Enteric permeability and inflammation associated with day of hatch Enterobacteriaceae inoculation

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ABSTRACT Early exposure to Enterobacteriaceae may result in inappropriate microbial colonization of the gastrointestinal (**GI**) tract, induce mild GI inflammation, alter immune system development, and predispose poultry to opportunistic infection. Four experiments were conducted to test Enterobacteriaceae isolates *Escherichia coli* LG strain (**LG**), *E. coli* Huff strain (Huff), *Salmonella* Enteritidis LB (**SE**) and *Salmonella* Typhimurium (**ST**) on ability to induce GI inflammation. All 4 experiments included a noninoculated control, and day of hatch (**DOH**) oral inoculation of LG, Huff, SE and ST in experiment 1, LG and SE in experiment 2, and LG, Huff, SE, and ST in experiment 3. Experiment 4 included LG, Huff, a noninoculated control (**NIC**), and *Clostridium perfringens* only (**NCP**) wherein birds received oral *C. perfringens* challenge on d15-16 to induce necrotic enteritis. Body weight was measured, yolk sacs and spleens were collected, and blood was obtained for serum fluorescein isothiocyanate dextran (**FITC-d**) recovery and alpha-1-acid glycoprotein (**A1GP**) concentrations. Samples were taken weekly through 2 wk of age in experiments 1 and 2, or 4 wk of age in experiments 3 and 4. Increased FITC-d recovery was observed for LG and SE on d13 in experiment 2 ($P < 0.05$), and *C. perfringens* only birds on d27 in experiment 4 ($P < 0.05$) as compared to noninoculated controls. Each experiment resulted in notable differences in A1GP serum concentrations over time, with fluctuations in A1GP patterns through d14 based on DOH inoculation ($P < 0.05$). Over time, A1GP was increased for DOH inoculated birds from d 22 to 29, the fourth wk of life, and d 2-29, the entire experiment, vs. noninoculated controls in experiment 3 ($P < 0.05$). Similarly, NCP and LGCP showed increased A1GP from d 20 to 27 and d 6 to 27, vs. NIC in experiment 4 ($P < 0.05$). In experiment 4, *C. perfringens* challenge resulted in earlier A1GP response in DOH inoculated birds, d 17-20, as compared to NCP birds, d 20-27 ($P < 0.05$). These results suggest early Enterobacteriaceae exposure may influence early inflammatory state in the GI tract and may also alter patterns of inflammation and responsiveness to pathogens.

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

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INTRODUCTION

The extent and effects of gastrointestinal (**GI**) inflammation due to inappropriate microbial colonization has been an often-overlooked concern throughout the poultry industry that requires greater attention and understanding. Exposure to Enterobacteriaceae on day of hatch (**DOH**) can result in mild GI inflammation that acts as a predisposing factor for opportunistic infections with little impact on growth performance (Wilson et al., 2018; Kubasova et al., 2019; Kempf et al., 2020). *Salmonella enterica* serovars and *Escherichia coli* have been associated with GI inflammation following DOH exposure with high impact in poultry, where microbial contact comes almost exclusively from the hatchery and farm environment (Bailey et al., 2002; Nava et al., 2005; Kubasova et al., 2019). These bacteria may influence developing microbiota populations and their interactions within the GI tract, resulting in a lasting inflammation, and altered immunological development (Ballou et al., 2016; Wilson et al., 2019; Rodrigues et al., 2020).

While *Escherichia coli* are commonly found within the GI tract of chicks, paratyphoid *Salmonella spp.* are not ubiquitous residents within poultry intestinal tracts, but are considered ubiquitous in the environment and are

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found in high proportions at hatcheries (Hassan and Curtiss, 1994; Bailey et al., 2002; Foley et al., 2008; Leimbach et al., 2013; Ballou et al., 2016). Due to their capacity to produce endotoxins, many Enterobacteriaceae, including *E. coli* and *Salmonella spp.*, can be classified as opportunistic pathogens that cause inflammation upon colonization of immature GI tracts on DOH (Wigley, 2015). Studies have shown that pioneer colonizers influence mature microbial populations, as well as intestinal and immune development of hosts, 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 changes in growth, intestinal microbial populations, pathogen susceptibility, and immune function (Teague et al., 2017; Wilson et al., 2019; Rodrigues et al., 2020).

Clostridium perfringens is a Gram-positive, spore forming, aerotolerant anaerobe that is ubiquitous in the GI tract of poultry and in the environment (Lacey et al., 2016). Known for its plasmid encoded arsenal of toxins capable of destroying cells, disrupting epithelial lining, and resulting in lesions characteristic of necrotic enteritis (Titball et al., 1999; Keyburn et al., 2008; Li et al., 2013). To induce necrotic enteritis, conditions within the GI tract need to favor overgrowth of *C. perfringens*, which has been proven to require a predisposing factor such as high protein, coccidiosis, or immunosuppression (Moore, 2016). Since DOH exposure to Enterobacteriaceae has the potential to cause mild pathology and GI inflammation, it may act as a predisposing factor for *C. perfringens* proliferation, which has been documented with DOH *S.* Typhimurium inoculation (Shivaramaiah et al., 2011).

Alpha-1-acid glycoprotein (**A1GP**) 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). With regard to its role in the inflammatory response, A1GP can be induced by stress, burns, infection, and other chronic inflammatory conditions, which results in uncertainty surrounding the trigger of elevated A1GP levels when used as a marker for GI inflammation (Fournier et al., 2000). Several studies in poultry have evaluated changes in A1GP associated with various bacterial diseases and inflammatory conditions, with A1GP levels consistently peaking 24 to 48 h postinjection or inoculation, with normal serum concentrations generally in the range of 150 to 400 μg/mL (Takahashi et al., 1994; Inoue et al., 1997; Adler et al., 2001; Buyse et al., 2007; O'Reilly et al., 2018). Although A1GP serum concentration is not exclusively related to GI inflammation, it can be used as a descriptive marker for inflammation associated with disruption of the GI tract, especially in research conditions with otherwise healthy animals.

Fluorescein isothiocyanate dextran (**FITC-d**) has been used as a maker of intestinal specific inflammation and damage (Duff et al., 2019). Serum FITC-d has been used extensively as a marker of mucosal barrier dysfunction to indicate GI-specific inflammation (Kuttappan et al., 2015; Vicuña et al., 2015; Gilani et al., 2016; Duff et al., 2019). Since FITC-d is specific to the intestine, it was used in the studies presented here to determine whether DOH Enterobacteriaceae exposure resulted in GI inflammation and altered intestinal permeability.

Infection of the yolk sac (**YS**), or omphalitis, has been associated with poor incubation conditions, hatchery or egg shell contamination, and improper closure of the navel leading to infection, increased rates of YS retention, early mortality, and depressed BW (Brandly, 1932; Reid et al., 1961; Gross, 1964). Day of hatch exposure to Enterobacteriaceae can also act as a source of YS infection and increase retention in birds, while serving as a potential pathogen reservoir in broilers (Cox et al., 2006). Since eggshell and hatchery contamination are a major source of pioneer colonizers, studying the influence of DOH oral exposure to pathogens on YS retention, and colonization or infection or the YS can provide insight into early microbial influence.

Gastrointestinal inflammation has the potential to disrupt various developmental and immunological processes within birds, leading to immeasurable growth performance losses and disease of poultry flocks. By developing an industry relevant, reliable model of GI inflammation, long-term effects of DOH exposure to Enterobacteriaceae can be studied. Therefore, 3 experiments were conducted to assess *E. coli* and *Salmonella* strains for their ability to induce mild GI inflammation and growth performance changes as measured by BWG, A1GP, FITC-d, YS retention, YS weight, and bacterial enumeration of YS. A fourth experiment assessed whether d1 inoculation with *E. coli* strains resulted mild GI inflammation and increased susceptibility to clostridial infection and necrotic enteritis by measuring BWG, A1GP, FITC-d, YS retention, YS weight and bacterial enumeration of YS, and necrotic enteritis lesions. Across all 4 experiments, BWG suppression, as well as increased A1GP, FITC-d, and YS metrics were hypothesized for chicks inoculated on DOH or d1 with Enterobacteriaceae, in addition to increased susceptibility to necrotic enteritis infection in experiment 4.

