Research Note

Selection for pro-inflammatory mediators produces chickens more resistant to *Campylobacter jejuni*

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ABSTRACT *Campylobacter spp.* are the second leading cause of bacterial-induced foodborne illnesses with an estimated economic burden of nearly $2B USD per year. Most human illness associated with campylobacteriosis is due to infection by *C. jejuni* and chickens are recognized as a reservoir that could lead to foodborne illness in humans resulting from handling or consuming raw or undercooked chicken. We recently developed a novel breeding strategy based on identification and selection of chickens with an inherently high and low phenotype of pro-inflammatory mediators including IL-6, CXCLi2, and CCLi2, hereafter referred to as the high and low lines, respectively. We have shown the high line chickens are more resistant to the foodborne and poultry pathogens *Salmonella enterica* serovar Enteritidis, *Eimeria tenella*, and *Clostridium perfringens*-induced necrotic enteritis compared to the low line. The objective of this study was to determine whether the same trend of enhanced resistance in the high line birds was observed for *C. jejuni*. Birds were challenged at 2 d of age by oral gavage (0.5 mL) with 5×10^6 colony forming units (cfu) of *C. jejuni*/mL, necropsied 4 d post challenge, and cecal content collected to determine if there was a difference in *C. jejuni* resistance between the high and low line chickens. There were fewer ($P = 0.01$) chickens from the high line ($28/40 = 71.8\%$) that were colonized by *C. jejuni* compared to the low line ($37/39 = 94.9\%$). The amount of *C. jejuni* recovered from the ceca of infected birds was quantified; however, no differences were observed ($P = 0.10$). Since the high line birds were also more resistant to *C. jejuni*, it provides additional validation of selection based on pro-inflammatory mediators producing a line of chickens with increased natural resistance against diverse foodborne and poultry pathogens. The poultry industry is moving towards reduced therapeutics and, as such, our breeding strategy would be a viable method to incorporate into traditional poultry breeding programs.

Key words: broiler, *Campylobacter jejuni*, pro-inflammatory, resistance, selection

2017 Poultry Science 96:1623–1627
http://dx.doi.org/10.3382/ps/pew465

INTRODUCTION

Campylobacter jejuni (*C jejuni*) is a Gram-negative, spiral, non-spore-forming, motile, rod-shaped bacterium that is ubiquitous in nature and grows mostly in microaerobic conditions, yet also can grow aerobically and anaerobically (Penner, 1988; Epps, et al., 2013). In the Centers for Disease Control and Prevention (**CDC**) 2012 annual report of foodborne disease outbreaks in the United States, *Campylobacter* is second only to *Salmonella* as the leading cause of bacterial-induced foodborne illnesses (CDC, 2014) with an estimated economic burden in the United States alone of $1.9B USD per year (Flynn, 2014). The 2 species of *Campylobacter* that are most often associated with human gastroenteritis are *C. jejuni*, which accounts for 80 to 85% of all infections, and *C. coli* which is responsible for 10 to 15% of the reported cases (Moore, et al., 2005). Food production animals including cattle, swine, and chickens are known host reservoirs and transmission sources of *Campylobacter*.

Poultry products account for an estimated 50 to 70% of human campylobacteriosis cases (Epps, et al., 2013) and illness typically results from consumption of undercooked or improper handling of contaminated poultry products including eggs and meat (Moore, et al., 2005). *Campylobacter* typically colonizes chickens early after hatch and most (>95%) remain carriers for life, and it is this carriage state that increases the likelihood of carcass contamination during slaughter and processing (Hermans, et al., 2011). Since *Campylobacter* readily infects chickens without eliciting significant clinical signs, it is often referred to as a commensal gut bacterium in the chicken, a natural host reservoir (Beery, et al., 1988; Moore, et al., 2005; Epps, et al., 2013). However, recent studies indicate *C. jejuni* can adversely impact

Received August 11, 2016.
Accepted December 5, 2016.

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animal health and welfare (Humphrey, et al., 2014) as well as growth and performance (Awad, et al., 2015). Furthermore, there are studies that show genetically different lines of chickens have distinct immunological responses to *C. jejuni* under experimental challenge conditions indicating some lines of chickens are more resistant than others (Li, et al., 2008; Li, et al., 2010; Connell, et al., 2012; Li, et al., 2012; Psifidi, et al., 2016).

