Growth performance and gastrointestinal responses of broiler chickens fed corn-soybean meal diet without or with exogenous epidermal growth factor upon challenge with *Eimeria* [1]

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ABSTRACT Epidermal growth factor (EGF), a protein known for its mitogenic and anti-apoptotic effects was fed to broiler chickens to evaluate growth performance, gastrointestinal measurements, and apparent retention (AR) of components upon challenge with *Eimeria*. A total of 216, d old male broiler chicks (Ross 708) were placed in cages (6 birds/cage) and allocated to treatments. The treatments were: 1) control (*Lactotobacilli lactis* fermentation supernatant without EGF), 2) 80 μg of EGF/kg BW/d, and 3) 160 μg of EGF/kg BW/d. A basal antibiotic-free corn-soybean diet containing TiO_2 was used. Birds were offered fresh feed with respective treatments on daily basis and had free access to drinking water for 14 d. On d 5, birds (6 replicates per treatment) were challenged with 1 mL of *E. acervulina* and *E. maxima* mixture via oral gavage and the other 6 replicates were given sham. Growth performance was measured in pre- (d 0 to 5) and post- (d 6 to 14) challenge periods. Two birds per cage were necropsied on d 10 for intestinal lesion scores and tissue samples for histomorphology and expression of select intestinal genes. Excreta samples for AR of components and oocyst shedding were taken d 10 to 13 and all birds were necropsied on d 14 for gastrointestinal weight. The EGF linearly ($P < 0.05$) increased BWG before challenge. There was no EGF and *Eimeria* interaction ($P > 0.05$) on growth performance, AR of GE, and intestinal histomorphology; the main effects were such that *Eimeria* depressed ($P < 0.01$) BWG, FCR, AR of DM, crude fat, and GE, and villi height to crypt depth ratio. An interaction between EGF and *Eimeria* ($P < 0.05$) on indices of gut function was such that EGF improved expression of genes for nutrient transporters and tight junction proteins in *Eimeria* challenged birds whilst no effect in non-challenged control. In conclusion, *Eimeria* challenge reduced growth performance and impaired gut function; EGF showed beneficial effects on growth pre-challenge and improved indices of gut function upon *Eimeria* challenge.

Key words: broiler, coccidiosis, *Eimeria*, epidermal growth factor, gut health and function

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INTRODUCTION

Nutritionists perceive a dysfunctional gastrointestinal tract as a potential rate-limiting factor in the survival and productivity of poultry. This perception has been fostered by the emergence of ideas and concepts concerning the development and function of the digestive tract in the light of advances in genetic improvement and restriction on the use of antibiotic growth promoters and anti-coccidial drugs (Roberts et al., 2015). Genetic selection for increased meat production efficiency has dramatically altered the physiological timeline of broilers (Ferket, 2012). For example, between 2005 and 2010, the length of time to raise 2.27 kg broiler reduced by 0.74 d per year (Gous, 2010). The significance of decreasing time to slaughter weight is that embryonic and early post-hatch periods will increasingly constitute a greater proportion of a bird's life. It has been estimated that 21-day incubation period and the 10-day post-hatch period accounts for about 50% of a 2.5 kg broiler (Ferket, 2012). The capacity of the intestine to absorb and assimilate nutrients may pose a constraint upon the rate of growth of newly hatched (Croom et al., 1999; Sklan and Noy, 2003; Gilbert et al., 2007). Studies have shown that up to 10 d post-hatch,

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the small intestine of the hatchling accounts for a larger percentage of whole body weight (Wijtten et al., 2012). This developmental pattern is believed to reflect a survival strategy in which great importance is placed on development of nutrient supply functions early in life so that post-absorptive growth functions can be maximized later in life cycle (Lilja, 1983; Ferket, 2012).

Epidermal growth factor (**EGF**) is made up of 53 amino acids single chain polypeptide and it is a critical component of mammalian colostrum and milk with a broad range of bioactivities on the intestinal epithelium, including stimulation of cellular proliferation, differentiation, and intestinal maturation in neonates (Jaeger et al., 1990; Playford and Wright, 1996). Epidermal growth factor is also produced and secreted from numerous sites along the intestinal tract as well as in saliva and bile secretions (Playford and Wright, 1996). The peculiarity is that the EGF binds to the EGF receptor (also known as ErbB1), a transmembrane receptor tyrosine kinase of the ErbB family (Thompson, 1988; Playford and Wright, 1996). This binding leads to autophosphorylation of tyrosine kinase receptor and subsequent activation of signal transduction pathways involved in regulating cellular survival, growth, proliferation, and differentiation (Playford and Wright, 1996; Oda et al., 2005). The EGF receptor is found along the length of the intestine on both the luminal and basolateral surfaces of the epithelial cells and exists in many species, including poultry (Lax et al., 1988; Playford and Wright, 1996). There is a body of literature showing gut health and function benefits of supplemental exogenous EGF in neonate or weaned mammals (James et al., 1987; Barnard et al., 1995; Kang et al., 2010). However, EGF has not been thoroughly assessed for its ability to stimulate gastrointestinal growth and function in broiler chicks post-hatch and how this relates to growth performance.

