Inclusion of high-flavonoid corn in the diet of broiler chickens as a potential approach for the control of necrotic enteritis

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ABSTRACT Avian necrotic enteritis (**NE**) is an infectious disease that impacts poultry worldwide causing economic losses. Discontinued use of antimicrobial growth promoters has been associated with high incidence of the disease, which has led to a necessity for finding new therapeutic alternatives. Flavonoids are polyphenolic compounds that have been studied for their health-promoting properties in animals and humans. This study presents a flavonoid-rich corn (**PennHFD**), as a potential alternative for ameliorating NE in broiler chickens. The effect of a diet formulated with PennHFD was compared to a diet based on commercially available corn in chickens subjected to a controlled challenge of NE based on a co-infection of *Eimeria maxima* and *Clostridium perfringens*. Birds fed on the PennHFD-based diet had lower incidence of intestinal lesions ($P = 0.048$), higher body weight gain ($P < 0.01$), lower feed conversion ratio ($P < 0.01$), and lower mortality rates ($P = 0.023$) compared to the control diet. Therefore, we concluded that the inclusion of the high-flavonoid PennHFD reduces the severity of an experimental challenge of NE in broiler chickens.

Key words: flavonoid, necrotic enteritis, poultry, phytogenic, performance

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INTRODUCTION

Avian necrotic enteritis (**NE**) is among the major diseases that affect the gastrointestinal health of broiler chickens (Kaldhusdal et al., 2016). It has been estimated that the disease could cost US$ 6 billion every year worldwide due to losses in productivity and costs involving treatment and prevention (Wade and Keyburn, 2015).

Necrotic enteritis is caused by pathogenic strains of *Clostridium perfringens* (**CP**), a spore-forming, anaerobic Gram-positive bacterium that can also be part of the indigenous microbiota of humans and animals. Clinical NE develops in broiler chickens between 2 and 5 wk of age causing, depression, ruffled feathers, inappetence, and mortality. The subclinical form of NE (**SNE**) is more frequent, and it often goes undetected in commercial settings. However, SNE negatively impacts body weight gain (**BWG**) and feed conversion ratio (**FCR**), claiming millions in revenues from producers around the world (Timbermont et al., 2011; Prescott et al., 2016a).

Several predisposing factors contribute to the development of NE, such as, high dietary non-starch polysaccharides and proteins, mycotoxins, and coccidiosis (Branton et al., 1987; Tsiouris et al., 2015; Broom, 2017). Coccidiosis is caused by the parasite *Eimeria* spp., and it is an important factor for the development of necrotic enteritis. *Eimeria* spp. disrupts the intestinal epithelium and creates an optimal environment for the multiplication of *C. perfringens* (Al-Sheikhly and Al-Saieg, 1980; Prescott et al., 2016b).

Necrotic enteritis was effectively controlled when in-feed antibiotic growth promoters were widely used in animal production. However, the increasing concern for antimicrobial resistance has led many countries to restrict the use of antibiotic growth promoters, which in turn has been associated with increasing outbreaks of NE (Kaldhusdal et al., 2016). Moreover, consumer preference is toward antibiotic-free poultry products (Brewer and Rojas, 2008). Therefore, alternative solutions to control infectious diseases have been studied, such as, probiotics, prebiotics, essential oils, organic

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acids, bacteriophages, and phytogenic compounds, including flavonoids (Caly et al., 2015; Panche et al., 2016; Adhikari et al., 2020).

Flavonoids are polyphenolic compounds found in many plants that provide protection against environmental challenges and also act as attractants for pollinators (Panche et al., 2016). In addition, flavonoids have been shown to have several health-promoting properties in humans and animals, including anti-inflammatory and antibacterial activities (Cushnie and Lamb, 2011; Abotaleb et al., 2019; Farhadi et al., 2019; Jin, 2019; Maleki et al., 2019; Kopustinskiene et al., 2020).

Flavonoids can modulate intracellular signaling pathways of both the innate and adaptive immune systems through several mechanisms of action, such as, inhibition of the COX-2 activity and the NF-κB and MAPK pathways (Chen et al., 2018). In addition, flavonoids can act as antibacterial compounds by disrupting bacterial cell membranes, inhibiting the enzymes that support DNA replication, and inhibiting production of bacterial toxins (Górniak et al., 2019).

