

Evaluation of day of hatch exposure to various Enterobacteriaceae on inducing gastrointestinal inflammation in chicks through two weeks of age

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ABSTRACT Inappropriate microbial colonization can induce gastrointestinal (GI) inflammation may predispose poultry to opportunistic infections and reduce growth performance. Four independent experiments were completed to test ability of select Enterobacteriaceae isolates to induce GI inflammation. Experiments 1 and 2 included a non-inoculated control (NC), and a low (L), medium (M), or high (H) day of hatch (DOH) oral inoculation level. In experiment 1, birds in L1, M1, and H1 received 10^2 to 10^4 CFU of a mixed dose of 2 species of *Citrobacter* and *Salmonella* Enteritidis LB (SE). In experiment 2, birds in L2, M2, and H2 received 10^3 to 10^5 CFU of *E. coli* LG (LG) and included NC. Body weight was recorded on d 0, 7, and 14, with blood collected for chicken serum alpha-1-acid glycoprotein (A1GP) measurements on d14. Neither experiment resulted in differences in BWG, however, A1GP was increased ($P < 0.05$) on d 14 when DOH inoculation dose 10^3 CFU/chick was used compared to NC. This observed increase in A1GP resulted in selection of 10^3

CFU/chick for DOH inoculation in experiments 3 and 4. Experiment 3 consisted of NC, *E. coli* Huff (Huff), and SE. On d 0, 7 and 15, BW was measured, with blood collected on d 15 for A1GP. Both d 15 A1GP and BWG from d 7 to 15 were reduced in inoculated chicks, Huff and SE, in experiment 3 ($P < 0.05$). Experiment 4 evaluated NC and LG with BW measured on d 0, 2, 7 and 14. Yolk sacs were evaluated for retention and bacterial enumeration, and blood for serum A1GP were collected on d 2 and 14. Experiment 4 resulted in no differences in yolk sac parameters or A1GP, whereas there was an increase in BWG for LG from d 0 to 14 ($P < 0.05$). When evaluated over time, serum A1GP increased between d 2 and d 14 by nearly 46% in LG, compared to negligible changes in NC ($P = 0.111$). Mild GI inflammation induced by early Enterobacteriaceae exposure may not drastically impact growth or inflammation parameters but may increase susceptibility to opportunistic infection necessitating further study of this model.

Key words: Enterobacteriaceae, gastrointestinal inflammation, alpha-1-acid glycoprotein, opportunistic disease

2021 Poultry Science 100:101193

<https://doi.org/10.1016/j.psj.2021.101193>

INTRODUCTION

Gastrointestinal (GI) inflammation due to inappropriate microbial colonization has been an often-overlooked concern throughout the poultry industry. Exposure to Enterobacteriaceae on day of hatch (DOH) can result in mild GI inflammation that acts as a predisposing factor for a variety of opportunistic infections with little impact on growth performance. *Salmonella enterica* serovars and *Escherichia coli* have been associated with GI inflammation following DOH exposure (Bailey et al., 2002; Nava et al., 2005). Early

exposure to pathogens often occurs at hatcheries due to contaminated hatchery equipment or egg shells (Cox et al., 1990; Cason et al., 1994; Byrd et al., 2007; Kim and Kim, 2010). This may influence the developing microbiome and its interactions within the GI tract, resulting in a lasting, mild GI inflammation (Ballou et al., 2016; Wilson et al., 2019).

Escherichia coli are commonly found within the GI tract of young chicks, many with the capacity to act as a pathogens (Leimbach et al., 2013; Ballou et al., 2016). On the other hand, paratyphoid *Salmonella* spp. are not ubiquitous residents within the poultry intestinal tract, but are considered ubiquitous in the environment and found in high proportions at hatcheries (Bailey et al., 2002), and result in mild pathology in chickens (Hassan and Curtiss, 1994; Foley et al., 2008). These facultative anaerobic Enterobacteriaceae can be classified as opportunistic pathogens that can result in

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Received January 26, 2021.

Accepted March 26, 2021.

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inflammation upon colonization of the immature GI tract of DOH chicks (Wigley, 2015). Studies have shown that early microbial exposure has the ability to influence microbial populations as well as intestinal and immune development of the host, highlighting the potential impact of inappropriate microbial inoculation (Ballou et al., 2016; Wilson et al., 2019; Rodrigues et al., 2020). Exposure to Enterobacteriaceae on DOH can manifest as altered body weight gain (BWG), serum concentration of acute phase proteins such as alpha-1-acid glycoprotein (A1GP), yolk sac retention, and bacterial habitation of the yolk sac.

