Selection for pro-inflammatory mediators produces chickens more resistant to *Clostridium perfringens*-induced necrotic enteritis

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ABSTRACT We developed a novel selection method based on an inherently high and low phenotype of pro-inflammatory mediators and produced "high" and "low" line chickens. We have shown high line birds are more resistant to *Salmonella enterica* serovar Enteritidis and *Eimeria tenella* compared to the low line. *Clostridium perfringens* is the fourth leading cause of bacterial-induced foodborne illness, and is also an economically important poultry pathogen and known etiologic agent of necrotic enteritis (NE). The objective of this study was to determine if high line birds were also more resistant to NE than low line birds using an established model. Birds were reared in floor pens and challenges were conducted twice (high line = 25/trial, 50 birds total; low line = 26/trial, 52 birds total). Day-old chicks were provided a 55% wheat-corn-based un-medicated starter diet. A bursal disease vaccine was administered at 10× the recommended dose via the ocular route at 14-d-of-age. Birds were challenged daily for 3 d beginning at 16-d-of-age by oral gavage (3 mL) with 10^7 colony forming units (cfu) of *C. perfringens*/mL then necropsied at 21-d-of-age. All birds had sections of the intestine examined and scored for lesions while the first 10 necropsied also had gut content collected for *C. perfringens* enumeration. Chickens from the high line were more resistant to *C. perfringens*-induced NE pathology compared to the low line, as indicated by reduced lesion scores. Ninety percent of the high line birds had lesions of zero or one compared to 67% of the low line birds. Wilcoxon rank sum test showed significantly higher lesion scores in the low line birds compared to the high line ($P < 0.0001$). There were no differences in the *C. perfringens* recovered ($P = 0.83$). These data provide additional validation and support selection based on elevated levels of pro-inflammatory mediators produces chickens with increased resistance against foodborne and poultry pathogens.

Key words: broiler, *Clostridium perfringens*, necrotic enteritis, pro-inflammatory, selection for resistance

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INTRODUCTION

Clostridium perfringens (*C. perfringens*) is a spore forming, Gram-positive rod-shaped bacterium that is ubiquitous in nature and grows in anaerobic conditions. Host reservoirs include humans, cats, cows, pigs, sheep, and chickens (Maier et al., 2000), and one way the bacteria can be transmitted to humans is through consumption of contaminated poultry products (Labbe, 1991). In the Centers for Disease Control and Prevention 2012 annual report of foodborne disease outbreaks in the United States, *C. perfringens* is the fourth leading cause of bacterial-induced foodborne illnesses behind *Salmonella*, *Campylobacter*, and *Escherichia coli* (CDC, 2014) with an estimated economic burden of $342 million USD per year (Flynn, 2014).

In addition to being a foodborne pathogen, *C. perfringens* is also an economically important poultry pathogen and is one of the known etiologic agents of necrotic enteritis (**NE**) (Timbermont et al., 2011). NE is categorized into clinical and subclinical infections with clinical signs including depression, decreased appetite, diarrhea, and severe necrosis of the intestinal tract (Ficken and Wages, 1997; Van Immerseel et al., 2004). Globally, the cost of clinical and subclinical NE infections is estimated at $2 billion USD taking into consideration reduced performance, disease treatment, and carcass condemnations (Van der Sluis, 2000; Paiva and McElroy, 2014); however, more resent estimates suggest the impact may be closer to $5—6 billion USD per year (Wadea and Keyburn, 2015). Understanding the disease progression of NE has been very difficult due to its complexity and several

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predisposing factors such as dietary components, immuno-suppression, mechanical irritation of the gut, and sudden gut microflora changes that appear to contribute to this disease (Smith, 1965; Ficken and Wages, 1997; Timbermont et al., 2011; Paiva and McElroy, 2014). To gain a better understanding of this highly important poultry disease, our laboratory developed an experimental model to study NE (McReynolds et al., 2004).

We recently developed a novel selection method based on identification and selection of chickens with an inherently high and low phenotype of pro-inflammatory mediators including IL-6, CXCLi2, and CCLi2. We have shown the high line of chickens is more resistant to the foodborne pathogen *Salmonella enterica* serovar Enteritidis (Swaggerty et al., 2014) and the poultry pathogen *Eimeria tenella* (Swaggerty et al., 2015) compared to the low line. If the high line of birds were also more resistant to *C. perfringens,* both a foodborne and poultry pathogen, it would provide additional validation of selection based on pro-inflammatory mediators and therefore be more appealing and valuable to the poultry industry. Therefore, the objective of this study was to determine whether the same trend of enhanced resistance in the high line of birds was observed in an experimental NE model following challenge with *C. perfringens*.