There is some evidence suggesting that a low inclusion of a flavonoid-rich muscadine pomace additive, can partially help ameliorate NE in broilers. However, muscadine pomace contains high concentrations of tannins that are known to negatively affect feed consumption in chickens (McDougald et al., 2008). Ingredients rich in flavonoids which could be used without a negative impact in zootechnical parameters may be more effective to control NE in poultry.

Corn (Zea mays L.) is commonly used source of energy for poultry in the United States (Dei, 2017). Some cultivars of corn have been genetically selected to contain higher concentrations of flavonoids, which have shown important antibacterial and anti-inflammatory properties (Nessa et al., 2012; Wu et al., 2020, 2021). A flavonoid-rich corn line (PennHFD) has been developed at The Pennsylvania State University. It was hypothesized that PennHFD decreases the negative impacts caused by necrotic enteritis in the performance and mortality of broiler chickens. The objective of this study was to compare the effects of a special diet based on PennHFD to a control diet on the incidence and severity of NE in broiler chickens.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted for 21 d using a completely randomized design with 4 treatments. A total of 400-day-old straight-run broiler chickens (Ross 308, Aviagen) were obtained from a local hatchery (Belleville, PA). Upon arrival, the birds were randomly allocated into 20 floor pens (2.6 m^2). The pens were randomly selected to receive one of the following treatments: CTL A (Uninfected birds fed a commercial corn-based diet); CTL B (Uninfected birds fed a PennHFD-based diet); INF A (Birds co-infected with *Eimeria maxima* and *Clostridium perfringens* and fed a commercial corn-based diet); INF B (Birds co-infected with *Eimeria maxima* and *Clostridium perfringens* and fed a PennHFD-based diet). Infected and noninfected controls were held in adjacent but identical rooms to avoid cross-contamination. All pens were equipped with a manual self-feeder and automatic nipple drinkers, and the birds had ad libitum access to feed and water. The brooding conditions were adjusted throughout the experiment based on recommendations of the genetic line (Aviagen, 2018). All procedures were previously approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University.

Diet Formulation

Feed used in this study was manufactured at the Poultry Education and Research Center at The Pennsylvania State University. Two diets were formulated including ingredients reported to be important predisposing factors for NE in broiler chickens: wheat and fishmeal (Prescott et al., 2016; Table 1). The diets were identical, except for the corn type. Diet "A" was formulated with a commercially available corn and "Diet B" was formulated with a proprietary corn (PennHFD), rich in flavonoid contents. The diets were formulated to meet or exceed requirements of broiler chickens as set by the National Research Council (1994).

To estimate the content of flavonoids, the relative flavylium ion concentration, a product derived from flavonoids during the extraction method, was measured to compare PennHFD and the commercially available corn lines, following a methodology previously described (Grotewold et al., 1998; Wu et al., 2021). Briefly, 100 mg of ground kernel was incubated in 1 mL of acidic butanol (HCL:butanol = 3:7, v/v) at 37°C for 1 hour. The samples were centrifuged for 20 s at 10,000 × *g* and the supernatant was removed. The supernatant was analyzed by spectrophotometry with a Cytation3 microplate reader (BioTek, Winooski, VT). Absorbance was measured at 550 nm, and the relative concentration was expressed as absorbance per gram of plant tissue.

Nutrients from both corn types were analyzed by proximate analysis performed at a third-party

Table 1. Composition of the diet treatments (Feed A and Feed B) fed to broiler chickens in an experiment of necrotic enteritis.

Item	Composition (%)
Corn*	31.53
Wheat	30.00
Soybean meal	20.20
Fish meal	15.00
Lard	2.57
Vitamin and mineral premix	0.40
Salt	0.30

*The source of corn varied in the two diets formulated for the experiment. Feed A (commercial corn); Feed B (high-flavonoid corn, PennHFD).

Table 2. Nutrient composition analysis of the two used sources of corn.