Alpha-1-acid glycoprotein is a major acute phase protein synthesized and released by the liver as part of the acute phase response (Chamanza et al., 1999; Fournier et al., 2000). Since A1GP can be induced by stress, burns, infection, and other chronic inflammatory conditions, a level of ambiguity surrounds the use of A1GP as a marker for GI inflammation (Fournier et al., 2000). Several studies in poultry have evaluated changes in A1GP associated with various diseases and inflammatory conditions, with peak A1GP consistently 24 to 48 h postinjection or inoculation, with normal serum concentrations generally in the range of 150 to 400 $\mu\text{g}/\text{mL}$ (Takahashi et al., 1994; Inoue et al., 1997; Adler et al., 2001; Buyse et al., 2007; O'Reilly et al., 2018). Although not exclusively related to GI inflammation, A1GP may provide a marker for GI inflammation alongside additional metrics.

Almost a century ago, Brandy (1932) tied infection of the yolk sac, or omphalitis, to poor incubation conditions, hatchery or egg shell contamination, and improper closure leading to infection of the navel. Egg shell contamination has also been connected to greater rates of yolk sac retention, early mortality, and depressed body weight (Reid et al., 1961; Gross, 1964). Day of hatch exposure to Enterobacteriaceae can also act as a source of yolk sac infection and increase retention in birds, while also serving as a potential pathogen reservoir in market-age broilers (Cox et al., 2006). Since eggshell and hatchery contamination can be considered a major source of pioneer colonizers, studying the influence of DOH oral exposure to pathogens on yolk sac retention, and colonization or infection of the yolk sac can provide insight into early microbial influence.

Microbial exposure to Enterobacteriaceae has the potential to disrupt various developmental and immunological processes within birds, which may lead GI inflammation as well as altered growth performance and

disease susceptibility of poultry flocks. By developing an industry relevant, reliable model of GI inflammation, as well as metrics with which to assess this inflammation, long-term effects of DOH exposure to Enterobacteriaceae can be studied. Therefore, experiments were completed to evaluate DOH dose of Enterobacteriaceae required to induce GI inflammation as measured by changes in BWG and A1GP at approximately 2 wk of age. Two additional experiments assessed *E. coli* and *Salmonella* strains for their ability to induce mild GI inflammation and growth performance changes as measured by BWG, A1GP, yolk sac retention, and bacterial enumeration of yolk sacs.

MATERIALS AND METHODS

Animals, Housing, and Experimental Design

A total of 4 experiments were completed at the Poultry Center of the Ohio Agricultural Research and Development Center, Wooster, Ohio under approved animal care protocols (2016A00000038 and 2018A00000074) from The Ohio State University Institutional Animal Care and Use Committee. In all experiments, DOH Ross 708 broiler chicks were obtained from a local hatchery, neck tagged, and randomly placed in wire floor cages, except in experiment 4, where chicks were randomly placed in floor pens with fresh pine shavings. Nutritionally complete feed and water were provided ad libitum, and ambient temperature and lighting were maintained at age-appropriate levels (NRC, 1994).

Bacterial Preparation

For each experiment, a frozen aliquot of *Salmonella* Enteritidis LB (SE), *Citrobacter freundii*, *Citrobacter sp.*, *E. coli* Huff (Huff), and *E. coli* LG (LG) were each thawed and individually inoculated into tryptic soy broth (Merck KGaA, EMD Millipore Cooperation, Billerica, MA) at 0.5% volume, which was incubated at 37°C for 24 h. Cells were washed three times in 0.9% sterile saline by centrifugation at 1,800 \times *g* for 15 min. Cultures administered via oral gavage were spectrophotometrically quantified (Spectronic 2000, Thermo Scientific, Waltham, MA). All inoculum CFU were retrospectively confirmed by serial dilution plating on tryptic soy agar and reported in Table 1.

Table 1. Enterobacteriaceae inoculation doses for experiments 1 and 2. In both experiments, chicks received no inoculation, or were orally administered one of three dose levels of either a mixed dose of Enterobacteriaceae in experiment 1 or *E. coli* LG in experiment 2 on day of hatch.

Treatment	n	Replicate Pens	Experiment 1 (Dose)	Experiment 2 (Dose)
Non-inoculated Control	12	3	None	None
Low	12	3	8.5×10^1 CFU/chick mixed Enterobacteriaceae ¹	9.0×10^2 CFU/chick <i>E. coli</i> LG
Medium	12	3	8.5×10^2 CFU/chick mixed Enterobacteriaceae	9.0×10^3 CFU/chick <i>E. coli</i> LG
High	12	3	8.5×10^3 CFU/chick mixed Enterobacteriaceae	9.0×10^4 CFU/chick <i>E. coli</i> LG

¹Mixed Enterobacteriaceae dose consisted of equal CFU of *Salmonella enterica* Enteritidis LB, *Citrobacter freundii*, and *Citrobacter sp.*

Serum A1GP Analysis

Birds were euthanized via CO₂ inhalation and blood was collected from the femoral vein, then allowed to clot at room temperature for approximately 3 h before serum collection, and stored at -20°C. Serum was diluted and A1GP serum concentrations were evaluated according to manufacturer instructions of the A1GP ELISA Kit (AGP-5, Life Diagnostics, Inc., West Chester, PA).