MATERIALS AND METHODS

Experimental Chickens

The high and low lines of broilers were produced following selection parameters previously described (Swaggerty et al., 2014). Briefly, sires that have inherently high levels of pro-inflammatory mediators (IL-6, CXCLi2, and CCLi2) were mated to randomly selected dams and their progeny are the high line. The low line is a product of sires with low levels of the pro-inflammatory mediators being mated to randomly selected dams. Fertilized eggs from the high and low lines were obtained from our industry partner and were incubated and hatched under standard conditions (Stromberg, 1975). Each line was coded to conceal their identity.

Experimental Design

On day-of-hatch, straight-run chicks from each line were placed in separate floor pens (3 m × 3 m) within the same room. Each pen contained wood shavings, supplemental heat, water, and a 55% wheat-corn-based un-medicated broiler starter diet ad libitum. The feed met or exceeded all established nutrient requirements (National Research Council, 1994). All birds were housed in the same room and each line was maintained in separate pens. The experimental challenge trials were conducted on two separate occasions with chickens from different hatches (high line = 25 per trial for a total of 50 birds; low line = 26 per trial for a total of 52 birds). All birds were scored for lesions; while the first 10 necropsied from each line for each experiment had gut content collected for *C. perfringens* enumeration.

Birds were administered a commercial bursal disease vaccine (Bursa-Vac, Merck Animal Health, Summit, NJ) at 10 times the recommended dose via the ocular route at 14-d-of-age to immunocompromise the birds and aid in the development of NE (McReynolds et al., 2004). Birds were challenged once daily for 3 d beginning at 16-d-of-age by oral gavage (3 mL per bird) with a stock culture of 10^7 colony forming units (cfu) of *C. perfringens*/mL. All birds were necropsied at 21-d-of-age and were terminated by cervical dislocation. The birds did not receive any medications during the study. All experiments were conducted according to guidelines established by the USDA animal care committee, which operates in accordance with established principles (National Research Council, 1996).

Clostridium Perfringens *Preparation, Isolation, and Enumeration*

Four field isolates of wild-type *C. perfringens* (type A) were obtained from active and confirmed outbreaks of NE in Georgia (two isolates), Texas (one isolate), and Virginia (one isolate). The isolates were cultured separately in thioglycollate medium (Becton Dickinson Co., Sparks, MD) for 12 h then combined to yield the challenge stock and *C. perfringens* was enumerated as previously described (McReynolds et al., 2007). Briefly, a 15.24-cm (6-in.) section of the small intestine cranial to Meckel's diverticulum was removed. The sample was placed in 10 mL of anaerobic thioglycollate, stomached for 30 s, and 0.5 mL of gut contents were removed and placed into 4.5 mL of thioglycollate medium. Three 10-fold serial dilutions were performed and plated onto Shahidi Ferguson Perfringens agar base plates supplemented with 50% egg yolk enrichment, Antimicrobic Vial K (12 mg), and Antimicrobic Vial P (30,000 U) (Becton Dickinson). After the sample was plated, 10 to 12 mL Shahidi Ferguson Perfringens agar base without egg yolk enrichment was overlaid and the plates incubated anaerobically for 24 h at 37°C. Colonies exhibiting typical *C. perfringens* morphology were counted and transformed into $\log_{10}$ values.

Lesion Scores

To evaluate gross lesions associated with NE, the jejunum and ileum of the small intestine were examined and scored as previously described (Prescott, 1979). To eliminate bias, one person blindly scored all tissues for lesions. Lesions were scored on a scale of 0 to 4. A score of 0 indicated normal healthy tissue with no gross lesions; a score of 1 was characterized by thin-walled or friable tissue with a grey appearance; a score of 2 was

Table 1. Distribution (no. of chickens) and rank score means of lesion scores.

	n	Lesion score[2] 0	1	2	3	4	Rank score means	*P*-value
High Line	50	34[1]	11	3	0	2	32.9	<0.0001
Low Line	52	15	20	15	2	0	69.3	

[1]Actual number of chickens in the experiment with a specific lesion score.

[2]Lesions were scored on a scale of 0 to 4. A score of 0 indicated normal healthy tissue with no gross lesions; a score of 1 was characterized by thin-walled or friable tissue with a grey appearance; a score of 2 was thin-walled, had focal necrosis, and grey in appearance with small amounts of gas production; a score of 3 had thin walls with sizable patches of necrosis, gas-filled intestine, and small flecks of blood; and a score of 4 was defined by severe extensive necrosis, marked hemorrhage, and large amounts of gas in the intestine (Prescott, 1979).