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 from The Ohio State University Institutional Animal Care and Use Committee (#2016A00000038, #2018A00000074, #2019A00000012). In all experiments, DOH Ross 708 broiler chicks were obtained from a local hatchery, neck tagged, and randomly placed in wire floor cages (experiment 1 and 2), or in floor pens with fresh pine shavings (experiments 3 and 4). Nutritionally complete corn-soy based feed and water were provided ad libitum, and ambient temperature and lighting were maintained at age-appropriate levels. At the end of each

experiment, birds were killed by CO_2 inhalation for sample collection, as described below.

Bacterial Preparation

E. coli LG was isolated from the retained YS of a chick with omphalitis and kindly shared by Dr. Billy Hargis from the University of Arkansas, while *E. coli* Huff strain has been previously utilized to induce colibacillosis in chickens (Huff et al., 1984, 2005). *Salmonella* Enteritidis LB (SE) was isolated from the GI tract of an apparently healthy adult chicken and identified by MALDI-TOF and PCR at the Ohio Agriculture Diagnostic Laboratory. *Salmonella* Typhimurium strain utilized was previously used as a predisposing factor for necrotic enteritis (Shivaramaiah et al., 2011). For each experiment, a frozen aliquot of *E. coli* LG, *E. coli* Huff, *S.* Enteritidis LB, and *S.* Typhimurium 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 overnight. *Salmonella* cultures were passaged every 8 h at 0.1% volume (Bielke et al., 2003). Cells were washed 3 times in 0.9% sterile saline by centrifugation at 1,800× *g* for 15 min. Cultures administered via oral gavage were spectrophotometrically quantified (Spectronic 2000, Thermo Scientific, Waltham, MA) and inoculum CFU were retrospectively confirmed by serial dilution plating on tryptic soy agar (**TSA**) as reported below.

In-Feed Administration of Clostridium perfringens CP641

Treatment inoculum for experiment 4 was prepared by 0.5% volume inoculation of a thawed aliquot of *C. perfringens* CP641 into tryptic soy broth with 0.5% sodium thioglycolate (thioglycolate acid sodium salt, Amresco. Solon, OH), and incubated in anaerobic jars at 37°C for up to 24 h (AnaerobicPack-Anaero, Mitsubishi, Japan; AnaeroPack Rectangular Jars, Mitsubishi, Japan), and fresh cultures were prepared each day. Five hundred milliliters of overnight *C. perfringens* CP641 culture was mixed with feed at 1:1 (w:v), then placed on a flat feed tray in each treatment pen immediately following a 6 h feed removal. Cultures of CP CP641 were spectrophotometrically quantified, retrospectively confirmed by serial dilution plating on TSA with sodium thioglycolate and were reported in Table 1.

Serum A1GP Analysis

Blood was collected from the femoral vein after euthanasia, allowed to clot at room temperature for approximately 3 h, centrifuged at 2000 × g for 15 min for serum separation and collection, then 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).

Serum FITC-d Recovery

Fluorescein isothiocyanate dextran (MW 3-5 kDa; Sigma Aldrich Co., St. Louis, MO) was used as a marker of increased paracellular transport and mucosal barrier dysfunction. Serum levels of FITC-d were measured similarly to Kuttappan et al. (2015) and Vicuña et al. (2015). Oral administration of FITC-d was standardized based on bird weight to approximate 4.17 mg/kg at time of dosing. At 2h following FITC-d administration, birds were euthanized, and blood was

Table 1. Day of hatch Enterobacteriaceae inoculation and *C. perfringens* challenge doses for experiments 1 through 4.

Experiment	Treatment	DOH Enterobacteriaceae CFU/chick[1]	d15 *C. perfringens* CP641 1:1 (w:v) in feed[2]	d16 *C. perfringens* CP641 1:1 (w:v) in feed[2]
1	Noninoculated control	None	None	None
	E. coli LG	3.00×10^2	None	None
	E. coli Huff	6.50×10^2	None	None
	Salmonella Enteritidis LB	7.00×10^2	None	None
	Salmonella Typhimurium	8.50×10^3	None	None
2	Noninoculated control	None	None	None
	E. coli LG	7.00×10^2	None	None
	Salmonella Enteritidis LB	6.50×10^2	None	None
3	Noninoculated control	None	None	None
	E. coli LG	4.33×10^2	None	None
	E. coli Huff	3.33×10^2	None	None
	Salmonella Enteritidis LB	7.67×10^2	None	None
	Salmonella Typhimurium	2.25×10^2	None	None
4	Noninoculated control	None	None	None
	C. perfringens only	None	3.05×10^6 CFU/mL	1.10×10^7 CFU/mL
	E. coli LG + *C. perfringens*	1.56×10^2	3.05×10^6 CFU/mL	1.10×10^7 CFU/mL
	E. coli Huff + *C. perfringens*	1.13×10^2	3.05×10^6 CFU/mL	1.10×10^7 CFU/mL

[1]Day of hatch Enterobacteriaceae administered by oral gavage at 0.25 mL/chick.

[2]500 mL of *C. perfringens* CP641 suspension was combined with 500 g of feed, and mixed for administration to birds, reported as CFU/mL.On day of hatch, or d 1 in experiment 4, chicks either received no inoculation, or were orally administered *E. coli* LG, *E. coli* Huff, *Salmonella* Enteritidis LB, or *Salmonella* Typhimurium. On d15 and 16 in experiment 1, all birds experienced a 6h feed withdrawal. Non-inoculated non-challenged control was provided access to regular feed immediately following feed withdrawal period. For *C. perfringens* challenged treatments, feed was mixed with *C. perfringens* CP641 at 1:1 (w:v) before being placed on a large tray and provided to birds for consumption.

collected from the femoral vein to quantify levels of FITC-d. Blood samples were left to clot at room temperature for approximately 3 h and centrifuged at 2000 × g for 15 min for serum separation and collection. Serum samples were diluted in phosphate buffered saline (1:4) and fluorescence was measured at 485 nm excitation and 528 nm emission (Synergy HTX, multimode microplate reader, BioTek Instruments Inc., Winooski, VT). Concentration of FITC-d in samples was determined based on a standard curve of known concentrations.

Bacterial Translocation and Recovery

To measure translocation of enteric bacteria into circulation and shifts in recoverable populations, spleens (experiment 2 only), and whole YS including stalks were collected aseptically into sterile bags, homogenized, and diluted 1:4 (w:v) with sterile 0.9% saline. Ten-fold serial dilutions were made in sterile 96-well plates and samples were plated on TSA (all experiments) and on MacConkey agar (experiments 3 and 4; Becton, Dickinson and Co., Difco, Sparks, MD), for total aerobic translocation or total Enterobacteriaceae translocation, respectively. All plates were incubated at 37°C for 24 h to determine bacterial shifts and recovery reported as Log_{10} CFU/g of tissue.

Experiment 1

A total of 180 DOH broiler cockerels were randomly placed into one of 5 treatment groups, noninoculated control (**NC**), SE, LG, Huff, and ST, with 12 birds per pen and 3 replicate pens for a total of 36 birds per treatment. On DOH, birds received 0.25 mL of 0.9% sterile saline for NC, or oral inoculation of 0.25 mL 3.00×10^2 CFU/chick LG, 6.50×10^2 CFU/chick Huff, 7.00×10^2 CFU/chick *S.* Enteritidis LB, or 8.50×10^3 CFU/chick ST, based on respective treatment, as described in Table 1. Oral inoculation doses of *S.* Enteritidis LB, *E. coli* LG, and *E. coli* Huff were based on previously established inoculation titrations of Enterobacteriaceae (Chasser et al., 2021) while oral inoculation dose for *S.* Typhimurium was determined based on a previous study by Shivaramaiah et al. (2011). Individual BW was measured on d0, 2, 8, and 15. Except for d 0, a total of 4 birds per pen were euthanized for blood collection to perform serum A1GP analysis. Remaining birds were necropsied for retained YS, which were aseptically collected for bacterial enumeration.