Coccidiosis is a disease of significant importance to the global poultry industry accruing more than US$6 billion dollars in morbidity and mortality losses (Chapman, 2014; Chapman et al., 2016). Pathogenesis entails protozoa *Eimeria* invading the intestinal cells as part of the life cycle. The resulting intestinal damage impairs nutrients digestion and absorption, gut barrier function, and ultimately leads to bacterial infections particularly necrotic enteritis (Chapman, 2014). Concerns over the development of resistant *Eimeria* species to existing anti-coccidial drugs and restrictive use of antibiotics to control secondary bacterial infections is making it imperative to explore alternative strategies for maintaining intestinal functionality. Administration of EGF has been shown to play a protective role in a variety of intestinal insults by either reducing injury (Buret et al., 1998), accelerating repair (Chao et al., 2003), or reducing enteropathogen colonization and translocation (Buret et al., 1998; Buret et al., 2002). Of particular interest to the context of the present study is the finding that oral administration of EGF altered colonization of *Campylobacter jejuni* in the intestinal tract of pullets and protected against pathogen-induced barrier defects (Lamb-Rosteski et al., 2008). There is dearth of data as to whether the EGF, a protein known for its mitogenic and antiapoptotic effects (Jaeger et al., 1990; Playford and Wright, 1996), could be beneficial in broiler chicks subjected to *Eimeria* challenge.

An EGF prototype from a microbial production system was shown to improve growth performance in newly weaned piglets linked to enhanced gastrointestinal development (Cheung et al., 2009; Kang et al., 2010; Bedford et al., 2012; Bedford et al., 2015). It was hypothesized that feeding this EGF prototype will promote gastrointestinal development in chicks and reduce negative effects of *Eimeria* challenge on growth performance, indices of gut health, and function. Therefore, the objective was to evaluate the effect of EGF supplementation on growth performance, nutrients retention, indices of gut health, and function in broilers subjected to *Eimeria* challenge.

MATERIALS AND METHODS

The experimental protocol (#3521) was reviewed and approved by the University of Guelph Animal Care Committee and birds were cared for in accordance with the Canadian Council on Animal Care guidelines (CCAC, 2009).

Epidermal Growth Factor, Production, and Preparation

Lactotobacilli lactis expressin porcine was generated via a recombinant approach as previously described (Cheung et al., 2009; Bedford et al., 2012; Bedford et al., 2015). Briefly, the mature EGF sequence was amplified from porcine RNA then ligated into an expression vector which was transformed into *L. lactis* and fermented in 3 L batches in M17 media (Oxoid, Basingstoke, United Kingdom) supplemented with 1% glucose and 0.001 g/mL erythromycin (Thermo Fisher Scientific, MA). Fermentations ran for 22 h in a Winpact fermentation system (Major Science, CA) at 32°C (Bedford et al., 2015). Fermentations were prepared for delivery to chicks by centrifuging the whole fermented culture at 10,000 × *g* for 15 min to pellet the bacterial cells and obtain the supernatant. Western blot analysis was performed to determine EGF concentration in the supernatant.

Birds and Housing

A total of 216 (males) day old broiler chicks were procured from a local hatchery (Maple Leaf Foods, New Humburg, ON, Canada) and placed in battery cages (6 birds/cage). The cages (each measuring 20″ × 30″; Ford Dickison Inc., Mitchell, Ontario, Canada) were housed in an environmentally controlled room. The room had a total of 64 cages installed in two rows

Table 1. Ingredients composition of the basal diet (%, as fed)

Ingredient, %	Amount %
Corn	38.54
Soybean meal	37.21
Rye	10.00
Soybean oil	8.70
Vitamin and trace mineral premix[1]	1.00
Mono calcium phosphate	1.50
Limestone	1.33
Sodium bicarbonate	0.51
Titanium dioxide	0.50
DL-Methionine	0.32
Lysine-HCL	0.14
L-Threonine	0.10
Tryptophan	0.10
Salt	0.04
Titanium dioxide	0.50
Calculated provisions	
AME, mcal/kg	3.10
CP, %	21.50
SID Lys, %	1.15
SID Met, %	0.60
SID Met + Cys, %	0.87
SID Thr, %	0.77
SID Trp, %	0.33
Ca, %	0.87
P, %	0.65
Avail. P, %	0.44
Na, %	0.16

Vitamin mineral premix provided per kilogram of diet: vitamin A, 880,000 IU; vitamin D3, 330,000 IU; vitamin E, 4,000 IU; vitamin B12, 1,200 mcg; biotin, 22,000 mcg; menadione, 330 mg; thiamine, 400 mg; riboflavin, 800 mg; pantothenic acid, 1500 mg; pyridoxine, 300 mg; niacin, 5,000 mg; folic acid, 100 mg; choline, 60,000 mg; iron, 6,000 mg; copper, 1,000 mg.

separated by a 36″ walkway and cages in a row stacked in two tiers of 16 cages each. For this study, 36 cages were used and the room temperature was set at 32°C on d 0 and gradually brought down to 29°C by d 13. The lighting program was 23 h of light (20+ LUX) from d 0 to 3 followed by 20 h of light (10 to 15 LUX) from d 4 onward. The cages were equipped with feeders and nipples.

Experimental Treatments and Feeding

A basal diet was formulated to meet breeder (Ross 708) nutrient specifications (Table 1). The diet was prepared in mash form, contained TiO_2 as indigestible marker, and had no antibiotics or anti-coccidial drugs. Three experimental treatments were tested: 1) control (fermentation supernatant without EGF), 2) EGF containing supernatant (80 μg/kg of BW/d), and 3) EGF containing supernatant (160 μg/kg of BW/d). The EGF was not added to the feed at the time of preparation but was rather supplied fresh on a daily basis in accordance with our previous piglet trials (Kang et al., 2010; Bedford et al., 2012; Bedford et al., 2015). Briefly, the absolute amount of EGF allocated to each pen was determined on a daily basis, based upon the number of chicks per cage and expected breeder's growth curve (Aviagen, Ross 708). The EGF chicks were given volumes of supernatant to achieve 80 and 160 μg EGF/kg BW/d throughout the study. The control chicks and chicks receiving 80 μg EGF/kg BW/d were fed matching volumes to 160 μg EGF/kg BW/d which was achieved by supernatant free of EGF.