We recently developed a novel selection strategy 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 are more resistant to *Salmonella enterica* serovar Enteritidis (Swaggerty, et al., 2014), *Eimeria tenella* (Swaggerty, et al., 2015), and necrotic enteritis-induced intestinal pathology associated with *Clostridium perfringens* (Swaggerty, et al., 2016) compared to the low line. If the high line birds were also more resistant to *C. jejuni,* it would provide additional validation that selection based on key pro-inflammatory mediators produces chickens that are naturally more resistant to diverse pathogens including those directly impacting food safety and animal health. Therefore, the objective of this study was to determine whether the same trend of enhanced resistance in the high line birds was observed following challenge with *C. jejuni.*

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 the commercial company 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 balanced, un-medicated corn and soybean meal based chick starter diet ad libitum. The feed contained 23% protein and 3,200 kcal of metabolizable energy/kg of diet, and all other nutrient levels met or exceeded established requirements (National Research Council, 1994). All birds were housed in the same room, but the lines were maintained in different pens. The experimental challenge trials were conducted on 2 separate occasions with chickens from different hatches (19 to 20 chicks per line per trial for a total of 39 to 40 birds per line).

Two-day-old chicks were challenged by oral gavage (0.5 mL per bird) with a stock culture of 10^7 colony forming units (cfu) of *C. jejuni*/mL (each chick received 5×10^6 cfu). Ten chicks from each line were mock-challenged with PBS to serve as negative controls. All birds were necropsied 4 days post challenge and were terminated by cervical dislocation. At necropsy, cecal content was collected for *C. jejuni* enumeration. 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).

Campylobacter Jejuni Preparation, Isolation, and Enumeration

The *C. jejuni* challenge strain was isolated from a broiler house in Mississippi during a field trial in which random birds were selected and necropsied and cecal content was collected and plated on selective media and individual colonies were then subjected to PCR analysis to confirm they were *C. jejuni.* The *C. jejuni* challenge stock was maintained and grown as previously described (Ziprin, et al., 2001). Cecal content was collected at necropsy to determine the number of *C. jejuni* recovered from each bird. Briefly, 0.25 g cecal content was transferred to 2.25 mL Butterfield's phosphate buffered water (Becton Dickinson Co., Sparks, MD) and 3 10-fold serial dilutions were performed and plated onto Campy-Cefex agar plates (Becton Dickinson Co.). The plates were then placed in plastic freezer bags and gassed with a mixture containing 5% O_2 and 10% CO_2 balanced with N_2 and then zipped closed trapping the gas in the bag, and incubated at 42° C for 48 hours. Colonies exhibiting typical *C. jejuni* morphology were counted and transformed into Log_{10} values.

Statistical Analyses

Two separate challenges were conducted using chickens from a different flock for each trial and 19 to 20 birds were used for each experiment. A total of 39 low and 40 high birds were used in the 2 experimental challenges. The data from the 2 challenge trials were combined for statistical analyses and data presentation. All analyses were performed in SigmaPlot 12 (Systat Software, Inc., San Jose, CA). The mean and SEM Log_{10} *C. jejuni* recovered were determined and statistical analyses performed (Student's t test). Significance was considered if $P \leq 0.05$.

RESULTS

Campylobacter Jejuni Challenged Birds

Two separate challenge trials were conducted using 2 separate hatches of chickens and the combined data

Table 1. *Campylobacter jejuni* experimental challenges.

	n[1]	No. *Campylobacter jejuni* positive chicks/total no. challenged	Percent positive	*P*-value	Average $\log_{10}$ cfu/g[2] ± SEM	*P*-value
High line	40	28/40	71.8	0.01	6.97 ± 1.15	0.10
Low line	39	37/39	94.9		6.64 ± 1.13	

[1]Data pooled and analyzed from 2 separate *C. jejuni* challenges.
[2]Colony forming units per gram.