Experiment 1

A total of 144 DOH broiler cockerels were randomly placed into one of 4 treatment groups, non-inoculated control (NC), Low (L1), Medium (M1), or High (H1), with 12 birds per pen and 3 replicate pens for a total of 36 birds per treatment. Day of hatch inoculations consisted of 10² to 10⁴ CFU of wild-type mixed Gram-negative bacteria containing SE, *Citrobacter freundii*, and *Citrobacter* spp. that were originally isolated from the GI tract of healthy adult chickens, and dosed according to treatment as described in Table 1. Individual BW was measured on d 0, 7, and 14. All birds were euthanized on d 14 via CO₂, blood was collected, and 11 samples per treatment were randomly selected for serum A1GP analysis.

Experiment 2

A total of 144 DOH broiler cockerels were randomly placed into one of 4 treatment groups, NC, Low (L2), Medium (M2), or High (H2), with 12 birds per pen and three replicate pens for a total of 36 birds per treatment. On DOH, birds received no inoculation, NC, or were orally inoculated with a low, medium, or high dose, treatment L2, M2, and H2 respectively, of 10³ to 10⁵ CFU LG according to treatment, as described in Table 1. Body weights were measured on d 0, 7, and 14. All birds were euthanized on d 14, blood was collected, and 11 samples per treatment were randomly selected for serum A1GP analysis.

Experiment 3

A total of 124 DOH broiler birds were randomly placed into one of 3 treatment groups split between 2 rooms. Due to concerns that *E. coli* Huff can spread from pen to pen and cause respiratory illness, pens were split across 2 rooms. Room 1 contained NC with 20 birds per pen, and SE with 11 birds per pen, with 2 replicate pens per treatment. Room 2 contained NC with 20 birds per pen, and Huff with 11 birds per pen, with 2 replicate pens per treatment. Since there was no room effects observed, the rooms were combined analyzed together. On DOH, birds received oral inoculation with 0.9% sterile saline, NC, or received 2.4 × 10³ CFU/chick of *Salmonella* Enteritidis LB or 5.0 × 10² CFU/chick of *E. coli* Huff. Body weights were measured on d 0, 8, and 15. Five birds per pen in NC and all birds in SE and Huff

were euthanized on d 15, and blood was collected for serum A1GP analysis.

Experiment 4

A total of 140 DOH broiler cockerels were randomly placed into NC or LG treatments, with 10 birds per pen and 7 replicate pens per treatment. On DOH, birds received oral inoculation of 0.9% sterile saline, NC, or 9.5 × 10² CFU/chick of *E. coli* LG. Feed and body weights were measured on d 0, 2, 7, and 14. On d 2 and d 14, a total of 5 birds per pen were killed for blood collection to perform serum A1GP measurements, and any retained yolk sacs were collected to quantify bacterial load within each yolk sac.

Statistical Analysis

In all experiments, BW, BWG, A1GP, and bacterial enumeration were subject to Analysis of Variance as a completely randomized design using the General Linear Models procedure of SAS (JMP Software, SAS Inc., 2016) and data are expressed as mean ± standard error. In experiment 4, A1GP data was further analyzed to determine treatment by time interactions, using the test slices option for mean separation. Retained yolk sac percentages were analyzed using Chi-squared analysis. Significant differences among the means were determined using Dunnett's test in experiments 1, 2, and 3, or *t* test in experiment 4, at *P* < 0.05.

RESULTS

Experiments 1 and 2 determined the optimal dose to induce mild inflammation at approximately two weeks of age. In both experiments, no differences in BWG were observed (Table S1), but a consistent elevation in A1GP was measured in chicks inoculated with 10³ CFU/chick on DOH (Table 2). In experiment 1, A1GP serum concentration of NC was 446.24 ± 56.80 µg/mL compared to M1 at 712.89 ± 116.09 µg/mL (*P* = 0.043; Table 2). In experiment 2, A1GP serum concentration of NC was 203.03 ± 47.61 µg/mL compared to L2 at 610.70 ±

Table 2. Serum concentration of alpha-1-acid glycoprotein (µg/mL) on d 14, experiments 1 and 2. In both experiments, chicks were orally inoculated on day of hatch with one of three dose levels of either a mixed dose of Enterobacteriaceae in experiment 1 or *E. coli* LG in experiment 2. Serum was collected from all chicks on d 14, with alpha-1-acid glycoprotein analysis completed on 11 randomly selected chicks/treatment within each experiment. All data represented as mean ± standard error.