Experiment 2

A total of 540 DOH broiler cockerels were randomly placed into one of 3 treatment groups, NC, LG, or SE, with 30 birds per pen and 6 replicate pens for a total of 180 birds per treatment. On DOH, birds received 0.25 mL of 0.9% sterile saline, NC, or oral inoculation of 7.00×10^2 CFU/chick *E. coli* LG or 6.50×10^2 CFU/chick *S.* Enteritidis LB, based on respective treatment as described in Table 1. Body weights were measured on d0, 2, 6, and 13, plus 4 birds per pen in NC and 5 birds per pen in SE and LG were orally administered FITC-d. Birds were euthanized 2h later for blood collection to perform serum FITC-d recovery and A1GP analysis, and spleens were aseptically collected to quantify bacterial translocation and recovery. Up to 5 additional birds per pen on d 6, and 10 additional birds per pen on d 13 were euthanized monitored for any retained YS, which were aseptically collected until a minimum of 10 YS per treatment were collected for bacterial enumeration.

Experiment 3

A total of 600 DOH broilers were randomly placed into one of 5 treatment groups, NC, SE, LG, Huff, and ST, with 40 birds per pen and 3 replicate pens for a total of 120 birds per treatment. On DOH, birds received 0.25 mL of 0.9% sterile saline for NC, or oral inoculation of 4.33×10^2 CFU/chick *E. coli* LG, 3.33×10^2 CFU/chick *E. coli* Huff, 7.67×10^2 CFU/chick *S.* Enteritidis LB, or 2.25×10^2 CFU/chick *S.* Typhimurium, based on respective treatment as described in Table 1. Body weights were measured on d 0, 2, 8, 15, 22, and 29. Oral administration of FITC-d to 3 birds per pen in NC and 4 birds per pen in SE, LG, Huff, and ST occurred on d 2, and 4 or 5 birds, respectively, on d 8, 15, 22, and 29. Two h following FITC-d administration, the birds were killed for blood collection to perform serum FITC-d recovery and A1GP analysis. Up to 5 additional birds per pen on d 8, and 10 additional birds per pen on d 15 were euthanized monitored for any retained YS, which were aseptically collected until a minimum of 10 YS per treatment were accumulated for YS weight and bacterial enumeration.

Experiment 4

A total of 480 DOH broiler cockerels were randomly placed into one of 4 treatments, noninoculated nonchallenged control (NIC), *C. perfringens* CP641 challenged control (NCP), LG CP CP641 (LGCP), and Huff CP CP641 (HCP), with 40 birds per pen and 3 replicate pens for a total of 120 birds per treatment. On d1, birds received 0.25 mL of 0.9% sterile saline, NIC and NCP, or oral inoculation of 0.25mL 1.56×10^2 CFU/chick *E. coli* LG for LGCP, or 1.13×10^2 CFU/chick *E. coli* Huff for HCP. On d 15 and 16, *C. perfringens* CP641 was administered in the feed at 1:1 (w:v) to NCP, LGCP, and HCP, as described in Table 1. Feed was removed from all pens 6 h prior to administration to ensure consumption of inoculated feed. Body weights were measured on d 1, 6, 14, 17, 20 and 27. Three birds per pen were orally inoculated with FITC-d on d 6, 14, 17, 20, and 27, and blood collected from 4 birds per pen 2 h following administration to perform serum FITC-d recovery and A1GP analysis. Up to an additional 5 or 10 birds per pen on d 6 or d 14, respectively, were euthanized and any retained YS were aseptically collected to obtain a minimum of 10 YS per treatment for weight and bacterial enumeration.

On d 17, in addition to blood collection, the 4 sampled birds were also evaluated for necrotic enteritis lesions based on the 0 to 4 scale described by Prescott and coauthors (1978).

Statistical Analysis

Birds were considered the experimental unit in all experiments, and time by treatment interactions were completed on BW, BWG, A1GP, FITC-d, and bacterial enumeration, included pen as random effect, with identified interactions using test slices option for means separation. When interactions were not identified, BW, BWG, FITC-d, and bacterial enumeration were subject to Analysis of Variance as a completely randomized design (JMP Software, SAS Inc., 2016), with data expressed as mean ± standard error. Retained YS percentages were analyzed using Chi-squared analysis. Significant differences among the means were determined using Student's t test in experiment 2 and Tukey's Honestly Significant Difference test in experiments 1, 3, and 4, at $P < 0.05$.

RESULTS

Experiment 1

While BWG showed no differences from NC from d 0 to 2, d 2 to 8, or d 8 to 15, BWG was decreased in LG compared to NC from d 0 to 15, at 261.81 ± 20.86 vs. 311.97 ± 23.97, respectively (Figure 1, $P = 0.031$). Yolk sac retention decreased steadily over time in NC, from 100% on d 2, to 64% on d 8, then to 33% on d 15, with about a 99% reduction in CFU/g of aerobic bacterial recovery in the YS, from 5.06 ± 0.50 Log_{10} CFU/g on d 2 to 2.92 ± 0.15 Log_{10} CFU/g on d8 in NC (Figure 1). Steady rates of YS retention between d 8 and d 15 were observed in all inoculated groups except LG where retention was 18%, though no groups was different from NC (Table 2, $P > 0.05$). On d 8, yolk sac aerobic bacterial recovery of DOH inoculated birds were approximately 3-6 Log_{10} CFU/g higher than that of NC, with SE and LG resulting in 9.02 ± 0.73 Log_{10} CFU/g, and 8.66 ± 0.10 Log_{10} CFU/g, respectively, compared to NC at 2.92 ± 0.15 Log_{10} CFU/g (Figure 1, $P = 0.044$ and $P = 0.060$, respectively). Due to low sample numbers of YS in NC and LG on d 15, bacterial enumeration could not be statistically analyzed for comparisons of