Composition	Commercial corn	PennHFD*
Moisture	8.8	9.1
Dry matter	91.2	90.9
Crude protein	13.2	12.9
Lignin (%DM)	2.02	2.23
Crude fat (%DM)	4.03	4.29
Ash (%DM)	1.85	2.07
Calcium (%DM)	0.01	0.02
Phosphorus (%DM)	0.33	0.40
Magnesium (%DM)	0.11	0.15
Potassium (%DM)	0.40	0.35
Sodium (%DM)	0.01	0.01
Iron (ppm)	31	95
Manganese (ppm)	10	17
Zinc (ppm)	26	27
Copper (ppm)	2	4
ME (Mcal/kg)	3.35	3.28

*PennHFD: flavonoid-rich corn cultivar developed at The Pennsylvania State University.

laboratory (Cumberland Valley Analytical Services, Waynesboro, PA; Table 2).

Necrotic Enteritis Model

Chicks at 13 d of age were infected by oral gavage with 5,000 *Eimeria maxima* oocysts. Aliquots of 3 strains of *C. perfringens*, 2 NetB positive and one NetB negative, isolated from field cases of NE, were inoculated into fluid thioglycolate medium (Neogen, Lansing, MI) and incubated anaerobically at 37°C for 24 h to reach a final inoculum concentration of 1×10^9 CFU/mL. Anaerobiosis was achieved with a pouch of AnaeroPack System (Mitsubishi Gas Chemical America, New York, NY).

Twelve hours before the first inoculation with *C. perfringens*, feed from all pens was removed. On d 18 and 19, the feed of all pens in the infected treatment was inoculated with 1 mL of 1×10^9 CFU of *C. perfringens* per bird.

Growth Performance and Mortality

On d 21, all animals and feed were weighed to calculate feed consumption (d 0−21), total body weight gain (d 0−21), and total feed conversion ratio (d 0−21). Mortality data was recorded throughout the experiment.

Sampling and Lesion Scoring

On d 21, a total of 103 birds (~ 5 birds/pen) were randomly selected, euthanized by cervical dislocation, and necropsied for intestinal evaluation. Lesions were classified according to a scoring system, ranging from 0 to 5 (Lorenzoni et al., 2019), modified from Gholamiandehkordi et al. (2007). Briefly, a score 0 was assigned to intestines with no sign of lesions. Score 1 was assigned to birds with one or 2 isolated areas of necrosis smaller than 3 mm in diameter. Score 2 was assigned to birds with one or 2 isolated patches of necrosis of 3 to 10 mm in diameter. Score 3 was assigned to the presence of three or more necrotic patches along the length of the small intestine. Score 4 was assigned to birds with more than 3 necrotic patches in close succession covering at least 10 cm of the intestine; and score 5 was assigned to birds with necrotic patches that fused together, covering at least 10 cm of the intestine.

Statistical Analysis

The statistical analyses were performed using the software Minitab 19 (Minitab Inc., State College, PA). To test the effects of diet (A and B) and infection (Infected and Control) on feed consumption, body weight gain, feed conversion ratio, and mortality, a general linear model procedure was used considering pen as the experimental unit, in which diet and group (control or infected) were assumed to be fixed effects. Optimal box-cox transformations were made for BWG and FCR. A statistical difference was claimed when $P \leq 0.05$, and pairwise comparisons were performed with Fisher least significant difference (**LSD**) to differentiate groups.

The incidence of intestinal lesions between the infected treatments (INF A and INF B) was tested with a Z-test for comparing 2 proportions, and a difference was claimed when $P \leq 0.05$. Since none of the animals sampled in the control treatments presented lesions, the difference between treatments CTL A and CTL B was not tested.

Ordinal logistic regression was carried out to test the severity of lesions based on the intestinal lesion scores. The two different diets were used as categorical predictors with Diet A used as the reference group. Significance was determined when $P \leq 0.05$.

RESULTS

Feed Consumption, Body Weight Gain, Feed Conversion Ratio, and Mortality

The means of feed consumption, total body weight gain, total feed conversion ratio, and mortality between treatments are shown in Table 3. There was a difference ($P < 0.01$) in the means of BWG between diets A and B. Birds from treatments INF A and CTL A obtained lower BWG than birds in the treatments INF B and CTL B, respectively. The FCR means were also different ($P < 0.01$). Birds in the treatments INF A and CTL A had higher FCR compared to birds in the treatments INF B and CTL B, respectively. In addition, mortality rate means were different ($P = 0.023$). As expected, the pairwise comparison did not show a difference in mortality between the treatments CTL A and CTL B. Birds from the treatment INF B had 42.86% less mortality compared to birds from the treatment INF A.