	Experiment 1	Experiment 2
Non-inoculated Control	446.24 ± 56.80	203.03 ± 47.61
Low	147.75 ± 44.68 ¹	610.70 ± 203.78 ¹
Medium	712.89 ± 116.10 ¹	232.20 ± 37.89
High	292.20 ± 62.08	346.77 ± 53.30
SEM	120.86	92.84
<i>P</i> -value	0.043	0.032

¹Mean values within a column indicate a significant difference from the Non-inoculated control (*P* < 0.05).

203.78 $\mu\text{g}/\text{mL}$ ($P = 0.032$; Table 2). Similar A1GP serum concentrations were observed for all other DOH inoculation doses on d 14 compared to NC, except L1, which resulted in decreased A1GP concentration, as seen in Table 2. Therefore, consistent A1GP elevation at 2 weeks of age using 10^3 CFU/mL Enterobacteriaceae on DOH compared to NC resulted in the selection of this dose for future experiments aimed to induce mild GI inflammation.

In experiment 3, BWG was depressed in Huff throughout the experiment, and in SE during the second week following DOH inoculation compared to NC (Table 3). From d 0 to 15, NC BWG averaged approximately 90 g greater, 304.97 ± 9.44 g, versus Huff at 212.43 ± 13.89 g, or SE at 216.72 ± 18.08 g ($P < 0.001$; Table 3). This clear reduction in BWG was not reflected in A1GP levels. The A1GP serum concentration in NC was above normal levels in poultry at 536.48 ± 122.45 $\mu\text{g}/\text{mL}$ on d 15, whereas A1GP in Huff and SE were extremely low, at 55.97 ± 17.93 $\mu\text{g}/\text{mL}$ and 68.58 ± 39.13 $\mu\text{g}/\text{mL}$, respectively ($P < 0.001$, Table 3). However, BWG seemed to be clearly influenced by DOH inoculation, which resulted in decreased BWG in both Huff and SE for the first 15 d.

Trends of improved BWG were observed d 2-7 and d 7-14 in experiment 4 ($P < 0.100$, Table 4), with LG displaying improved BWG throughout the experiment from d0-14, at 313.33 ± 14.34 g, compared to NC at 268.69 ± 15.78 g ($P = 0.040$; Table 4). These results contrasted those found in experiment 3, where the inoculated treatments resulted in depressed BWG compared to NC. Yolk sac retention, yolk sac weight, and bacterial enumeration showed almost no difference, but some interesting trends were observed. There were no changes

observed in yolk sac retention at either d 2 or d14 (Table S2). When yolk sac weights were considered over time, NC showed a 70% reduction in yolk sac weight from d 2 to d 14, from 0.64 ± 0.04 g to 0.17 ± 0.04 g, whereas LG exhibited about a 30% reduction in yolk sac weight, from 0.39 ± 0.06 g to 0.27 ± 0.13 g, in the same period (d 2 $P = 0.001$, d 14 $P = 0.515$; Table 5). Neither total aerobic nor Enterobacteriaceae bacterial enumerations of the yolk sac resulted in differences between NC and LG on d 2 or d 14. However, Enterobacteriaceae encompassed the majority of quantified bacteria on d2 in LG, with 1.76 ± 0.24 Log_{10} CFU/g Enterobacteriaceae comprising the 3.70 ± 0.33 Log_{10} CFU/g total aerobic bacteria in NC compared to 2.16 ± 0.22 Log_{10} CFU/g Enterobacteriaceae comprising the 3.67 ± 0.24 Log_{10} CFU/g total aerobic bacteria in LG ($P > 0.05$; Table 5). This may hint toward a reason for the minimal reduction in yolk sac weight compared to NC. No differences were observed between treatments in A1GP concentration in the serum at either time point (Table 4). However, when evaluated over time, LG demonstrated a nearly 46% increase in serum concentration between d 2, at 858.97 ± 90.92 $\mu\text{g}/\text{mL}$, and d 14, at $1,252.90 \pm 238.31$ $\mu\text{g}/\text{mL}$ ($P = 0.111$; Table 4). No change was observed in NC between d 2 and d 14 from $1,102.16 \pm 140.17$ $\mu\text{g}/\text{mL}$ to $1,112.63 \pm 186.06$ $\mu\text{g}/\text{mL}$, respectively ($P = 0.966$; Table 4).