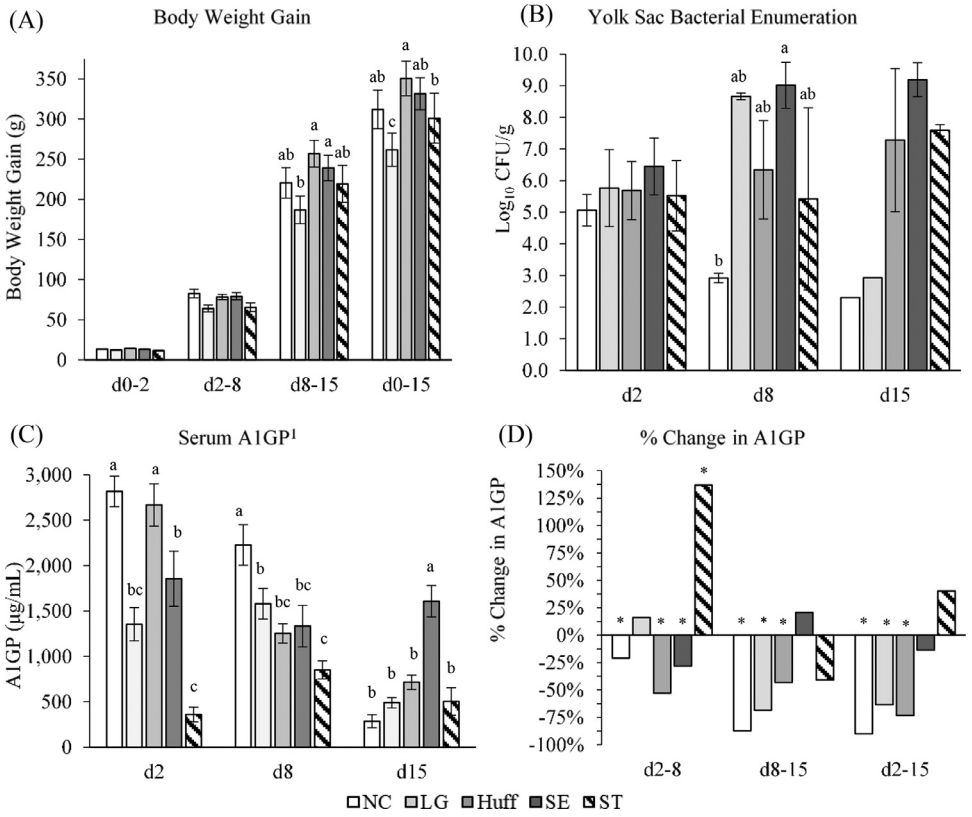

Figure 1. Body weight gain, yolk sac aerobic bacterial enumeration, serum concentration of alpha-1-acid glycoprotein, and time by treatment interaction on change in serum concentration of alpha-1-acid glycoprotein for experiment 1. Chicks either received no inoculation on DOH or were inoculated with *E. coli* LG at 3.00×10^2 CFU/chick, *E. coli* Huff at 6.50×10^2 CFU/chick, *Salmonella* Enteritidis LB at 7.00×10^2 CFU/chick or *Salmonella* Typhimurium at 2.13×10^2 CFU/chick. Body weight was measured on d0, 2, 8, and 15, and was used to calculate body weight gain (A). All retained yolk sacs were aseptically collected for total aerobic bacterial enumeration on tryptic soy agar (B). Blood was collected from all chicks on d2, 8, and 15 to measure serum concentrations of alpha-1-acid glycoprotein (A1GP, µg/mL, C). Time by treatment interactions ($P < 0.001$) was analyzed for changes in serum A1GP over time (D). All data represented as mean ± standard error, except A1GP time by treatment interaction, represented as percent change in average serum A1GP concentration for specified time interval within treatment. [a,b,c]Mean values with different superscript letters within a column and within a block indicate a significant difference ($P < 0.05$). [1]±2 standard deviations used for outlier removal *Indicates percent change of alpha-1-acid glycoprotein over time as significant across the specified time interval ($P < 0.05$).

Table 2. Yolk sac retention for experiments 1 and 2.

Experiment 1	d 2	d 8	d 15
Noninoculated Control	100% (12/12)[1]	64% (9/14)	33% (3/9)[ab]
E. coli LG	100% (12/12)	50% (6/12)	18% (2/11)[b]
E. coli Huff	92% (11/12)	50% (6/12)	42% (5/12)[ab]
Salmonella Enteritidis LB	100% (12/12)	50% (6/12)	50% (5/10)[ab]
Salmonella Typhimurium	100% (12/12)	42% (5/12)	58% (7/12)[a]
SEM	2%	4%	7%
P-value	0.232	0.247	0.044
Experiment 2	d2	d6	d13
Noninoculated Control	100% (60/60)	35% (20/57)[b]	31% (18/58)
E. coli LG	100% (60/60)	54% (31/57)[a]	36% (21/58)
Salmonella Enteritidis LB	100% (60/60)	30% (18/61)[b]	45% (26/58)
SEM	0%	8%	4%
P-value	1.000	0.007	0.125

[a,b]Mean values with different superscript letters within a column and within a block indicate a significant difference ($P < 0.05$).

[1]Yolk sac retention percentage analyzed using Chi-squared analysis.In experiment 1, chicks either received no inoculation on DOH or were inoculated with *E. coli* LG at 3.00×10^2 CFU/chick, *E. coli* Huff at 6.50×10^2 CFU/chick, *Salmonella* Enteritidis LB at 7.00×10^2 CFU/chick or *Salmonella* Typhimurium at 2.13×10^2 CFU/chick. In experiment 2, chicks either received no inoculation on DOH or were inoculated with *E. coli* LG at 7.00×10^2 CFU/chick, or *Salmonella* Enteritidis LB at 6.50×10^2 CFU/chick. All retained yolk sacs were aseptically collected to determine yolk sac retention percentage.

inoculated groups to NC. Serum A1GP displayed extreme elevation in NC on both d 2 and d 8, 2,817.24 ± 167.61 μg/mL and 2,226.41 ± 223.39 μg/mL, respectively, but returned to normal levels by d 15, 285.77 ± 71.59 μg/mL (Figure 1). While only SE presented increased A1GP on d 15 compared to NC, at 1,607.63 ± 171.80 μg/mL compared to 285.77 ± 71.59 μg/mL, respectively, all inoculated treatments did maintain numerically increased A1GP serum concentrations (Figure 1, $P < 0.001$). When evaluated by treatment over time, A1GP serum concentrations of ST increased by 137% from d 2 to 8 while NC, SE, and Huff decreased by 21%, 28%, and 53%, respectively during the same time period (Figure 1, $P = 0.043$, $P = 0.011$, $P = 0.035$, $P < 0.001$, respectively). Only ST increased significantly, while NC, SE, and Huff decreased, and LG showed inconsequential change.

Experiment 2

In experiment 2, no differences in BWG were observed in d6 and d 13, and only a 2.5 g statistically significant difference between NC and SE was measured on d 2 (Table S1). Yolk sac retention decreased from 100% in all treatments on d 2 to 54% in LG, 30% in SE, and 35% in NC on d6 (Table 2, $P < 0.05$). No differences were observed for YS retention on d 13. Bacterial enumeration of the YS had little variation throughout the experiment, with no treatment differences observed at any time point, but there was an observational increase in bacterial recovery for all groups from d 2 to d 13. Aerobic bacterial enumeration of the spleen on d 13 showed that NC was elevated compared to SE and LG at 3.23 ± 0.25 Log_{10} CFU/g vs. 2.07 ± 0.23 Log_{10} CFU/g and 2.35 ± 0.24 Log_{10} CFU/g, respectively (Figure 2, $P = 0.001$ and 0.011, respectively). On d 2, serum A1GP was only reduced numerically for SE vs. NC, at 475.23 ± 70.53 μg/mL vs. 746.64 ± 52.57 μg/mL, respectively, whereas on d 6 serum A1GP was elevated for SE vs. NC, at 2,769.62 ± 175.00 μg/mL vs. 1,049.58 ± 283.29 μg/mL, respectively (Figure 2, $P = 0.471$, $P < 0.001$, respectively). By d 13, no differences were observed in serum A1GP concentration between treatments. However, when analyzed over time, both SE and LG had a statistically significant increase of serum A1GP between d 2 and d 6 with changes of 483% and 89%, respectively, whereas the increase for NC was not significant at 41% (Figure 2, $P < 0.001$, $P = 0.009$, and $P = 0.201$, respectively). Over the course of the experiment, d 2 through d 13, A1GP increased for all treatments, with SE representing the greatest change of 89%, at $P = 0.035$, whereas NC increased by 36% and LG increased by 50% (Figure 2, $P = 0.253$, $P = 0.092$, respectively). Serum FITC-d recovery was elevated in both SE and LG, compared to NC, on d 13, with serum concentrations of 358.06 ± 7.29 ng/mL and 364.90 ± 9.42 ng/mL, vs. 328.32 ± 11.88 ng/mL, respectively (Figure 2, $P = 0.033$, $P = 0.009$, respectively).