Table 3. Means of feed consumption (kg), total body weight gain (kg), total feed conversion ratio, mortality of the pens of broilers fed a commercial corn (Feed A) or PennHFD corn (Feed B) with (infected) or without (control) a challenge of necrotic enteritis.

	Control				Infected				
	Feed A		Feed B		Feed A		Feed B		
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	*P*-value
FC	24.520^{a}	0.412	24.796^{a}	0.190	19.223^{b}	0.655	20.729^{b}	0.385	0.463
BWG	19.483^{B}	0.274	20.519^{A}	0.143	9.320^{D}	1.135	13.293^{C}	0.643	<0.01
FCR	1.253^{C}	0.012	1.208^{D}	0.004	1.470^{A}	0.026	1.400^{B}	0.024	<0.01
Mortality	0.040^{bc}	0.018	0.000^{c}	0.000	0.375^{a}	0.083	0.161^{b}	0.042	0.023

Different superscripts in the same row denote statistical differences ($P \leq 0.05$). Lowercase superscript (^{abc}) denotes $P \leq 0.05$, and uppercase superscripts (^{ABCD}) indicate $P \leq 0.01$ or less. First alphabetical letters indicate the largest mean in the row.

Infected birds received a co-infection with *E. maxima* and *C. perfringens*.

Abbreviations: FC, feed consumption; BWG, total body weight gain; FCR, total feed conversion ratio.

Lesions

The incidence of NE lesions is presented in Figure 1. As expected, no necrotic lesions were found in the intestines of the 52 birds sampled from the control treatments (CTL A and CTL B). In the infected treatments, 43.13% (22 of 51) of the sampled birds presented lesions characteristic of necrotic enteritis. Birds from the treatment INF A had an incidence of lesions of 55.55%, while birds from the treatment INF B had an incidence of 29.16% ($P = 0.048$). There was no difference in the severity of NE lesions (lesion scores) between the treatments INF A and INF B.

Proximate Analysis and Flavylium Ion Concentration

The proximate analysis indicated that the commercial corn line contained 13.2% of crude protein and 3,351 kcal/kg of ME. The PennHFD contained 12.9% of crude protein and 3,285 kcal/kg of ME (Table 2). The relative flavylium ion concentration, measured at 550 nm, was 1.98 absorbance/g for the PennHFD, and 0.17 absorbance/g for the commercial corn line.

DISCUSSION

The use of antibiotics as growth promoters has been steadily decreasing in poultry production (Brewer and Rojas, 2008; Millet and Maertens, 2011) and alternatives such as flavonoids have been presented as a potential replacement for these compounds. In our experiment, a corn line rich in flavonoids reduced mortality and the presence of lesions indicative of NE in broiler chickens. To our knowledge, this is the first study that tested the effects of a high-flavonoid corn variety on broilers undergoing necrotic enteritis.

Flavonoids can be found in many seed-bearing plants and have antibacterial and anti-inflammatory properties (Farhadi et al., 2019; Maleki et al., 2019). In fact,