DISCUSSION

The importance of pioneer colonizing bacteria has been established through various studies, with a focus on the positive influence of probiotic strains (Jin et al.,

Table 3. Body weight gain (g) and serum concentration of alpha-1-acid glycoprotein ($\mu\text{g}/\text{mL}$), experiment 3. Chicks either received no inoculation on DOH or were inoculated with *Salmonella* Enteritidis LB at 2.4×10^3 CFU/chick or *E. coli* Huff at 5.0×10^2 CFU/chick. Body weight was measured on d 0, 7, and 15, and was used to calculate body weight gain (BWG). Serum was collected from all chicks on d 15 to measure concentrations of alpha-1-acid glycoprotein ($\mu\text{g}/\text{mL}$). All data represented as mean \pm standard error.

	BWG (g)			A1GP ($\mu\text{g}/\text{mL}$) d 15
	d 0-7	d 7-15	d 0-15	
Non-inoculated Control	67.59 ± 2.33	236.87 ± 7.69	304.47 ± 9.44	536.48 ± 122.45
<i>Salmonella</i> Enteritidis LB	60.60 ± 4.60	150.07 ± 15.92^1	216.72 ± 18.08^1	55.97 ± 17.93^1
<i>E. coli</i> Huff	48.15 ± 3.45^1	164.27 ± 12.08^1	212.43 ± 13.89^1	68.58 ± 39.13^1
SEM	5.68	26.88	29.99	158.11
P-value	<0.001	<0.001	<0.001	<0.001

¹Mean values within a column indicate a significant difference from the Non-inoculated control ($P < 0.05$).

Table 4. Body weight gain (g) and serum concentration of alpha-1-acid glycoprotein ($\mu\text{g}/\text{mL}$), experiment 4. Chicks either received no inoculation on DOH, or were orally administered *E. coli* LG at 9.5×10^2 CFU/chick. Body weight was measured on d 0, 2, 7, and 14, and was then used to calculate body weight gain (BWG) in grams. Serum was collected from 3 chicks/pen ($n = 21/\text{treatment}$) on d 2 and 14, to evaluate concentrations of alpha-1-acid glycoprotein ($\mu\text{g}/\text{mL}$). All data are represented as mean \pm standard error.

	BWG (g)				A1GP ($\mu\text{g}/\text{mL}$)		Time X Trt P-value
	d 0-2	d 2-7	d 7-14	d 0-14	d 2	d 14	
Non-inoculated Control	16.10 ± 0.43	64.66 ± 4.45	188.53 ± 11.75	268.69 ± 15.78^b	$1,102.16 \pm 140.17$	$1,112.63 \pm 186.06$	0.966
<i>E. coli</i> LG	16.64 ± 0.46	76.10 ± 4.09	220.07 ± 10.83	313.33 ± 14.34^a	858.97 ± 90.92	$1,252.90 \pm 238.31$	0.111
SEM	0.27	5.72	15.77	22.32	121.6	70.13	
P-value	0.391	0.063	0.052	0.04	0.155	0.645	

^{a,b}Mean values with different superscript letters within a column indicate a significant difference ($P < 0.05$).

Table 5. Yolk sac weight (g) and bacterial enumeration of the yolk sac on tryptic soy agar and MacConkey Agar for total aerobic and Enterobacteriaceae bacterial quantification (Log_{10} CFU/g), respectively, experiment 4. Chicks either received no inoculation on DOH, or were orally administered *E. coli* LG at 9.5×10^2 CFU/chick. A total of 5 chicks/pen ($n = 35/\text{treatment}$) were sampled on d 2 and 14. All retained yolk sacs were aseptically collected at each time point, wherein all retained yolk sacs were weighed, homogenized in 0.9% sterile saline at a 5-fold dilution, then plated on tryptic soy agar (TSA) for total aerobic bacterial content and on MacConkey Agar for Enterobacteriaceae concentrations within each yolk sac. Yolk sac weights are represented as mean \pm standard error in grams, and bacterial enumeration is represented as mean \pm standard error of the Log_{10} CFU/g.

	d 2			d 14		
	Yolk Sac Weight (g)	Tryptic Soy Agar	MacConkey	Yolk Sac Weight (g)	TSA	MacConkey
Non-inoculated Control	0.64 \pm 0.04 ^a	3.70 \pm 0.33	1.76 \pm 0.24	0.17 \pm 0.04	5.70 \pm 1.14	3.24 \pm 0.65
<i>E. coli</i> LG	0.39 \pm 0.06 ^b	3.67 \pm 0.24	2.16 \pm 0.22	0.27 \pm 0.13	6.32 \pm 1.82	4.52 \pm 2.08
SEM	0.13	0.02	0.20	0.05	0.31	0.64
<i>P</i> -value	0.001	0.941	0.231	0.515	0.783	0.593

^{a,b}Mean values with different superscript letters within a column indicate a significant difference ($P < 0.05$).