Experiment 3

In experiment 3, there were generally no differences, except d 2-8 in which all inoculated treatments had numerically higher BWG than NC, but only ST was different at 112.64 ± 2.33 g vs. NC at 102.31 ± 2.33 g (Figure 3, $P = 0.019$). No differences were observed for YS retention, weight, or bacterial enumeration on both TSA and MacConkey agar (Table S2). Throughout the experiment A1GP was exceptionally high in all treatments compared to normal serum levels in chickens (Takahashi et al., 1994; O'Reilly et al., 2018), with differences observed between treatments and NC at every time point, except d29 (Figure 3, $P < 0.05$). On d 29, ST approached a significant increase in serum A1GP compared to NC, at 2,670.34 ± 105.39 μg/mL vs. 2,194.77 ± 196.70 μg/mL, respectively (Figure 3, $P = 0.069$). Interestingly, when treatment was evaluated over time, A1GP serum concentrations of ST increased by 93% from d 2 to 8 while NC decreased by 39% during the same period (Figure 3, $P < 0.001$, $P = 0.001$). Only ST increased significantly, while NC, and LG decreased, and SE experienced insignificant change. The decrease for Huff during this period was 33% (Figure 3, $P = 0.072$). There was also a distinct increase in serum A1GP for LG, Huff, SE, and ST for d 22-29, with values of 139%, 52%, 108%, and 32%, respectively, compared to NC at 0% (Figure 3, $P < 0.001$, $P = 0.001$, $P < 0.001$, $P = 0.008$, and $P = 1.000$, respectively). Across the entire experiment, d 2 through d 29, A1GP only increased significantly for all DOH inoculated treatments, LG, Huff, SE, and ST, with values of 65%, 84%, 67%, and 192% (Figure 3, $P < 0.001$). In this

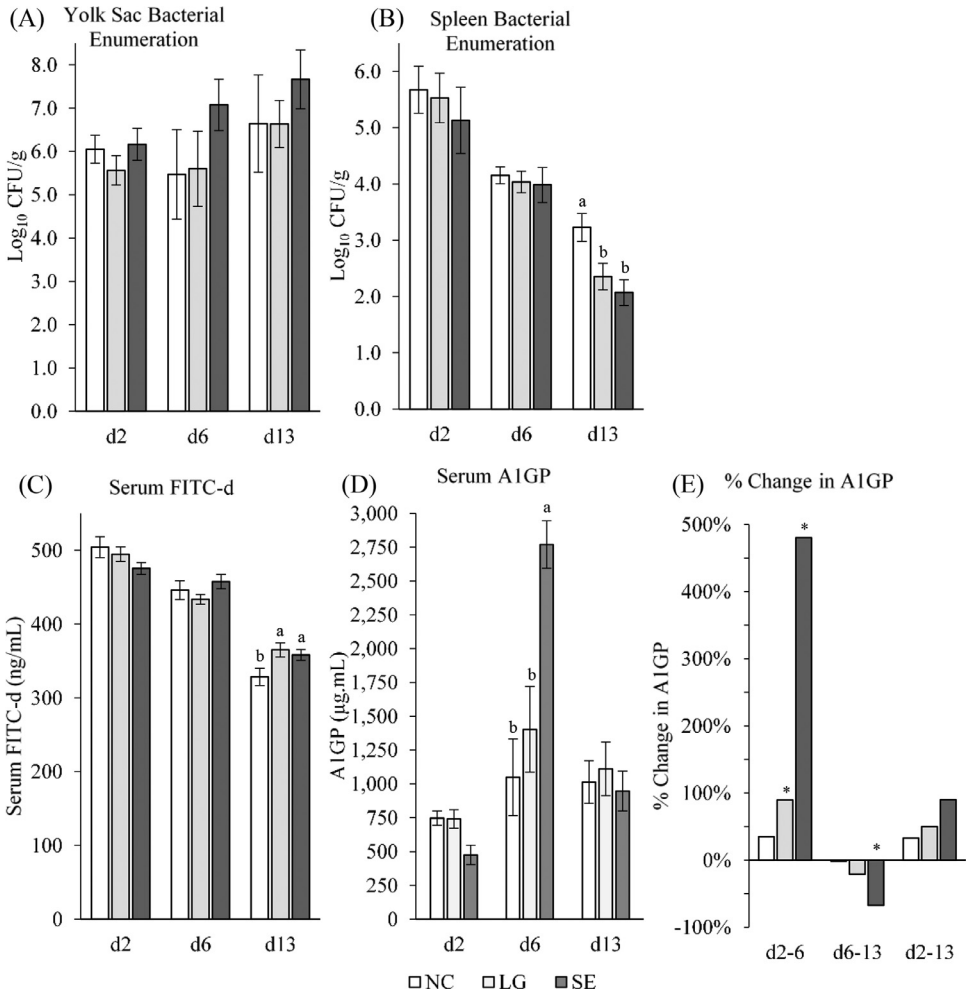

Figure 2. Body weight gain, yolk sac aerobic bacterial enumeration, spleen aerobic bacterial enumeration, serum recovery of fluorescein isothiocyanate dextran, serum concentration of alpha-1-acid glycoprotein, and time by treatment interaction on change in serum concentration of alpha-1-acid glycoprotein for experiment 2. Chicks either received no inoculation on DOH or were inoculated with *E. coli* LG at 7.00×10^2 CFU/chick, or *Salmonella* Enteritidis LB at 6.50×10^2 CFU/chick. All retained yolk sacs (A) and spleens (B) were aseptically collected for total aerobic bacterial enumeration on tryptic soy agar. Blood was collected from all chicks on d2, 6, and 13 to measure serum recovery of fluorescein isothiocyanate dextran (FITC-d, ng/mL, C) and serum concentrations of alpha-1-acid glycoprotein (A1GP, μg/mL, D). Time by treatment interactions ($P < 0.001$) was analyzed for changes in serum A1GP over time (E). All data represented as mean ± standard error, except A1GP time by treatment interaction, represented as percent change in average serum A1GP concentration for specified time interval within treatment. [a,b]Mean values with different superscript letters indicates a significant difference ($P < 0.05$). *Indicates percent change of alpha-1-acid glycoprotein over time as significant across the specified time interval ($P < 0.05$).

experiment, no statistical differences in FITC-d recovery were measured. However, from d 22 to d 29, LG, Huff, SE, and ST were all numerically greater in FITC-d recovery, with Huff having the greatest difference compared to NC, at 516.54 ± 30.48 ng/mL vs. 425.49 ± 31.01 ng/mL, respectively (Figure 3, $P = 0.097$).

Experiment 4

In experiment 4, no differences were observed in BWG, YS retention, YS weight, bacterial enumeration of the YS on TSA or MacConkey agar, or LS (Table S3). While classical necrotic enteritis lesions were not observed, subclinical nonhemorrhagic clostridiosis was noted while scoring in all *C. perfringens* inoculated groups. This has been classified as a discoloration of amorphous material that adheres to the mucosal surface (Goossens et al., 2020). In HCP, serum A1GP was decreased on d 17 at 440.78 ± 47.22 μg/mL, compared to NCP 1,036.88 ± 87.24 μg/mL and approached significance compared to NIC at 838.19 ± 61.89 μg/mL, respectively (Figure 4, $P = 0.062$ and $P = 0.008$, respectively). On d 20, LGCP serum A1GP was increased relative to NCP, but not to NIC, with values of 1,435.77 ± 151.57 μg/mL for LGCP, 897.64 ± 88.24 μg/mL for NCP, and 1,065.85 ± 62.05 μg/mL for NIC (Figure 4, $P = 0.015$, $P = 0.081$, respectively). Serum A1GP was elevated again for LGCP on d 27, at 1,647.97 ± 216.75 μg/mL, compared to NIC, at 613 ± 52.83 μg/mL (Figure 4, $P < 0.001$). After inoculation with *C. perfringens*, NCP serum A1GP on d27 was increased to 1,383.85 ± 127.28 μg/mL, which was significantly higher than NIC and statistically similar to LGCP and HCP (Figure 4). Time by treatment interaction showed a surge in A1GP for LGCP and HCP between d 17 and d 20 that was not paralleled by NIC and NCP.