Experimental Procedures and Sampling

The three treatments were allocated to cages in a completely randomized block (row of cages) design to give 12 replicates per treatment. Birds had free access to treatments and drinking water for 14 d; feed was replenished throughout the day and feed refusals were weighed daily, for determining cage feed intake. On d 5, birds (6 replicates per treatment on the left row of cages) were challenged with 1 mL of *Eimeria* culture (25,000 oocysts of *E. acervulina* and 5,000 oocysts of *E. maxima*) in distilled water suspension via oral gavage and the other 6 replicates (non-challenged control, on the right row of cages) were given equal volume of distilled water. The separation of right and left cages was an effort to minimize cross-contamination of non-challenge cages. Moreover, daily checks and servicing of the birds started with non-challenged birds followed by challenge birds. The *Eimeria* culture and challenge protocols were provided by Dr. John Barta of Department of Pathobiology, University of Guelph. Body weight and feed intake was monitored during pre- (d 0 to 5) and post- (d 6 to 14) challenge periods for calculation of BWG and FCR. Two birds per cage were Necropsied on d 10 for intestinal tissue samples. Jejunum was immediately located and excised at duodenal loop and 2 cm anterior to Markel diverticulum. Segments (~3 cm) of mid-jejunum were excised and placed in buffered formalin for histomorphology analysis (Kiarie et al., 2007). Additional segments of mid-jejunum (~1 cm) were placed in a 2 mL tube filled with 1.2 mL Ambion RNAlater (Life Technologies Inc., Burlington, ON, Canada). These samples were placed on ice and immediately transported to the lab and stored at −20°C until required for mRNA analysis of digestive enzymes, nutrients transporters, tight junction proteins, and cytokines. Lesion scores in intestinal regions (duodenum, jejunum, ileum, and ceca) were assessed blindly as described by Price et al. (2014) using a scale of 0 (none) to 4 (high) (Johnson and Reid, 1970). Excreta samples for apparent retention (**AR**) of components and oocyst shedding were collected from d 10 to 13. The excreta samples for oocyst counts were collected, stored at 4°C, and processed in accord with Price et al. (2014). The excreta samples for AR of components were frozen at −20°C until required for analyses. All birds were Necropsied by cervical dislocation on d 14 for gastrointestinal weight measurements

Sample Processing and Laboratory Analysis

Fixed jejunal tissues were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin

for morphological examinations. In each cross-sectioned tissue, at least 4 to 5 complete villous-crypt structures were examined under a Leica DMR microscope (Leica Microsystems, Wetzlay, Germany). Villous height and crypt depth were measured using a calibrated micrometer (Kiarie et al., 2007). The number of oocysts were determined by the McMaster counting chamber technique using saturated NaCl as the flotation medium (Price et al., 2014). Each sample was counted twice and the 2 counts were averaged to provide a single mean count; the mean count was then divided by the fecal weight in grams to calculate the oocysts per gram of excreta.

The excreta samples were thawed, pooled by cage, and subsequently freeze dried. Samples of the basal diet and freeze-dried excreta samples were finely ground. All samples were analyzed for DM, gross energy, crude fat, and titanium. Dry matter determination was carried out according to standard procedures (AOAC International., 2005), method 930.15). Gross energy was determined in a bomb calorimeter (IKA - WERKE bomb calorimeter [C7000, GMBH & CO., Staufen, Germany]) using benzoic acid as a calibration standard. Crude fat content was determined following Soxhlet extraction procedure. Briefly, fat in samples were dissolved by repeatedly washing with petroleum-ether by refluxing in a Soxtec apparatus (Soxtec System HT 1043 Extraction Unit, Höganäs, Sweden). The solubilized fat was then collected in the distillation flask and the increase in weight of the flask represented the dissolved fat. Titanium content was measured on a UV spectrophotometer following the method of Short et al. (1996).

Jejunal tissue samples were used to measure expression of genes for digestive enzymes, nutrient transporters, cytokines, and tight junction proteins. The assessed digestive enzymes were maltase and sucrase. Transporters were neutral AA transporter, high affinity glutamate transporter (**Glut**), cationic AA transporter (**CAT1**), peptide transporter 1, sodium glucose transporter 1 (**SGLT1**), and sodium-independent Cys-Glu antiporter (**xCT**). Additional genes assessed were toll like receptors (**TLR 2** and **TLR 4**), tight junction proteins: occuludin (**OCLN**), and zonola occludens. Proliferating cell nuclear antigen (**PCNA**) was also assessed. The Total RNA was isolated from 50 mg of homegnized jejunal samples using RNAqueous total RNA isolation kit (Life Technologies Inc.) according to the manufacturer's instructions. The RNA samples were treated with DNase and the quality was checked by 1% agarose gel electrophoresis with bands stained with SYBR Green (Life Technologies Inc.). The concentration and OD260:OD280 ratio of extracted RNA sample was measured using Nanodrop UV-Vis spectrophotometer and OD260:OD280 ratios were between 1.9 and 2.1. The RNA samples were stored −80°C until further analysis. A total of 1 μg RNA was used to synthesize the first strand cDNA using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) according to the manufacturer's instructions.