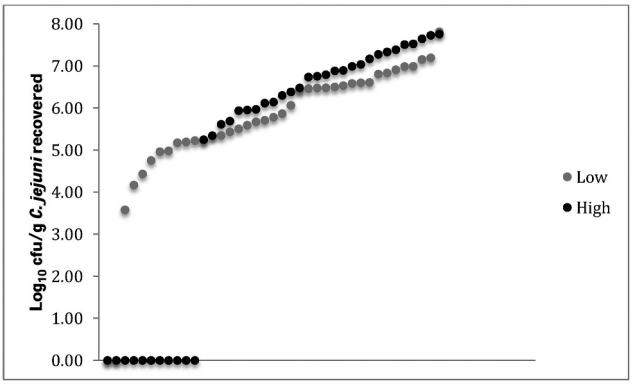

Figure 1. Distribution of *Campylobacter jejuni* recovered from high and low line chickens. Two-day-old chicks were challenged by oral gavage (0.5 mL per bird) with a stock culture of 10^7 colony forming units (cfu) of *C. jejuni*/mL. Birds were necropsied 4 d post challenge and cecal content was collected for *C. jejuni* enumeration. The amount ($\log_{10}$ cfu/g) of *C. jejuni* recovered from individual high (black circles) and low (gray circles) line birds was quantified. There were no differences in the average number of *C. jejuni* recovered from the high (6.97 ± 1.15) and low (6.64 ± 1.13) lines ($P = 0.10$).

are presented herein. High (n = 40) and low (n = 39) chicks were challenged with 5×10^6 cfu *C. jejuni*. Significantly ($P = 0.01$) fewer high line chicks (28/40 = 71.8%) were colonized by *C. jejuni* compared with the low line (37/39 = 94.9%; Table 1). In each challenge trial, mock-infected control chicks (n = 10) from each line were administered sterile PBS and were negative for *C. jejuni* (data not shown).

Campylobacter Jejuni Recovered

The amount ($\log_{10}$ cfu/g) of *C. jejuni* recovered from the ceca of challenged birds was quantified for each line (n = 19 to 20 per experiment per line for a total of 39 to 40 birds per line; Table 1). There were no differences in the amount of *C. jejuni* recovered from the high (6.97 ± 1.15) and low (6.64 ± 1.13) lines ($P = 0.10$). The distribution of the *C. jejuni* recovered from individual birds for each line is shown in Figure 1.

DISCUSSION

There are 3 basic control strategies to control *Campylobacter* at the farm level including reducing environmental exposure and enhanced biosecurity measures, increasing host resistance through genetic selection, or the use of antimicrobial alternatives to reduce or eliminate *Campylobacter* infected chickens (Lin, 2009). Studies using divergent lines of chickens show there is a genetic basis that contributes to birds being either resistant or susceptible to *Campylobacter* (Stern, et al., 1990; Boyd, et al., 2005; Li, et al., 2008), and evaluation of 2 widely studied experimental lines of chickens recently confirmed *C. jejuni* colonization is a quantitative trait (Psifidi, et al., 2016). Others also suggest that "the possibility of breeding for natural resistance to *Campylobacter*" should be investigated (Gormley, et al., 2014).

In 2010, the Food Safety and Inspection Service (**FSIS**) released new performance standards for *Salmonella* and *Campylobacter* in young chickens and turkeys aimed at reducing the numbers of bacteria that can be present on a carcass with the ultimate goal to reduce the instance of foodborne illness (FSIS, 2016). The current study demonstrated our novel breeding program produced a line of birds that was more resistant to *C. jejuni*. Incorporating lines of birds that are naturally more resistant, such as the high line described herein, could potentially reduce the likelihood of carcass contamination during processing, and subsequently fewer illnesses associated with handling and/or consuming undercooked contaminated poultry products because of fewer *Campylobacter*-positive birds entering the food chain. This theory is supported by quantitative risk assessment studies that speculate reducing contamination could yield a 97% reduction in the number of campylobacteriosis cases (Rosenquist, et al., 2003).

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 to *Salmonella enterica* serovar Enteritidis (Swaggerty, et al., 2014), *Eimeria tenella* (Swaggerty, et al., 2015), and necrotic enteritis resulting from *Clostridium perfringens* (Swaggerty, et al., 2016) compared to the low line. To the authors' knowledge, there are no other experimental or commercial lines of poultry that have undergone specific selection pressures for pro-inflammatory mediators or any other innate immune-based phenotype with the exception of the birds described herein.