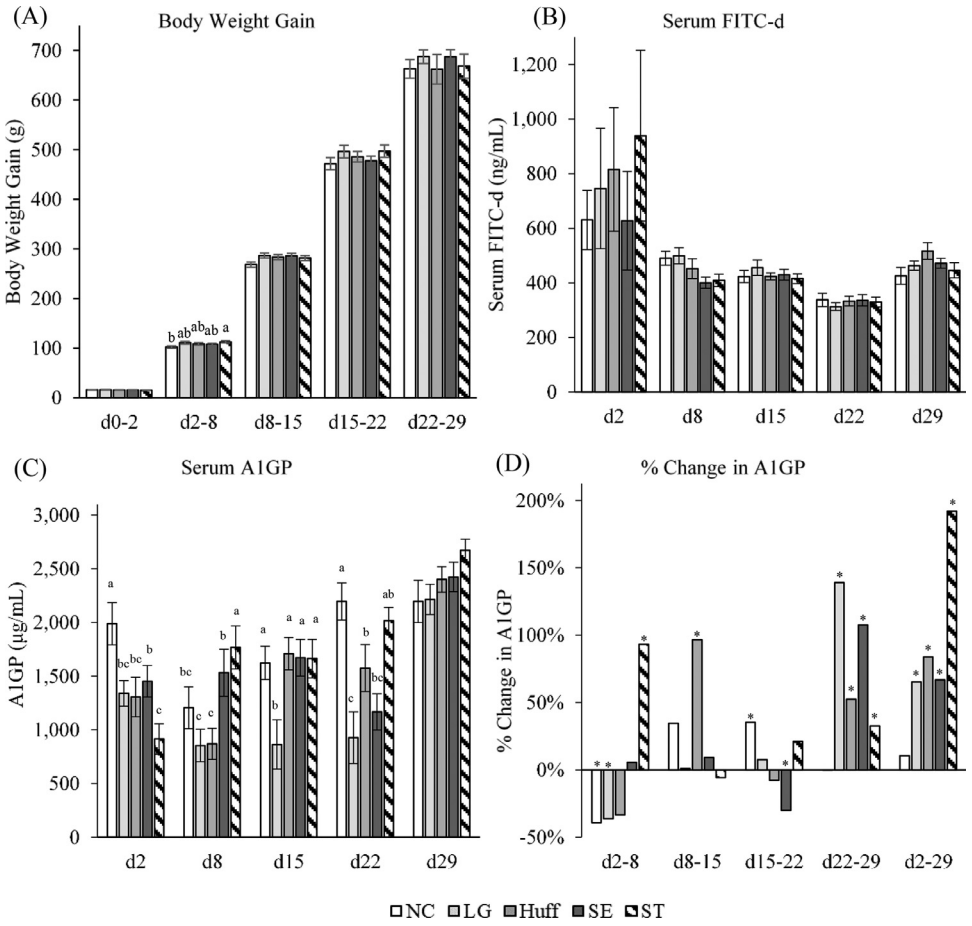

Figure 3. Body weight gain, serum recovery of fluorescein isothiocyanate dextran, serum concentration of alpha-1-acid glycoprotein, and time by treatment interaction on change in serum concentration of alpha-1-acid glycoprotein for experiment 3. Chicks either received no inoculation on DOH or were inoculated with *E. coli* LG at 4.33×10^2 CFU/chick, *E. coli* Huff at 3.33×10^2 CFU/chick, *Salmonella* Enteritidis LB at 7.67×10^2 CFU/chick or *Salmonella* Typhimurium at 2.25×10^2 CFU/chick. Body weight was measured on d0, 2, 8, 15, 22, and 29 and was used to calculate body weight gain (A). Blood was collected from all chicks on d2, 8, 15, 22, and 29 to measure serum recovery of fluorescein isothiocyanate dextran (FITC-d, ng/mL, (B) and serum concentrations of alpha-1-acid glycoprotein (A1GP, μg/mL, (C). Time by treatment interactions ($P < 0.001$) were analyzed for changes in serum A1GP over time (D). All data represented as mean ± standard error, except A1GP time by treatment interaction, represented as percent change in average serum A1GP concentration for specified time interval within treatment. [a,b]Mean values with different superscript letters indicates a significant difference ($P < 0.05$). *Indicates percent change of alpha-1-acid glycoprotein over time as significantly different from previous collection ($P < 0.05$).

Both LGCP and HCP sharply increased A1GP by 107% and 139%, compared to 27% in NIC and a reduction of 14% in NCP (Figure 4, $P < 0.001$, $P < 0.001$, $P = 0.361$, $P = 0.118$, respectively). Between d 6 and d 27, A1GP for both NCP and LGCP increased significantly by 180% and 127%, while HCP increased by 36% and NIC decreased by 19% during the time interval (Figure 4, $P < 0.001$, $P < 0.001$, $P = 0.078$, $P = 0.334$, respectively). Serum FITC-d recovery was greater in NCP, LGCP and HCP compared to NIC on d6 at, 114.25 ± 1.47 ng/mL, 114.48 ± 1.47 ng/mL, and 114.45 ± 2.34 ng/mL, compared to 107.51 ± 1.47 ng/mL, respectively (Figure 4, $P = 0.016$, $P = 0.014$, and $P = 0.013$, respectively). The following week, serum FITC-d recovery was reduced in NIC and HCP compared to NCP on d 14, with 125.21 ± 1.99 ng/mL and 124.12 ± 1.34 ng/mL vs. 133.29 ± 2.14 ng/mL (Figure 4, $P = 0.004$ and $P = 0.002$, respectively), which paralleled A1GP concentrations observed on d 14. Following *C. perfringens* challenge there were no differences in serum FITC-d recovery on d17, d20, and d27 (Figure 4).

DISCUSSION

These experiments were performed to elucidate effective methods to induce and measure mild GI inflammation by exposing broilers to Enterobacteriaceae on DOH and whether YS parameters, FITC-d, and A1GP serve as reliable measures for microbially-induced changes. Pioneer colonizing bacteria have the capacity to influence not only early microbial populations, but also early immune development and growth, with potential for long lasting impact (Wilson et al., 2019; Rodrigues et al., 2020). Extensive research has been afforded to the benefits of probiotic supplementation to improve poultry health, yet pioneer colonizing bacteria, such as Enterobacteriaceae, may exert influence on microbial populations, growth, immune development, and disease susceptibility, and can begin to be understood in the context of probiotic effects (Jin et al., 1997; Nava et al., 2005; Smith, 2014). Probiotic microbes exploit a wide variety of mechanisms to benefit their survival while simultaneously benefiting poultry and, research has