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Statistical Analyses

Two separate challenges were conducted using chickens from a different flock for each trial and 25 to 26 birds were used for each experiment. A total of 52 and 50 low and high birds, respectively, were used in the two experimental challenges. The data from the two challenge trials were combined for statistical analyses and data presentation. All analyses were performed in JMP 12 (SAS, Inc., Cary, NC). Since a disease score is a non-numeric categorical response of disease severity, the scores for each bird were transformed to numeric ranks 1 to 102. Non-parametric analysis (assumptions of normality are not made) of the rank response was performed using the Wilcoxon rank sum test (Agresti, 2010). A one-way test with a Chi-Square approximation was used. Log_{10} *C. perfringens* recovered were determined and statistical analyses performed (Student *t* test). Significance was considered at a *P*-value of ≤ 0.05.

RESULTS

Lesion Scores

To determine if the high and low line chickens differed in susceptibility to pathological intestinal damage caused by NE, sections of the intestine were evaluated and any lesions were scored on a scale of zero to four. Chickens from the high line were more resistant to *C. perfringens*-induced NE pathology compared to the low line as indicated by reduced NE-associated lesion scores (Table 1). Forty-five/50 (90%) high line birds had lesions of zero or one, compared to 35/52 (67.3%) of the low line birds while the remaining 5/50 (10%) and 17/52 (32.7%), respectively, had lesion scores of two or greater. Wilcoxon rank sum test supported our hypothesis that high line birds would have lower disease severity than low line birds on average ($P < 0.0001$; rank score means 32.9 and 69.3, respectively). No mortality was observed.

Table 2. Average log_{10} *Clostridium perfringens* recovered from intestinal content.

	n	Average log_{10} cfu/g[1] ± SEM	*P*-value
High Line	20	5.41 ± 0.85	0.83
Low Line	20	5.58 ± 0.77	

[1]Colony forming units per gram.

Clostridium Perfringens Recovered

In addition to lesion scores, the amount (Log_{10} cfu/g) of *C. perfringens* recovered from the intestines of challenged birds was quantified for each line (n = 10 per experiment per line for a total of 20 birds per line; Table 2). There were no differences in the amount of *C. perfringens* recovered from the high (5.41 ± 0.85) and low (5.58 ± 0.77) lines ($P = 0.83$). There were 3 high line birds that had undetectable levels of *C. perfringens* while one bird from the low line had undetectable levels. All birds were included in the data analysis.

DISCUSSION

As poultry breeding companies keep up with global consumption, consumer and regulatory demands reducing antimicrobial use while striving to improve robustness and livability, methods to produce birds with natural resistance to poultry and foodborne pathogens will become more important. The high and low line chickens used in the present study are selected for an inherently high and low phenotype of IL-6, CXCLi2, and CCLi2, respectively. The high line birds have increased resistance against organ invasion by *Salmonella enterica* serovar Enteritidis (Swaggerty et al., 2014) and reduced cecal lesions following challenge with *Eimeria tenella* (coccidiosis) (Swaggerty et al., 2015) compared to the low line. To the authors' knowledge, there are no other experimental or commercial lines of poultry that have undergone selection pressures for pro-inflammatory mediators or any other innate immune-based phenotype. The current study demonstrated high line birds were also more resistant to NE-induced intestinal pathology following challenge with *C. perfringens*, one of the most costly diseases facing the modern poultry industry.

Withdrawal of growth-promoting antibiotics from poultry feed has been accompanied by an increase in the incidence of *C. perfringens* carriage levels and NE outbreaks (Gaucher et al., 2015; Williams, 2005). The complex nature of NE in poultry is exacerbated by numerous pre-disposing factors including, but not