Primers for real-time PCR analysis were designed with Primer-Blast (Table 2) and synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Real-time PCR was carried out using SYBR Green Supermix on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). One μL of cDNA was added to a total volume of 25 μL containing 12.5 μL SYBR Green mix, and 1 μM each of forward and reverse primers. We used the following conditions: denaturation 15 s at 95°C, annealing 15 s at 56°C, extension 30 s at 72°C, repeating 45 cycles. We used β-actin and Glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) as the internal control to normalize the amount of starting RNA used in the real-time RT-PCR for all the samples. A melting curve program was conducted to confirm the specificity of each product and the size of products was verified on SYBR-stained 2% agarose gels in Tris acetate–EDTA buffer. Real-time RT-PCR analyses were all performed in duplicate for each sample. Threshold cycle (**Ct**) values were obtained at the cycle number at which the gene is amplified beyond the threshold of 30 fluorescence units. Real-time PCR efficiencies were acquired by amplification of dilution series of DNase-treated RNA according to the formula $10(-1/\text{slope})$ (Pfaffl, 2001). The efficiencies of all primers used this study were between 96 and 105%. The target gene expression was normalized with that of a selected reference gene and relative gene expression was determined by using $R = 2(\text{Ct(reference)}-\text{Ct(test)})$ (Kleta et al., 2004). The average of Ct values of two reference genes (GAPDH and β-actin) was used for the normalization of the expression of target genes.

Calculations and Statistical Analysis

The AR of components was calculated according to Kiarie et al. (2014). Pre-challenge growth performance data was subjected to 1-way ANOVA of the GLM procedures (SAS Inst. Inc., Cary, NC), with diet as a fixed factor. Post-challenge growth performance (with d 5 BW as co-variate), gastrointestinal measurements, and AR of components data were subjected to 2-way ANOVA with fixed effects of treatment, *Eimeria* challenge and their interactions. There was no oocyst shedding detected in non-challenged birds, in this context data for the oocyst shedding in challenged birds were analyzed using the Proc Mixed procedure of SAS as repeated measurements with the fixed effects of treatment, time, and treatment × time interaction. Similarly, lesion scores were not detected in non-challenged birds and as such the data for challenged birds was subjected to 1-way ANOA with treatment as fixed effect. Linear and quadratic responses of EGF were also assessed. An α level of $P \leq 0.05$ was used as the criterion for statistical significance.

Table 2 Forward and reverse primers for quantitative PCR[1]

Genes	Sequence (5'-3')[2]	Genbank ID
Neutral amino acid transporter	FP:GCTCTACAGTGTTTGGAACCC RP:AAACTAGGCACACCAGCGAT	XM_419056
High affinity glutamate transporter	FP:GATTGTTCTGAGCGCTGTCG RP:ACCAAAGGCATCTCCCAAG	XM_424930.5
Cationic amino acid transporter 1	FP:AACTGGGTTTCTGCCAGAGG RP:AACCCATGATGCAGGTGGAG	NM_001145490.1
Peptide transporter 1	FP:CTTTGGCTACCCCTTGAGCA RP:AAAGTTGTCATCCCACCGCA	NM_204365
Sodium glucose transporter 1	FP:ATGCTGCGGACATCTCTGTT RP:TCCGTCCAGCCAGAAAGAAT	NM_001293240.1
Sodium-independent Cys-Glu antiporter	FP:TGAGCTGGGAACGTGCATTA RP:AGGGCGAATAACCAGCAGTT	XM_426289.5
Maltase	FP: AAGAACCTCTGCAACCTCCG RP:TCTCCGTCCACCCTATAGC	XM_015273018.1
Sucrase	FP:GCAACAAGACAAGCCATCGA RP:AGCCAGTGTCCTGTGTGCTTT	XM_015291762
Toll like receptor 2	FP:TGGTGGTCGTTGGGTACAAG RP:AGCAGATGTCTTTCGTGGGG	NM_204278.1
Toll like receptor 2	FP:AGGCACCTGAGCTTTTCCTC RP:TACCAACGTGAGGTTGAGCC	NM_001030693.1
Occuludin	FP:ACGGCAGCACCTACCTCAA RP:GGGCGAAGAAGCAGATGAG	NM_205128.1
Zonula occludens-1	FP:TATGCACAAGGAGGTCAGCC RP:TTGGCCGAAGCATTCCATCT	XM_015278981.1
Zonula occludens-2	FP:ATCCAAGAAGGCACCTCAGC RP:CATCCTCCCGAACAATGCCT	NM_2040918
Proliferating cell nuclear antigen	FP:GCCATGGGCGTCAACCTAAA RP:AGCCAACGTATCCGCATTGT	NM_204170.2
β-actin	FP:AATGGCTCCGGTATGTGCAA RP: GGCCCATACCAACCATCACA	NM_205518.1
Glyceraldehyde 3-phosphate dehydrogenase	FP:ACTGTCAAGGCTGAGAACGG RP:CACCTGCATCTGCCCATTTG	NM_204305

[1](Cheled-Shoval et al., 2011);(Zhang et al., 2012). The primers had similar Tm values and therefore 56°C was the annealing temperature for all primers.
[2]FP, forward primer; RP, reverse primer.