1998; La Ragione et al., 2004; Mountzouris et al., 2007; Pedroso et al., 2016; Wilson et al., 2019). Probiotic microbes exploit a wide variety of mechanisms to benefit their survival while simultaneously benefiting poultry, with one of the most commonly recognized mechanisms being minimizing pathogen invasion through competitive exclusion (Rantala and Nurmi, 1973). Due to the abundance of nutrients and resources in the GI tract, probiotic bacteria are thought to outcompete pathogens for attachment sites and nutrients, preventing pathogen proliferation (Rantala and Nurmi, 1973; Nurmi et al., 1992; Jin et al., 1997). Introduction of probiotic strains have been shown to impede attachment of pathogens, such as *Salmonella* spp., *E. coli*, and *Campylobacter*, 3 genera that constitute a food safety concern in poultry (Jin et al., 1996a; b, 1998; La Ragione et al., 2004; Ma et al., 2006). Taking competition one step further, probiotic strains attach directly to pathogens to prevent proliferation (Ohashi and Ushida, 2009). Although competitive exclusion has been proven time and again, bacterial strains have the ability to produce their own inhibitory compounds and metabolites, specific to genera, species, and strain (Jin et al., 1997; Smith, 2014). Many of the genera used as direct fed microbials have the ability to produce antimicrobial peptides and other metabolites that act as bacteriostatic or bactericidal compounds against pathogens (Belkaid and Hand, 2014; Smith, 2014; Belkaid and Harrison, 2017; Ma et al., 2018). In addition to the ways in which direct fed microbials can inhibit pathogen proliferation, they also interact with the host and modulate the immune response (Lebeer et al., 2010; Kogut and Swaggerty, 2012; Belkaid and Harrison, 2017). Adequate control of early microbial exposure can provide a positive foundation for poultry intestinal development. While research has focused on how probiotics influence intestinal health and microbial colonization, this view has expanded to include the influence of probiotics on immune development and response. By studying the influence of Enterobacteriaceae on GI microbiota, inflammation, and development, the importance of pioneer colonizers can be more greatly appreciated.

Exposure to potentially pathogenic bacterial strains can lead to colonization of these populations, which may

negatively impact intestinal inflammation and overall poultry health. Some of the mechanisms utilized by probiotic strains can also be used by opportunistic pathogens in order to establish a foothold in the GI tract, resulting in immune tolerance of bacteria such as Enterobacteriaceae (Leimbach et al., 2013; Ghareeb et al., 2016). Exposure to Enterobacteriaceae on DOH has the capacity to induce low level inflammation that may not be reflected in changes in BWG, but still elicit an immune response (Kogut et al., 2018), such as elevated A1GP. In these experiments, sterile inflammation, or chronic, low level inflammation (Rubartelli, 2013) was achieved through DOH exposure to Enterobacteriaceae which resulted in various effects on BWG and expression of acute phase protein A1GP.

During the innate immune response, hepatic cells are stimulated to express various acute phase proteins, including A1GP (Chamanza et al., 1999). The specific biological function of A1GP is not well understood, but it performs several physiological functions during acute phase response (Bteich, 2019). This negatively charged glycoprotein can transport various hormones and contaminants, depress the inflammatory immune response, and may bind lipopolysaccharides to neutralize the toxin (Adler et al., 2001; O'reilly and Eckersall, 2014; Bteich, 2019). In poultry, A1GP has been found to be one of the most responsive acute phase proteins to inflammation (Adler et al., 2001). Although A1GP expression is not specific to intestinal inflammation, its sensitivity to inflammatory stimuli provided a baseline with which to measure inflammation affected by treatment. Several poultry studies have evaluated changes in A1GP associated with various diseases and inflammatory conditions, with peak A1GP consistently 24 to 48 h postinjection or inoculation, with normal serum concentrations of A1GP generally in the range of 150 to 400 $\mu\text{g}/\text{mL}$ (Takahashi et al., 1994; Inoue et al., 1997; Adler et al., 2001; Buyse et al., 2007; O'Reilly et al., 2018). Dose response of A1GP in relation to DOH inoculation was observed in experiments 1 and 2, with serum A1GP elevated at 10^3 CFU/chick inoculation compared to NC in both experiments ($P < 0.05$; Table 2), even though A1GP concentration was outside the normal range in experiment 1. Sensitivity of A1GP allowed for

the dose response to be captured to determine the appropriate inoculation dose of Enterobacteriaceae on DOH to induce inflammation at approximately 2 wk of age. Expression of A1GP has been shown to increase in response to acute exposure to various pathogens and pathogenic components, such as infectious bursal disease virus, infectious bronchitis, and *E. coli* lipopolysaccharides (Inoue et al., 1997; Takahashi et al., 1998; O'reilly and Eckersall, 2014). Typically, peaks in serum A1GP concentration occur 24 to 48 h postinfection, but may remain elevated in response to chronic infection or inflammatory conditions (Fournier et al., 2000; Bteich, 2019). The only experiment that measured A1GP two days following inoculation was experiment 4, but no differences were measured in A1GP concentrations between NC and LG (Table 4). However, the slight increase in A1GP between d 2 and d 14 for LG suggests chronic inflammation is likely influencing the acute phase protein expression. Since this increase was not observed for NC over the same time frame, DOH inoculation seems to influence A1GP expression and mild chronic GI inflammation over time.