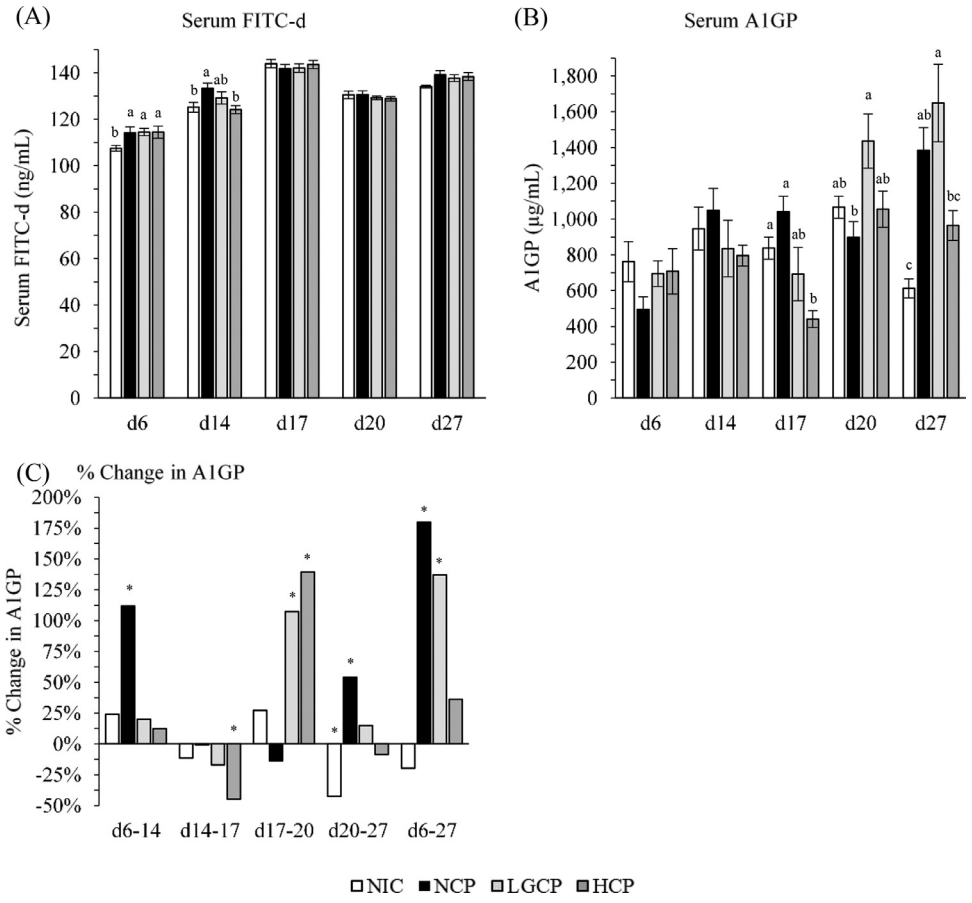

Figure 4. Serum recovery of fluorescein isothiocyanate dextran, serum concentration of alpha-1-acid glycoprotein, and time by treatment interaction on change in serum concentration of alpha-1-acid glycoprotein, experiment 4. Chicks either received no inoculation on DOH or were inoculated with *E. coli* LG at 1.56×10^2 CFU/chick, or *E. coli* Huff at 1.13×10^2 CFU/chick. On d15 and 16, *C. perfringens* CP641 was provided to birds in all *C. perfringens* challenge treatments at 1:1 (w:v) in the feed. Blood was collected from all chicks on d6, 14, 17, 20, and 27 to measure serum recovery of fluorescein isothiocyanate dextran (FITC-d, ng/mL, (A) and serum concentrations of alpha-1-acid glycoprotein (A1GP, μg/mL, (B). Time by treatment interactions ($P < 0.001$) was analyzed for changes in serum A1GP over time (C). All data represented as mean ± standard error, except A1GP time by treatment interaction, represented as percent change in average serum A1GP concentration for specified time interval within treatment. [a,b,c]Mean values with different superscript letters indicates a significant difference ($P < 0.05$). *Indicates percent change of alpha-1-acid glycoprotein over time as significantly different from previous collection ($P < 0.05$).

generally focused on the positive role probiotics play in both humans and agriculture, with numerous studies on probiotics and their positive effects (Nava et al., 2005; Crane et al., 2020; Zommiti et al., 2020). Additionally, early microbial colonizers play an important role contributing to the early microbial development and health of the GI tract, as well as the overall bird health. While research has centered around how probiotics influence intestinal health, recent focus has broadened to include their role in immune development and response. Conversely, Enterobacteriaceae and other bacteria of the gut not classified as probiotics can also affect microbiota, inflammation, and GI development. By studying the influence of Enterobacteriaceae on these processes, an improved understanding and appreciation of the role these pioneer colonizers have will enable optimization of poultry health.

In addition to the ways in which probiotics 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; Rodrigues et al., 2020). Proteomic analysis of in ovo inoculated chicks from our lab has identified the capacity of a lactic acid based probiotic to upregulate and enhance the cellular response to inflammation, whereas chicks that received in ovo inoculation of Enterobacteriaceae did not promote these differentially regulated systemic immune response processes (Rodrigues et al., 2020). While the 2 Enterobacteriaceae used were both *Citrobacter sp.*, the proteomic analysis predicted increased inflammation alongside suppression of the ability to respond to inflammation (Rodrigues et al., 2020; Wilson et al., 2020). This uncontrolled inflammation can result in a nonbeneficial intestinal environment, suppression of the immune system, and in turn, increased risk of opportunistic infection (Meirow and Baniyash, 2017). Further, several studies have indicated early microbial exposure, including Enterobacteriaceae, can shift microbial populations in the GI tract of poultry, which was cited as a potential cause for change in protein expression regarding immune response (Ballou et al., 2016; Wilson et al., 2019; Rodrigues et al., 2020). Here, elevated A1GP was observed in the week after *Salmonella* infection (Figures 1, 2, and 3). *Salmonella enterica* are well documented to take advantage of inflammation pathways, and these results demonstrate

the importance of parent flock and hatchery contamination of poultry (Kaiser et al., 2000; Setta et al., 2012). Though it was not further investigated here, the Wilson et al. (2019) and Rodrigues et al. (2020) reports suggest that this early inflammation can lead to downstream effects within the immune system, especially an ability to respond to inflammation, noted by pathway analysis that revealed decreases in leukocyte migration despite increased levels of inflammatory markers. The A1GP levels measured in these experiments may be indicative of the same type of condition, which resembles immune tolerance (Kogut and Arsenault, 2017).

Alpha-1-acid glycoprotein is a major acute phase protein that has been used as an indicator to predict changes in other acute phase proteins (Adler et al., 2001; Iseri and Klasing, 2013). Elevated levels of A1GP were generally associated with lasting infection, and were cited as nutritionally costly, with the potential to result in depressed growth (Chamanza et al., 1999; Iseri and Klasing, 2013). Throughout these experiments, A1GP was elevated, even in NC, compared to expected baseline levels in chickens of under 500 μg/mL (Takahashi et al., 1994; Iseri and Klasing, 2013; O'Reilly et al., 2018), which not only indicated consistent exposure to an unknown inflammatory stimulus but may also be responsible for the minimal differences in BWG observed across all experiments (Figures 1−4). Although A1GP was elevated in control groups across all experiments, some interesting patterns were still observed, particularly when interactions between treatment and time were assessed.

Both ST and SE displayed a significant increase in A1GP from d 2 to 8 while NC decreased in experiments 1 and 3, or showed no change in experiment 2, indicating *S.* Typhimurium and *S.* Enteritidis may manipulate early A1GP expression (Figures 1, 2, and 3). Since bacterial insult tends to result in a strong acute phase response (Gruys et al., 2005), evidence would indicate chickens were responding to *S.* Typhimurium exposure for these DOH inoculated birds while NC was decreasing its acute phase response. The opposite effect was observed in Huff, however, with a significant decrease in A1GP from d 2 to 8 in experiment 1 (Figure 1), and a decrease approaching significance in experiment 3 (Figure 3), which paralleled NC. This would indicate the birds were not responsive to DOH oral exposure, or experienced a shift in protein expression which resulted in a suppressed inflammatory response (Meirow and Baniyash, 2017; Rodrigues et al., 2020). Variable changes in expression of A1GP over the first wk for both LG and SE in experiments 1, 2, and 3 may also indicate an alteration in response to inflammation, resulting in a modified acute phase response early in life (Rodrigues et al., 2020).