Table 3. Growth performance of broiler chicken fed corn diet without or with epidermal growth factors, non-challenge birds

		Day 0 to 5			Day 0 to 14		
EGF[1], μg	Initial BW, g	BWG, g	Feed intake, g	FCR	BWG, g	Feed intake, g	FCR
0	40.8	45.7[b]	82.8	1.813	320	675	2.16
80	41.0	48.6[a]	84.1	1.738	331	678	2.09
160	41.2	49.9[a]	85.6	1.719	356	655	1.84
SEM	0.36	1.00	1.61	0.054	15.56	21.80	0.145
P- value	-	0.029[L]	0.484	0.237	0.121	0.715	0.147

[1]Epidermal growth factor, μg/kg BW based on projected growth curve of Ross 708.
Means assigned different letters (a, b) within a response criteria are significantly different, $P < 0.05$.
[L]Linear effects of EGF.

RESULTS

Growth Performance and Nutrient Retention

The EGF linearly ($P = 0.03$) increased BWG before challenge (Table 3). There was no ($P > 0.05$) effect of EGF on feed intake and FCR before challenge. When growth performance data for the non-challenged birds was examined from d 0 to 14, there was no effect of EGF on BWG, feed intake, and FCR (Table 3). Growth performance data for the post-challenge period (d 6 to 14) are shown in Table 4. There was no ($P > 0.05$) interaction between *Eimeria* challenge and treatment or the main effects of EGF.*Eimeria* challenge depressed ($P < 0.01$) BWG by 38% and feed intake by 21% and increased FCR by 31% (Table 4). There was no EGF or EGF and *Eimeria* interaction ($P > 0.05$) on AR of DM, crude fat, and gross energy; the main effects were such that *Eimeria* depressed ($P < 0.01$) AR of these components (Table 4).

Oocyst Shedding, Lesion Scores, Gut Weight, and Histomorphology

Oocyst shedding and intestinal (duodenum, jejunal, and ileum) lesion scores were only observed in birds challenged with *Eimeria* and there was no EGF effects ($P > 0.05$) on these parameters (data not shown). The oocyst shedding was 87,479, 910,449, 428,583, and 57,868 (SEM = 48,074) oocysts per gram of excreta for d 5, 6, 7, and 8 post-challenge, respectively. The

Table 4. Growth performance and apparent retention of components in broiler chickens fed corn-soybean meal based diet without or with epidermal growth factor and challenged with *Eimeria*

Treatments		Final BW, g	BWG, g	Feed intake, g	FCR	AR, %		
Eimeria[1]	EGF[2], μg					DM	Crude fat	Gross energy
No	0	373	284	589	2.113	74.7	82.4	75.9
No	80	372	282	594	2.171	73.1	82.0	74.6
No	160	390	301	570	1.922	74.2	81.0	75.5
Yes	0	264	174	460	2.699	58.0	−18.1	48.6
Yes	80	277	186	469	2.813	55.5	−10.8	50.9
Yes	160	265	176	462	2.640	61.5	−9.0	53.1
	SEM	15.3	15.3	23.3	0.270	2.05	4.45	1.64
Main effect, *Eimeria*								
	No	379[a]	289[a]	585[a]	2.069[b]	74.0[a]	81.8[a]	75.3[a]
	Yes	268[b]	179[b]	464[b]	2.717[a]	58.3[b]	−12.6[b]	50.9[b]
	SEM	8.70	8.72	13.29	0.150	1.19	2.57	0.95
Main effect, EGF, μg								
0		319	229	525	2.406	66.3	32.1	62.3
80		324	234	532	2.492	64.3	35.6	62.7
160		328	238	516	2.281	67.8	36.0	64.3
SEM		10.8	10.8	16.44	0.190	1.45	4.02	1.16
Probabilities								
Eimeria		<0.01	<0.01	<0.01	0.006	<0.01	<0.01	<0.01
EGF		0.836	0.844	0.810	0.744	0.247	0.633	0.421
*Eimeria**EGF		0.619	0.630	0.892	0.971	0.452	0.473	0.311

[1]Chicks were orally gavaged with a 1 mL mixture of 25,000 *E. acervulina* and 5,000 *E. maxima* on d 5.
[2]Epidermal growth factor, μg/kg BW based on projected growth curve of Ross 708.
Means assigned different letters (a, b) within a factor of analysis (*Eimeria*, EGF and their interactions) are significantly different, $P < 0.05$.

Table 5. Gastrointestinal weight and jenunal histomorphology of broiler chickens fed corn-soybean meal based diets without or with epidermal growth factor and challenged with *Eimeria*

Eimeria[1]	EGF[2], μg	BW[3], g	Relative weight, mg/g BW			Jejunal histomorphology[4]		
			Gizzard	Small intestine	Ceca	VH, μm	CD, μm	VH:CD
No	0	394	26.5	48.8	8.11	906	144	6.454
No	80	403	26.2	45.5	7.61	967	155	6.296
No	160	431	25.4	48.0	7.60	940	154	6.457
Yes	0	278	33.5	76.2	10.9	577	286	2.012
Yes	80	277	33.7	78.3	10.3	593	285	2.186
Yes	160	280	33.6	73.8	10.5	530	306	1.790
SEM	17.7	1.10	2.23	0.44	33.1	14.7	0.310	
Main effects, *Eimeria*								
	No	409[a]	26.0[b]	47.4[b]	7.78[b]	935[a]	151[b]	6.402[a]
	Yes	278[b]	33.6[a]	76.1[a]	10.6[a]	567[b]	292[a]	1.996[b]
	SEM	10.2	0.63	1.29	0.25	19.1	8.51	0.18
Main effects, EGF								
	0	336	30.02	62.5	9.49	742	215	4.233
	80	340	20.0	61.9	8.93	777	220	4.241
	160	356	29.5	60.9	9.08	735	230	4.124
	SEM	12.5	0.774	1.58	0.311	23.4	10.4	0.220
Probabilities								
Eimeria		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
EGF		0.515	0.889	0.766	0.433	0.390	0.613	0.916
EGF**Eimeria*		0.595	0.845	0.278	0.941	0.473	0.756	0.664