While the A1GP tends to peak 24 to 48h following acute exposure to a pathogen or pathogenic components, low pathogen exposure following a vaccine has resulted in significantly lower A1GP concentrations compared to unvaccinated controls (Sylte and Suarez, 2012). This may be reflective of the results observed in experiment 3, where A1GP for SE and Huff were not only lower than NC but were also below normal A1GP levels. It would follow that DOH chicks inoculated with Enterobacteriaceae may have been provided some form of protection and limited A1GP production in these birds. Inconsistencies observed between BWG and A1GP results in experiment 3 may be confounded by various factors that influence A1GP serum concentration. Since A1GP can be affected by multiple factors, beyond GI inflammation due to DOH inoculation with Enterobacteriaceae, A1GP alone cannot be used to interpret GI inflammation, but only generalized inflammation within the bird. Another concern observed with A1GP concentrations were consistently elevated levels in NC across multiple experiments. This may have been due, in part, to exposure to a *Proteus* spp. at the hatchery since the bacteria was detected within intestinal samples of DOH chicks, identified by its characteristic swarming morphology (data not shown). *Proteus* have been characterized as a ubiquitous bacterium within the environment that can act as an opportunistic pathogen (O'Hara et al., 2000), and has been increasingly isolated from poultry and other food animals (Wong et al., 2013; Nahar, 2014; Yeh et al., 2018). As a result of exposure to this opportunistic pathogen at the hatchery, A1GP concentrations of NC birds may have been impacted, resulting in variations of A1GP throughout the experiments.

Although A1GP is one of the more sensitive acute phase proteins that can be measured during inflammation, the literature consistently cites peak serum concentrations of A1GP to occur 24 to 48 h postinfection, with A1GP returning to normal levels within 7 to 14 d

(Nakamura et al., 1998; O'reilly and Eckersall, 2014). In addition, acute phase proteins have been cited as being minimally influenced by changes in the mucosal barrier in chickens, with a lack of specificity as an intestinal health marker (Ducatelle et al., 2018). Collecting blood to measure serum A1GP concentrations 2 wk following DOH inoculation does not fit within the pattern of peak A1GP concentration, but can provide insight into the inflammatory status of the bird at that time point, and over the course of the experiment when applicable. Consequently, A1GP may be a useful tool for monitoring inflammatory status given various inoculation species and strains on DOH.

Routes of bacterial entry to the yolk sac include egg shell and hatchery contamination, as well as entry directly from the environment in cases of improper closure of the navel (Brandy, 1932). The DOH inoculation model used in these experiments was designed to mimic low level pathogen exposure during the neonatal period. This inoculation prior to any exogenous feed would increase chances of intestinal colonization with potential transmission to the yolk sac since the yolk stalk is unimpeded for the first 72 h post hatch (Noy et al., 1996). Evidence of yolk sac content movement through the proximal GI tract via antiperistaltic pulses (Noy et al., 1996) provided further support that pioneer colonizing bacteria would have greater opportunity for proliferation and colonization within the GI tract and to move into the yolk sac, resulting in yolk sac infection. Colonization of the yolk sac has been tied to oral inoculation, with potential pathogens, including *E. coli*, *Salmonella* spp. and *Campylobacter* spp., colonizing and residing in the yolk sac of broilers and layers from experimental and commercial conditions (Bailey et al., 2005; Buhr et al., 2006; Cox et al., 2006). Although no differences were observed in bacterial enumeration of the yolk sac in experiment 4, the numerically greater percentage of Enterobacteriaceae that comprised LG compared to NC on d 2 suggests that there may have been some proliferation by LG within the yolk sac. Enterobacteriaceae remained the numerically greater percentage of bacteria that comprised total aerobic bacteria in the yolk sac through d 14 in LG compared to NC. Though no further classification of the microbes within the yolk sacs were completed in experiment 4, studies have found the most common isolate of Enterobacteriaceae to be *E. coli* (Dzoma and Dorrestein, 2001; Cox et al., 2006). This furthers the idea that DOH LG inoculation resulted in proliferation within the GI tract and potential colonization of the yolk sac.