An earlier study using high and low line birds indicates the high line is more resistant to *C. perfringens* associated necrotic enteritis (Swaggerty, et al., 2016). In that study, there was reduced intestinal pathology even though the number of recoverable *C. perfringens* was comparable between lines. Similarly, in the current study, there were no differences in the number of recoverable *C. jejuni* between the 2 lines following challenge (Table 1; Figure 1), which is a common finding with

C. jejuni in various breeds of chickens (Humphrey, et al., 2014). Once the first bird in a flock becomes colonized by *Campylobacter*, the remaining birds are typically infected within d (Lin, 2009; Gormley, et al., 2014). In the current study, the birds were sacrificed 4 d post challenge, which should have been sufficient time for horizontal transfer as high levels of cecal colonization can occur within 20 to 48 h post infection (Shaughnessy, et al., 2009), suggesting the high line birds with undetectable levels of *C. jejuni* successfully eliminated the challenge bacteria and subsequently prevented further colonization due to horizontal transfer. Future studies could be carried out for longer periods of time to confirm these initial findings.

The reduced percentage of *C. jejuni*-positive birds in the high line was also similar to what we observed following challenge with *Salmonella* (Swaggerty, et al., 2014). Taken together, these data point to activation of a common protective mechanism that limits these 2 foodborne pathogens. A recent study using inbred experimental lines of birds identified multiple quantitative trait loci (**QTL**) associated with resistance to both *Salmonella* and *Campylobacter* colonization and include NRAMP1 (SLC11A1), TRAP1, and AXIN1 (Psifidi, et al., 2016). To date, only challenge studies have been conducted with the high and low lines to show enhanced resistance to diverse pathogens could be achieved using commercial birds. Now that the concept has been proven and there are apparent common genes for resistance against *Salmonella* and *Campylobacter*, the specific mechanism(s) and signaling pathway(s) that result from the selection pressures warrant additional molecular and biochemical evaluations to dissect the upstream protective pathways associated with increased resistance against these highly important foodborne pathogens.

Possible insight into the protective mechanisms could be similar to what our laboratory previously found after conducting extensive studies characterizing 2 parental broiler lines that showed one line (A) was more resistant than the other line (B) to diverse challenges, including *C. jejuni* (Li, et al., 2008) and *Salmonella* (Ferro, et al., 2004). In depth transcriptome analysis of tissues from *C. jejuni*-infected birds and cells from *S.* enteritidis-infected line A and B chickens revealed distinct immunological responses, including those associated with receptor activation, cytokines, chemokines, and regulatory signaling pathways, contribute to increased resistance (Chiang, et al., 2008; Li, et al., 2010; Li, et al., 2011; Kogut, et al., 2012; Li, et al., 2012). Additional studies also show differential expression of intestinal immune genes following *C. jejuni* colonization including Toll-like receptor (**TLR**)4, TLR21, CXCLi1, and CXCLi2 (formerly IL8) (Shaughnessy, et al., 2009; Connell, et al., 2012; Psifidi, et al., 2016). Structural equation modeling (**SEM**) comparing cytokine responses and interactions in 2 different lines of broiler chickens showed the expression of IL1ß and IL6 positively influenced IL17A, which could be important in protecting mucosal surfaces from colonization by *C. jejuni* (Reid, et al., 2016). Though unknown at this time, it is possible that similar immunological responses are occurring in the high and low lines.

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 ability of *C. jejuni* to colonize the ceca of broiler chickens, potentially reducing the load of foodborne pathogens entering the food chain and adversely impacting consumers. Taken together with our previous studies, the current findings provide additional evidence to support that 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.

ACKNOWLEDGMENTS

The authors recognize the late Dr. Pete Kaiser for his countless contributions to the field of avian immunology; you'll be missed professionally and personally as a great friend... Cheers, Pete! The authors also thank Denise Caldwell and M. Reiley Street Jr. for expert technical assistance. Mention of commercial products is for the sole purpose of providing specific information, not recommendation or endorsement by the USDA.

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