[1]Chicks were orally gavaged with a 1 mL mixture of 25,000 *E. acervulina* and 5,000 *E. maxima* on d 5.
[2]Epidermal growth factor, μg/kg BW based on projected growth curve of Ross 708.
[3]Average bodyweight of two birds.
[4]VH, villi height; CD, crypt depth.
Means assigned different letters (a, b) within a factor of analysis (*Eimeria*, EGF and their interactions) are significantly different, $P < 0.05$.

duodenal lesion scores were 2.33, 2.50, and 2.75 (SEM = 0.26) for the control, 80 μg of EGF/kg BW/d, and 160 μg of EGF/kg BW/d, respectively; the respective values for the jejunum were 1.55, 1.55, and 1.67 (SEM = 0.21) and ileum 0.35, 0.42, and 0.33 (SEM = 0.18). There was no ($P > 0.05$) interaction between *Eimeria* and EGF or the main effect of EGF on gastrointestinal weight and jejunal histomorphology (Table 5). The main effects of *Eimeria* challenge was such that challenged birds exhibited heavier ($P < 0.01$) gizzard (33.6 vs. 26.0 mg/g BW), small intestine (76.2 vs. 47.4 mg/g BW), and ceca (10.6 vs. 7.78 mg/g BW)

Table 6. Jejunal expression of genes for nutrient transporters and digestive enzymes in broiler chickens fed corn-soybean meal based diets without or with epidermal growth factor and challenged with *Eimeria*

Treatments		Nutrient transporters[3]						Enzymes	
Eimeria[1]	EGF[2], μg	NAA	Glut	CAT1	PEPT1	SGLT1	xCT1	Maltase	Sucrase
No	0	0.35	1.32^{a}	0.29^{b}	1.57	59.3^{a}	0.09^{ab}	4.59^{ab}	26.2^{ab}
No	80	0.28	1.09^{ab}	0.44^{b}	0.98	48.4^{ab}	0.16^{ab}	3.88^{ab}	17.3^{ab}
No	160	0.48	1.29^{a}	0.41^{b}	1.41	50.7^{ab}	0.21^{ab}	2.12^{b}	16.9^{b}
Yes	0	0.13	0.27^{b}	0.49^{b}	0.73	32.8^{b}	0.07^{b}	2.18^{b}	18.3^{ab}
Yes	80	0.77	1.09^{ab}	0.80^{ab}	2.11	40.2^{ab}	0.18^{ab}	4.32^{ab}	35.8^{a}
Yes	160	0.69	1.20^{ab}	1.26^{a}	1.64	57.0^{a}	0.40^{a}	6.15^{a}	33.5^{ab}
	SEM	0.25	0.32	0.23	0.54	8.41	0.10	1.07	6.37
Main effect, *Eimeria*									
	No	0.37	1.23	0.47^{b}	1.32	52.8	0.16	3.53	20.1
	Yes	0.53	0.85	0.85^{a}	1.49	43.3	0.22	4.22	29.2
	SEM	0.15	0.22	0.15	0.31	4.86	0.06	0.62	3.68
Main effect, EGF, μg									
	0	0.24	0.79	0.39	1.15	46.05	0.08^{b}	3.38	22.2
	80	0.52	1.09	0.62	1.54	44.29	0.17^{ab}	4.10	26.5
	160	0.58	1.24	0.83	1.52	53.82	0.30^{a}	4.14	25.2
	SEM	0.19	0.23	0.17	0.38	5.95	0.07	0.76	4.51
Probabilities									
Eimeria	0.415	0.182	0.020	0.701	0.177	0.478	0.437	0.094	
EGF	0.338	0.427	0.170	0.705	0.493	0.038^{L}	0.736	0.785	
*Eimeria**EGF	0.064	0.043	0.009^{L}	0.060	0.034	0.028	0.020^{L}	0.039^{Q}	

[1]Chicks were orally gavaged with a 1 mL mixture of 25,000 *E. acervulina* and 5,000 *E. maxima* on d 5, intestinal samples collected 5 (day 10 of life) d after challenge.

[2]Epidermal growth factor, μg/kg BW based on projected growth curve of Ross 708.

[3]NAA, neutral amino acids transporter; Glut, glutamate transporter; CAT1, cationic amino acids transporter; SGLT1, sodium dependent glucose transporter; PEPT1, Peptide transporter 1; xCT1, sodium independent Cys-Glu antiporter.

Means assigned different letters (a, b) within a factor of analysis (*Eimeria*, EGF and their interactions) are significantly different, $P < 0.05$.

^{L}linear and ^{Q}quadratic effects of EGF.

than non-challenged control. *Eimeria* challenge reduced ($P < 0.01$) villi height and increased ($P < 0.01$) crypt depth resulting in decreased ($P < 0.01$) villi to crypt depth ratio (Table 5).