In addition to yolk sac retention and bacterial enumeration, yolk sac weight was used as an additional measurement of mild GI inflammation. Yolk sac weight has not commonly been measured beyond the first week of life since yolk sac weight has been used to illustrate yolk sac utilization in chicks (Chamblee et al., 1992; Noy et al., 1996; Jamroz et al., 2004; Şahan et al., 2014). However, yolk sac weight was observed to decrease steadily over time for both chickens and ostriches through 2 wk of age when it became almost negligible

(Noy et al., 1996; Dzoma and Dorrestein, 2001). In experiment 4, yolk sac weight of LG decreased by about 30% between d 2 and d 14, as compared to about a 70% reduction in NC. The reduction in yolk sac weight of NC was in line with the expected reduction in yolk sac weight described by Dzoma and Dorrestein (2001), which suggested an influence of DOH LG inoculation on yolk sac resorption of these chicks. Although not immediately clear, evaluation of yolk sac weight contributed to the interpretation of the effects of DOH inoculation on yolk sac parameters.

Exposure to Enterobacteriaceae on DOH may not result in mild GI inflammation as measured by distinct and consistent changes in BWG, serum A1GP, or yolk sac parameters, but disruption caused by DOH exposure may provide opportunistic pathogens with an environment suitable to cause disease around 2 wk of age. During the first one to two weeks of age, chicks are protected by passive immune transfer of maternal antibodies within the egg yolk (Gharaibeh and Mahmoud, 2013). Maternal antibody protection has been demonstrated to be most effective during the first week of life, with no estimable protection against most pathogens, except infectious bursal disease virus and Newcastle disease virus, by 10 to 15 d of age (Gharaibeh and Mahmoud, 2013). Several studies demonstrated similar patterns of decline in maternal antibodies within the first two weeks (Hamal et al., 2006; Leandro et al., 2011; Friedman et al., 2012; Lu et al., 2019). Upregulation in endogenous antibody production and immune function occurs with a clear wave beginning at four days of age, and resulting in a functionally mature immune system around 14 d (Bar-Shira et al., 2003; Friedman et al., 2003, 2012). However, various endogenous antibodies, including IgM, IgA, and IgY, only began to approach adult levels around 21 d (Hamal et al., 2006), suggestive of a gap in immune protection from 10 to 21 d. This coincides with the idea that birds would be most susceptible to opportunistic pathogens at approximately 2 wk of age. With the added incidence of mild GI inflammation, birds would be even more susceptible to these pathogens. Several disease induction models for coccidiosis and necrotic enteritis capitalize on this increased susceptibility to infection, with acute exposure to *Eimeria* or *Clostridium perfringens* generally falling between 10 and 21 d (Park et al., 2008; Lee et al., 2010; Singh et al., 2015; Barrios et al., 2017; Wilson et al., 2018). Further, early *Salmonella* Typhimurium exposure has resulted in increased susceptibility to NE infection (Shivaramaiah et al., 2011), suggestive of a disruption to the GI tract that resulted in inflammation and pathogen susceptibility. Therefore, understanding the influence of early exposure to Enterobacteriaceae on GI inflammation and vulnerability to pathogens can provide targets to improve early intestinal development and health of poultry.

In assessing the influence of early exposure to Enterobacteriaceae and its role in mild GI inflammation, measures of BWG, A1GP, and yolk sac parameters were more valuable when combined than when analyzed

separately. In addition to various measures for GI inflammation, timing also proved to be a valuable component of the assessment. By adding more time points in future experiments, trends in A1GP and yolk sac parameters can be evaluated over time to track the influence of DOH exposure to Enterobacteriaceae. As evidenced by inconsistencies with BWG and A1GP concentrations between experiments, mild GI inflammation may not produce demonstrable shifts in inflammatory measures.

Therefore, GI specific measures can be added to reveal GI specific inflammation, which may be more sensitive to mild GI inflammation, as opposed to generalized inflammation measures. Since Enterobacteriaceae exposure on DOH may disrupt GI microbial populations just enough to result in minor, nearly undetectable, GI inflammation, utilizing multiple methods, including measures of GI specific and general inflammation, as well as various time points, would strengthen understanding of mild GI inflammation. Future studies should focus on including additional measures of GI inflammation to characterize the influence of DOH exposure to Enterobacteriaceae on mild GI inflammation through 2 wk of age.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Hafiz Abdullah, Ms. Cheryl Patterson, Mr. Jordan Welsh, Ms. Reiley Murphy, and Mr. Jack Sidle for their technical assistance during these experiments. This research was partially supported by USDA Hatch Funds Project #OHO01471.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2021.101193](https://doi.org/10.1016/j.psj.2021.101193).

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