and 1×10^{-4} dilutions.

The efficacy of the administered PECV is further supported by the embryo developmental stage and site of injection results. In the current study, all 21 AC injections were successfully administered without penetration of the inner AC membrane, and 19 of the 21 (90.5%) AM injections were successfully administered in the AM after penetration through the inner AC membrane. Although the mean embryo stage score at 18 DOI of 1.10 was lower than the 2.09 score reported by Sokale et al. [22] for Ross 708 broiler embryos at 18.5 DOI, it was similar to the 1.65 score reported by Elliott et al. [35] for layer embryos at 18 DOI that also experienced 83.3% AM injections. According to Avakian [36], embryos injected at 18 DOI with an Inovoject machine have a greater than 90% chance of receiving their injections in the AM, with approximately 5.79% of their injections occurring in the allantoic sac, AC, or yolk sac. The current results of this and previous studies indicate that most of the injections administered at 18 DOI were successfully administered in the AM.

The HI values were significantly lower in eggs injected in the AM in comparison to those injected in the AC for all 4 dosages of the PECV. These results are like those of Elliott et al. [27], who investigated the AM injection of the F-strain of *Mycoplasma gallisepticum* in Hy-Line W-36-layer embryos at 18 DOI. In that study, in which a full dosage as well as 1×10^{-2}, 1×10^{-4}, and 1×10^{-6} dilutions of the Poulvac Myco F vaccine were used, it was found that HI decreased as dosage increased, with an approximate 60% HI observed in the full dosage treatment [27]. Likewise, administration of a 1.73×10^{4} CFU (high) dosage of a 6/85MG vaccine in the AM of Hy-Line W-36-layer embryo hatching eggs has been shown to reduce HI when compared to a similar dosage administered in the AC [17].

Increased PEIS and PEPE values in response to the injection of the PECV in the AM rather than in the AC indicates that the *E. coli* of the PECV at the dosages delivered compromised late embryonic viability, thereby preventing their ability to complete a normal hatching process. The increases in PEIS and PEPE led to the significant decrease in HI at all dosage levels due to an AM injection. Elliott et al. [35] also reported a lower hatch of embryonated eggs when higher levels of *Mycoplasma gallisepticum* bacteria were injected into the AM. More specifically, there was a significantly higher percentage of embryos that died while pipping after receiving a medium (1×10^{4} CFU) or full (1×10^{6} CFU) dosage of the Poulvac Myco F Vaccine. Triplett et al. [37] administered 3 different probiotic strains of bacteria (*Lactobacillus acidophilus*, *Bacillus subtillus*, and *Bifidobacterium animalis*) by in ovo injection to broiler hatching eggs at 18 DOI. In the *Bacillus subtillus* treatment, there was a significant increase in the percentage of embryos that died during the pipping process. Upon consideration of various possible reasons for the increase in the number of embryos that died while pipping, Triplett et al. [37] suggested that the embryos in that treatment group may have lacked sufficient energy reserves to immunologically resist the bacterial

challenge. This same type of response to the *E. coli* bacterial challenge in the current study may also have occurred.

The negative effects of an AM in ovo injection on embryo and chick survival could more specifically be linked to the immaturity of their immune systems. It is well documented that the immune systems of chicken embryos begin to develop after the first week of incubation, with full development being completed during the first 10 days of posthatch age [38]. Thus, chicken embryos are more susceptible to enteric pathogenic agents during the incubational and early posthatch periods. Williams [14,15] reported that administration of the Marek's disease vaccine via an AM injection in broiler hatching eggs resulted in approximately 90% immune protection while AC injections elicited no immune protection. These results suggest that the lack of negative effects of AC injection of the PECV on layer embryo development and hatchability are related to low transmissibility and efficacy. Further research is needed to confirm PECV presence in embryos and posthatch chicks after AC and AM injections using DNA detection and serological testing methods.

Furthermore, a comparison of the PFCH values in the AM and AC treatments within the dosages tested showed that at the 6.5×10^1, 6.5×10^3, and 6.5×10^4 CFU dosages, fewer females hatched from eggs that received PECV injections in the AM. This would indicate that female embryos may be more susceptible than male embryos to the AM administration of the PECV, suggesting a sex-biased level of mortality in response to the administration of the PECV in the AM. Elliott et al. [35] also noted a sex-biased response to an F-strain *Mycoplasma gallisepticum* vaccination injected in the AM at 18 DOI. It was reported that male Hy-Line W-36 embryos had a higher level of mortality than females, indicating that males were more susceptible to the F-strain of *Mycoplasma gallisepticum* when it was administered via the AM. Furthermore, IgG levels have been shown to increase when birds are challenged with *Salmonella typhimurium*, whereas they decreased in response to a Luria-Bertani broth, indicating that different humoral immune responses can occur to different pathogenic agents [39]. The possible reason for the differential susceptibilities of male and female embryos to pathogenic agents such as FMG or PECV could be linked to differences in their systematic inflammation or humoral immunity responses to different pathogens. Higher nitric oxide levels have been shown to be linked to decreased growth and immune responses [26,40–43]. Mousstaaid et al. [44] reported that plasma nitric oxide concentrations were higher in male than female broilers at hatch and at 14 days of age.

Mean chick BW at 22 DOI was significantly lower in the AM-injected eggs compared to their AC counterparts. These results reflect the effects of the AM injections of the PECV on late embryonic mortality and HI. This establishes that the detrimental effects of AM injections of PECV on embryo viability, particularly at the higher dosages employed, continue through hatch with lingering detrimental effects on hatchling BW. Although the PECV treatments decreased mean chick BW, suggesting that these chicks were physiologically compromised due to the bacterial challenge, further investigations on BW over time are needed to establish the duration of the negative effect of AM PECV injections on chick growth and development. It would be of interest to determine if chicks that received AM PECV injections are able to recover physiologically at some point in their posthatch development and to possibly exhibit compensatory growth.

5. Conclusions

In conclusion, it was shown in this study that the PECV can be successfully delivered to the AC or AM of Hy-Line W-36-layer hatching eggs at 18 DOI. Nevertheless, the injection of the PECV into the AM at 18 DOI at the dosages employed in this study may incur detrimental effects on late embryo livability and subsequent HI and hatchling BW. Further exploration of the in ovo administration of the PECV at higher dosages in the AC or at lower dosages in the AM as potentially less incursive methods for the effective prevention of *E. coli* colonization in layers should be considered. Additionally, further research is needed to determine the posthatch effects of in ovo administration of PECV on layer pullets.

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Abbreviations

AC: air cell; AM: amnion; APEC: Avian pathogenic *E. coli*; BW: body weight; CFU: colony forming units; DOI: days of incubation; HI: chicks that fully hatched as a percentage of injected eggs that contained live embryos as of 18 DOI; PECV: Poulvac *E. coli* vaccine; PEIS: embryos that failed to pip externally and remained in the shell (live and dead) as a percentage of injected eggs that contains live embryos as of 18 DOI; PEPE: embryos that pipped externally but did not complete hatch (live and dead) as a percentage of injected eggs that contains live embryos as of 18 DOI; PFCH: percentage of female chicks hatched; 6/85MG: 6/85 strain of *Mycoplasma gallisepticum*.

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