Another interesting pattern in A1GP expression arose in experiment 3, where A1GP increased significantly for all DOH inoculated birds from d 22 to 29 (Figure 3). While the initially proposed model was aimed at capturing mild GI inflammation at 2 wk of age, this time frame may be too short. With the synchronous spike observed to occur during the fourth wk, from d 22 to 29, birds may in fact be more susceptible to opportunistic pathogens and inflammatory agents beyond 2 wk of age. Maternal antibodies have been found to confer protection to chickens up to 10 d of age for most pathogens (Gharaibeh and Mahmoud, 2013). While this evidence supported the hypothesis that birds would be most susceptible opportunistic pathogens and exhibit signs of mild GI inflammation at about 2 wk of age, these experiments did not consistently support this idea. In fact, the elevation between d 22 and 29 in experiment 3 indicated increased susceptibility beyond 2 wk (Figure 3). Experiment 4 echoed this idea, with a direct bacterial assault on the intestine expected to trigger an acute phase response during the challenge period, d 14 to 17, but A1GP was reduced for all treatments following *C. perfringens* challenge which occurred on d 15 and 16, with a significant reduction of A1GP observed in HCP (Figure 4). From d 17 to 20, the period immediately following *Clostridium* inoculation, significantly increased A1GP was observed for LGCP and HCP, while NIC and NCP did not change significantly (Figure 4). The increase observed for DOH *E. coli* inoculated groups had an apparently faster inflammatory response to *C. perfringens* exposure than NCP, and indicated an increased susceptibility to inflammation, with maintained elevation of A1GP during the remainder of the experiment to d 27 (Figure 4). These results would indicate increased susceptibility to opportunistic infection, especially those pathogens that can take advantage of inflammation. In the context of *Clostridium perfringens*, this revelation deserves further investigation to determine the role of early colonizing bacteria at establishing gut conditions that favor clostridial growth.

Beyond weekly fluctuations in A1GP, experiments 3 and 4 revealed a general pattern of increased serum A1GP for all treatments except noninoculated controls in each experiment (Figure 3 and 4). In experiment 3, A1GP was considerably elevated for all DOH inoculated birds between d 2 and d 29, whereas NC only changed by 10% from d 2 to d 29 (Figure 3). In experiment 4, NCP, LGCP, and HCP all increased in contrast to the decrease observed in NIC from d 6 through d 27 (Figure 4). Though not significant across all treatments, this pattern was suggestive of the influence DOH inoculation and later CP challenge could have on inflammation. This stark contrast between A1GP changes in DOH inoculated birds compared to NC or NIC in experiments 3 and 4, respectively, highlights the influence Enterobacteriaceae had in triggering an immune response shift toward a state of low level inflammation. Exposure to *Salmonella* Enteritidis on DOH has been shown to result in an immune response shift from resistance on d2 postinoculation through increased production of proinflammatory cytokines IL-1β, IL-6, and TNF-α, as well as anti-inflammatory IL-10, to one of tolerance with decreased production of IL-1β and IL-6, and production IL-10 and TNF-α by d 4 following inoculation (Lee et al., 2020). While production of IL-1β and IL-6 was reduced, the maintained level of TNF-α,

which has been noted as a cytokine involved in acute phase response (Asasi et al., 2013), may provide insight to increased A1GP over the duration of the experiment. While Enterobacteriaceae manipulation of the immune response was made evident by changes in A1GP over time in experiments 3 and 4, this change may be explained by a shift in cytokine stimulation toward a tolerant immunological state, which may manifest as higher homeostatic levels of inflammation (Lee et al., 2020).

Traditionally, FITC-d has been a useful tool in measuring intestinal barrier function and intracellular permeability (Kuttappan et al., 2015; Vicuña et al., 2015; Gilani et al., 2016). Experiment 2 resulted in elevated FITC-d recovery on d 13 in LG and SE compared to NC, indicative of increased intestinal permeability (Figure 2). Experiment 4 also demonstrated changes in intestinal permeability as measured by FITC-d, as NIC and HCP showed a decrease in FITC-d recovery compared to NCP on d 14 (Figure 4). However, since NIC and NCP were ostensibly the same at this point, the combined values of NIC and NCP on d 14 resulted in no differences between LGCP, HCP, and NIC/NCP (Table S4). Considered in parallel with A1GP, sustained inflammation may be linked to the observed increase in intestinal permeability captured by FITC-d. The minimal differences observed in experiments 2, 3 and 4 reinforced the idea that FITC-d does not easily detect low levels of inflammation, as there generally would need to be more damage for disruption of tight junctions and paracellular transport of the molecule (Kuttappan et al., 2015; Gilani et al., 2016; Duff et al., 2019). *C. perfringens* is known as the etiological agent of necrotic enteritis, and uses a variety of toxins that result in extensive tissue damage and necrosis (Titball et al., 1999; Keyburn et al., 2008; Lacey et al., 2016). Models for necrotic enteritis have typically included a predisposing factor that would provide an ideal environment for CP proliferation, such as high protein, high nonstarch polysaccharides, coccidial infection, or immunosuppression (Shojadoost et al., 2012; Moore, 2016). While DOH Enterobacteriaceae may shift the microbial population of the GI tract and manipulate the immune development to induce some mild immunosuppression, this likely did not result in a large enough shift to provide *C. perfringens* with an ideal environment to induce hemorrhagic necrotic enteritis. However, nonhemorrhagic clostridial enteritis was noted when lesion scoring birds, as described by Goossens et al. (2020). Discoloration of the mucosa was noted, along with amorphous material that adhered to the intestinal surface which suggested proliferation of *C. perfringens* in the intestinal tract though no gross necrotic lesions were present. Therefore, while FITC-d would have been expected to increase on d 17, immediately following *C. perfringens* challenge, DOH Enterobacteriaceae had not acted as enough of a predisposing factor for intestinal barrier impairment to occur (Figure 4). Though FITC-d provided an enteric specific detection method, it was not sensitive enough to consistently evaluate differences in GI inflammation. This method of detection should, therefore, be reserved for instances of moderate to severe inflammation or intestinal barrier disruption.

Yolk sac retention was evaluated as a factor indicative of infection since birds generally resorb their YS by about 2 wk of age, therefore, increased incidence of retained YS would be indicative of infection (Buhr et al., 2006). The overall lack of difference in YS retention, YS weight, and aerobic and Enterobacteriaceae recovery of the YS was surprising, since the YS was anticipated to indicate intestinal infection. There were 2 exceptions noted. In experiment 1, aerobic bacterial recovery of the YS was increased in SE, while also being between 2 and 5 Log_{10}CFU/g greater in LG, Huff, and ST (Figure 1). In experiment 2, YS retention of LG was greater than NC and SE on d 6 (Table 2). These differences represented a possible bacterial reservoir or omphalitis (Gross, 1964; Cox et al., 2006), which may have provided opportunistic pathogens a location within the intestine to reside and await ideal conditions to cause infection. The lack of consistency across experiments led to questions of possible hatchery contamination, which resulted in the discovery of swarming colonies, characteristic of *Proteus spp.* in DOH intestinal samples, and specifically noted in experiment 4, on d 6 across all treatments (Figure S1). *Proteus spp.* are known to cause disease in poultry while also presenting a public health concern (Yeh et al., 2018; Sanches et al., 2020). While additional analysis must be completed to confirm the bacteria as *Proteus spp.*, and that the contamination occurred at the hatchery, it may clarify some of the inconsistencies of results. Yolk sac parameters have the potential to provide intestinal specific indication of GI inflammation, but these results suggest that DOH Enterobacteriaceae inoculation may result in too mild of an infection or inflammatory status to warrant stark changes in these parameters.

These studies have demonstrated the capacity of DOH exposure to Enterobacteriaceae to induce some changes to inflammatory markers throughout the first few weeks of life. While the initial hypothesis was that these changes would be seen through 2 wk of age, evidence suggested a change to acute phase response, as indicated by fluctuations in A1GP over time, to last through at least the fourth wk of life. After *C. perfringens* inoculation, A1GP increased more rapidly in birds inoculated with *E. coli* on DOH, suggesting conditions in the intestine favored the pathogen, and highlight the need to control inflammation-causing bacteria within hatching environments. While minimal differences in BWG, FITC-d recovery, YS retention, YS weight, and both aerobic and Enterobacteriaceae recovery of the YS were noted, it is important to consider that early, low level pathogen exposure may not have major effects on these parameters. Additional analysis on how hatchery Enterobacteriaceae exposure influences microbial and immune development should be considered, since this was likely where DOH Enterobacteriaceae inoculation had the greatest impact.

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DISCLOSURES

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101298.

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