Jejunal Expression of Genes for Digestive Enzymes, Nutrient Transporters, Cytokines, and Tight Junction Proteins

There was an interaction between *Eimeria* challenge and EGF on expression of maltase (linear, $P = 0.02$) and sucrase (quadratic, $P = 0.04$) such that EGF improved expression of maltase and sucrase in *Eimeria* challenged birds and not in non-challenged birds (Table 6). However, the expression of maltase in challenged birds fed the highest dose of EGF was higher (6.15 vs. 2.12, $P = 0.020$) than in the non-challenged birds fed similar dose. The data for nutrient transporters are shown in Table 6. Interaction ($P < 0.05$) between *Eimeria* and EGF was observed for the expression of Glut, CAT1, SGLT1, and xCT. In this context, birds fed EGF maintained similar ($P > 0.05$) pattern of Glut, SGLT1, and xCT without or with *Eimeria* challenge whilst the expression of these genes were down regulated ($P < 0.05$) by *Eimeria* challenge in the control birds. With respect to CAT1, EGF linearly increased expression. The main effects on nutrients transporters was such that *Eimeria* challenge increased ($P = 0.020$) the expression of cationic amino acids transporter and EGF linearly ($P = 0.04$) increased the expression of sodium-independent Cys-Glu antiporter.

Interaction between *Eimeria* and EGF on TLR and tight junction proteins was such that EGF linearly increased the expression of TLR4 ($P = 0.03$) and OCLN ($P = 0.02$) in challenged birds and not in non-challenge birds (Table 7). The expression of OCLN in challenged birds receiving highest dose of EGF was much higher than in non-challenged birds receiving this dose. A similar observation was made for ZO2, where the challenged birds fed EGF showed higher ($P = 0.03$) expression than non-challenged birds fed EGF. The interaction ($P = 0.05$) between *Eimeria* and EGF on PCNA was such that the expression of PCNA in challenged birds receiving highest dose of EGF was much higher than in non-challenged birds receiving this dose. In general, *Eimeria* challenge increased the expression of ZO2 ($P = 0.02$) and PCNA ($P = 0.03$).

DISCUSSION

Perinatal nutrition of the chick is becoming very critical for the growth and feed efficiency of the modern broiler genotypes (Uni and Ferket, 2004; Ferket, 2012). Early nutrients and factors access not only promote greater speed in growth and feed efficiency, but also favors development of the immunological system, making the birds more resistant to pathogenic organisms (Croom et al., 1999; Sklan and Noy, 2003; Gilbert et al., 2007). The mucosal epithelium of the chick small

Table 7. Jejunal expression of genes for cytokines, tight junction proteins, and proliferating cell nuclear antigen in broiler chickens fed corn-soybean meal based diets without or with epidermal growth factor and challenged with *Eimeria*

Treatments		Toll like receptors[3]		Tight junction proteins[4]			PCNA[5]
Eimeria[1]	EGF[2], μg	TLR2	TLR4	Occuludin	ZO1	ZO2	
No	0	0.33	0.23^{ab}	1.21^{ab}	1.31	1.36^{ab}	3.69^{ab}
No	80	0.53	0.36^{ab}	0.73^{ab}	1.43	1.27^{b}	3.55^{ab}
No	160	0.62	0.40^{ab}	0.56^{b}	1.32	1.26^{b}	3.47^{b}
Yes	0	0.33	0.12^{b}	0.64^{b}	1.23	1.38^{ab}	3.57^{ab}
Yes	80	0.89	0.43^{ab}	0.93^{ab}	1.33	1.96^{a}	5.81^{a}
Yes	160	1.26	0.84^{a}	1.29^{a}	2.24	1.87^{a}	5.88^{a}
	SEM	0.34	0.22	0.22	0.37	0.22	0.81
Main effect, *Eimeria*							
	No	0.49	0.33	0.83	1.36	1.30^{b}	3.57^{b}
	Yes	0.83	0.46	0.96	1.60	1.74^{a}	5.08^{a}
	SEM	0.19	0.13	0.13	0.21	0.13	0.47
Main effect, EGF							
	0	0.33	0.18	0.93	1.27	1.37	3.63
	80	0.71	0.39	0.83	1.38	1.62	4.68
	160	0.94	0.62	0.93	1.78	1.56	4.68
	SEM	0.238	0.157	0.156	0.261	0.155	0.575
Probabilities							
Eimeria		0.235	0.472	0.504	0.429	0.021	0.031
EGF		0.077	0.054	0.878	0.172	0.489	0.335
*Eimeria**EGF		0.636	0.026^{L}	0.021^{L}	0.055	0.026	0.048

[1]Chicks were orally gavaged with a 1 mL mixture of 25,000 *E. acervulina* and 5,000 *E. maxima* on day 5, intestinal samples collected 5 (day 10 of life) d after challenge.
[2]Epidermal growth factor, μg/kg BW based on projected growth curve of Ross 708.
[3]Toll-like receptor 2 and 4.
[4]Zonula occludens 1and 2.
[5]Proliferating cell nuclear antigen.
Means assigned different letters (a, b) within a factor of analysis (*Eimeria*, EGF and their interactions) are significantly different, $P < 0.05$.
[L]Linear effects of EGF.

intestine constitutes a highly dynamic interface with the external environment through the delivery, processing and absorption of nutrients. Enterocytes undergo rapid development from non-polar cellular structure to fully functioning, elongated, polar, enterocyte structure in the first two weeks of life (Uni et al., 1999; Geyra et al., 2001). Epidermal growth factor was first isolated from mouse salivary glands and recognized by its ability to accelerate the eruption of mouse teeth and the opening of eyelids of newborn mice (Cohen, 1962). EGF is a heat- and acid-stable peptide that produces a variety of biologic responses, most of which involve regulation of cell replication, cell movement, and cell survival (Playford and Wright, 1996; Oda et al., 2005). In the gastrointestinal tract, EGF enhances proliferation and differentiation of epithelial cells, but also has significant effects on healing of damaged mucosa or on intestinal adaptation after injury (James et al., 1987; Barnard et al., 1995; Kang et al., 2010). Although, we did not assess the effects of EGF on gut development on d 5, the observed improvement of d 5 BWG could be linked to EGF positive effects on gut development. The EGF assessed in the present study was shown to improve growth performance in piglets linked to enhanced gastrointestinal tract development and function (Kang et al., 2010; Bedford et al., 2012; Bedford et al., 2015). However, we did not observe EGF effects on 6 to 14 d growth performance, d 10 jejunal histomorphology and d 14 gastrointestinal weight. Comprehensive review of avian gastrointestinal development showed, the small intestine of the hatchling accounts for a larger percentage of whole body weight peaking at d 7 to 10 post-hatch (Wijtten et al., 2012). Perhaps, taking the gastrointestinal tract measurements at earlier age would have been ideal to assess the effects of EGF on gastrointestinal development in our study.