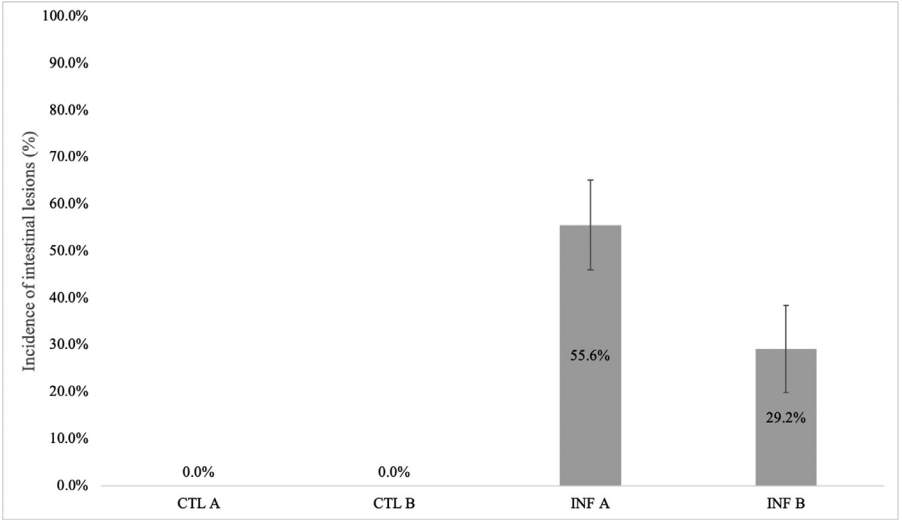

Figure 1. Incidence of birds that presented intestinal lesions characteristic of necrotic enteritis. Necrotic enteritis was defined as the finding of at least one area of necrosis in the small intestine of broiler chickens. INF A (Birds co-infected with *E. maxima* and *C. perfringens* and fed a commercial corn-based diet, n = 27, SE: 0.095); INF B (Birds co-infected with *E. maxima* and *C. perfringens* and fed PennHFD-based diet, n = 24, SE: 0.092); CTL A (Uninfected fed a commercial corn-based diet, n = 27); CTL B (Uninfected birds fed PennHFD-based diet, n = 25).

McDougald et al. (2008) studied the effects of including 0.5 or 2% of muscadine pomace, a flavonoid-rich in-feed additive derived from the production of wine, in broiler chickens undergoing NE. When compared to a diet without additives, birds consuming muscadine pomace had lower mortality and lesion scores and improved feed conversion. However, muscadine pomace contains high concentrations of tannins which are known for limiting feed consumption and affecting the productive performance of birds (Chung et al., 1998). Since corn can be included at a high rate in the diets of animals, lines of corn with high concentrations of flavonoids may represent an ideal alternative for the control of intestinal diseases in livestock production.

In our experiment, birds receiving PennHFD and challenged with *C. perfringens* (INF B) had decreased mortality rates and decreased incidence of intestinal lesions, as well as improved BWG and FCR compared to the treatment INF A. These results are also in agreement with previous studies that presented an increased growth performance, and a reduction in mortality and C. perfringens counts after the inclusion of phytogenic in-feed additives in birds undergoing necrotic enteritis (Granstad et al., 2020). In contrast, Leusink et al., 2010 did not see an effect on growth performance and mortality on broilers challenged with NE after including up to 0.016% of a flavonoid-rich cranberry fruit extract in the diet. This may indicate that the inclusion of the active compound was not in a sufficient level to produce the desired effects in the challenged birds.

Lines of corn genetically selected to express high flavonoid contents have shown important anti-inflammatory activity in vitro and in vivo in mice subjected to intestinal inflammation (Wu et al., 2020, 2021). Factors promoting intestinal inflammation, have been correlated with decreased animal performance, multiplication of *C. perfringens* and development of NE (Timbermont et al., 2011). The diets used in our experiment contained ingredients reported to induce inflammation, such as wheat and fishmeal (Branton et al., 1987; Prescott et al., 2016). The uninfected treatment receiving high flavonoid corn (CTL B), had improved BWG and FCR which could be an effect of the reduction of a subclinical intestinal inflammation. This agrees with reports showing that flavonoid-rich feed additives can improve the growth performance and immunity of birds in the absence of clinical disease (Zhou et al., 2019).

It is important to underscore that PennHFD has lower crude protein percentage (12.9%) and lower metabolizable energy (3,285 kcal/kg) compared to the commercial line (13.2% and 3,351 kcal/kg), which shows that the improved results in growth performance may not be explained by these nutritional aspects of PennHFD.

Although the mechanisms of action of the high-flavonoid corn on ameliorating NE in chickens were not investigated in our study, we speculate that the anti-inflammatory and antibacterial properties of flavonoids could play a key role in the control of this disease. Further experiments need to be conducted to understand the mechanisms of action that resulted in amelioration of the inflammatory process seen in this study.

In conclusion, the addition of a flavonoid-rich corn in the diets of broilers undergoing experimental NE resulted in reduced mortality, reduced incidence of intestinal lesions and improved growth performance. In addition, birds that were not challenged with NE also had improved growth performance when fed a high-flavonoid corn. Therefore, high-flavonoid corn may serve as a potential alternative for improving health and performance in the absence of antimicrobials in birds challenged with NE or undergoing subclinical enteritis.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. A provisional patent has been filed to protect the intellectual property covered in this work.

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