limited to, diet composition, immunosuppression in the flock, and likely one of the most influential being mucosal damage caused by coccidiosis (Van Immerseel et al., 2004; Paiva and McElroy, 2014). NE is highly correlated with coccidiosis, which causes immunosuppression and severe gastrointestinal damage, which, in turn, may give rise to overgrowth by *C. perfringens* (Al-Sheikhly and Al-Saieg, 1980; Broussard et al., 1986). An earlier study using high and low line birds indicate the high line is more resistant to coccidial-induced intestinal pathology (Swaggerty et al., 2015). With respect to *C. perfringens*, the reduced pathology observed in the current study was not a result of reduced bacterial numbers as there were no differences in the bacteria recovered from the high and low line (Table 2). It is possible there is a mechanism(s) or signaling pathway(s) that has been enhanced by the elevated levels of pro-inflammatory mediators selected for in the high line that protects the gastrointestinal tract from damage. Additional molecular and biochemical studies are required to delineate any common intestinal protection strategies in the high line. Additionally, due to the importance of these two pathogens, future studies to evaluate the high and low lines with a co-infection of coccidia followed by challenge with *C. perfringens* should be considered. Typically, the co-infection NE models use *E. maxima* followed by challenge with *C. perfringens* (Williams et al., 2003; Gholamiandehkordi et al., 2007). However, our findings with the high and low lines report on *E. tenella* (Swaggerty et al., 2015), but preliminary data suggests a similar trend with *E. maxima* (C. L. Swaggerty and M. H. Kogut, unpublished data). If the high line birds continue to maintain less intestinal damage following exposure to coccidia that we observed in previous studies, it would be possible that they would also be less susceptible to the secondary NE outbreaks that are often observed in commercial rearing conditions.

Most studies that address differences in resistance or susceptibility to NE employ the dual infection model described above. Evaluation of two commercially available broiler lines showed differences in tissue expression of pro-inflammatory cytokines in the spleen following NE but not in the intestine (Hong et al., 2012). As might be expected, these data suggest there is a systemic response outside the local intestinal response. Additional studies indicate either a protective or pathological role that cytokines and chemokines play in NE in chickens (Sumners et al., 2012; Lee et al., 2013). Recent findings indicate other immunological pathways are involved and indicate "genetic determinants outside the chicken B complex" have a significant impact on birds being either resistant or susceptible to NE (Kim et al., 2014). To date, cytokine and chemokine levels in the tissues of high and low birds have not been examined as all selection was based on the mRNA expression levels of key pro-inflammatory mediators in the peripheral blood leukocytes (Swaggerty et al., 2014). Clearly, pro-inflammatory mediators including cytokines and chemokines have a pivotal role in determining the outcome and severity of NE in broilers albeit either in the tissues as reported by other investigators or as shown in this report by selecting for inherently higher levels in peripheral blood leukocytes.

The lesion scores observed in this study were more consistent with sub-clinical NE, even though the strains of *C. perfringens* used in the challenges were isolated from active outbreaks of clinical NE. The lesions in the low line are similar to what we have recorded in previous studies employing this model (McReynolds et al., 2004; McReynolds et al., 2009) further demonstrating the complexity of reproducing NE in a laboratory setting. Others also report little-to-no changes or "subclinical inflammatory responses" with limited intestinal damage in a laboratory NE model (Olkowski et al., 2006). In the above-mentioned studies, the commercial broilers were all from different sources, which further supports the fact that NE is a multi-factorial disease that is difficult to fully reproduce experimentally. The lower lesion scores we observed are also in agreement with the emerging pattern of sub-clinical enteric disorders in modern poultry strains compared to the more acute clinical forms of the disease (Olkowski et al., 2006; Timbermont et al., 2011). Broiler performance including feed conversion ratio and live weight at 6-wk-of-age was not evaluated in the present study, but should be incorporated into subsequent experiments to determine if the lower lesion scores observed in the high line birds also corresponds to enhanced performance. This would be of great interest as sub-clinical NE is very costly to the poultry industry.

The *C. perfringens* strains used herein were type A α toxin positive, which are believed to contribute to mucosal necrosis associated with NE (Van Immerseel et al., 2004; Sumners et al., 2012). However, other toxins including NetB and TpeL may also contribute to the onset of NE (Timbermont et al., 2011; Coursodon et al., 2012; Shojadoost et al., 2012; Paiva and McElroy, 2014) but in some instances may be dependent on geographical location (Bailey et al., 2015). Outside screening for the α toxin, additional toxin screening was not performed on the isolates used in the present study, but since the strains were from commercial NE cases it could be suggested that at least one of the strains possessed one or both of these additional toxins. Though outside the scope of the current manuscript, a more detailed characterization of the field isolates should be considered in the future to further define the molecular makeup of *C. perfringens* strains that lead to NE outbreaks in commercial broiler flocks.

Collectively, these studies would support the hypothesis that an effective innate immune response accompanied with a strong and efficient pro-inflammatory response could limit the pathology and potentially reduce the loss of productivity associated with NE. The previous reports that demonstrate the high line of birds

have increased resistance against *Salmonella* Enteritidis (Swaggerty et al., 2014), coccidiosis (Swaggerty et al., 2015), and now NE in this report provide additional evidence to support selection based on inherently elevated levels of key pro-inflammatory cytokines and chemokines could be used to produce chickens with increased natural resistance against foodborne and poultry pathogens.

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