Three essential phases characterize the life cycle of *Eimeria* species (multiplication and sexual reproduction in the intestine and sporulation in the external environment) (Chapman, 2014; Chapman et al., 2016). The key aspects of the biology of these organisms was described in early 20th century and includes the liberation of sporozoites from the sporocysts, cell penetration by sporozoites, schizogony and merozoite formation (asexual multiplication), formation of macro- and microgametocytes (sexual reproduction), oocyst wall formation, and sporogony (spore formation) as reviewed by Chapman (2014). In the chicken, different species of *Eimeria* are known to develop in different regions in the gut where, depending on the magnitude of infection, they can cause mild to severe lesions and significant pathology including death (Chapman, 2014; Chapman et al., 2016). Thus *E. acervulina* and *E. praecox* develop in the duodenum extending in heavy infections to the mid-intestine, *E. mitis, E. maxima*, and *E. necatrix* develop in the mid-intestine

extending to the posterior intestine, and *E. tenella* develops in the ceca (Joyner et al., 1978). The hallmark of coccidiosis is the fluid loss and malabsorption of nutrients, inflammation of the intestinal wall with pinpoint hemorrhages and sloughing of epithelia, or complete villi destruction resulting in extensive hemorrhage (Chapman, 2014; Chapman et al., 2016). These characteristics have been utilized in the widely accepted visual system for scoring the severity of lesions in different regions of the gut (Johnson and Reid, 1970). In agreement to this well-known biology, challenge with a culture containing *E. acervulina* and *E. maxima* induced greater lesion scores in upper-mid intestine in the present study. Complemented with oocyst shedding our model arguably induced coccidiosis. Previous research showed that EGF prevented *C. jejuni* colonization of the chicken intestinal tract by preventing *C. jejuni* from invading the intestinal epithelium (Lamb-Rosteski et al., 2008). Lack of EGF effects on lesion scores and oocyst shedding in the present study suggest negligible effects on attenuating *Eimeria* invasion of the intestines to complete its life cycle.

Eimeria invasion of the intestinal epithelium give rise to weight loss, poor feed conversion, diarrhea, and, in extreme cases, death. The failure of parasitized animals to gain weight at normal rates may be partially attributed to anorexia and partially to other factors, including malabsorption (Major and Ruff, 1978; Su et al., 2014, 2015). It has been suggested that diarrhea, as a clinical entity, may be caused by various intestinal malfunctions, including reduced absorption and impaired digestive processes (Chapman, 2014; Chapman et al., 2016). *Eimeria* challenge resulted in structural and functional damages in the small intestine as demonstrated by the histomorphology, digestive enzymes, nutrients transporters, and DM retention data. There is a body of literature linking growth depression effects of coccidiosis to negative effects on the digestive and absorptive capacity in the small intestine (Chapman, 2014; Chapman et al., 2016). For example, the effect of coccidiosis on the absorption of nutrients in birds infected with *E. acervulina* is a well investigated phenomenon and in most cases impaired absorption demonstrated (Turk and Stephens, 1969; Southern and Baker, 1983). Recent research demonstrated *E. acervulina and E. maxima* infection down regulated the expression of digestive and nutrient transporters in poultry (Su et al., 2014, 2015). We hypothesized that EGF, a protein known for its mitogenic and anti-apoptotic effects would remedy *Eimeria* structural and functional damage to the gut. Although we did not observe the effects of EGF on jejunal histomorphology we observed improved expression of nutrients transporters (CAT1, SGLT1, and xCT1) and maltase. These observations extended our previous observations in piglets in which feeding EGF supernatant increased the expression of SGLT1, and glucagon-like peptide-2, mucin2, goblet cell number (Bedford et al., 2015). Collectively suggesting EGF could play a role in attenuating some of the negative effects of *Eimeria* invasion on chicken digestive and absorptive capacity.

The intestinal immune system develops under the dual pressure of protecting the host from pathogenic infections and co-existence with the myriad commensal organisms in the lumen. Toll-like receptors are important components of the avian gut innate immune system that sense conserved microbial patterns and endogenous danger signals (Abreu et al., 2005; Satoh and Akira, 2016). Toll-like receptors (**TLR**) are a class of proteins that play a key role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors usually expressed in sentinel cells such as macrophages and dendritic cells, which recognize structurally conserved molecules derived from microbes (Satoh and Akira, 2016). The TLR convert the recognition of pathogen-associated molecules in the gut into signals for anti-microbial peptide expression, barrier fortification, and proliferation of epithelial cells (Abreu et al., 2005). Healing of injured intestinal epithelium and clearance of intra-mucosal bacteria require the presence of intact TLR signaling (Abreu et al., 2005). Chickens fed EGF showed better expression of TLR4 in the presence of *Eimeria* challenge. The TLR4 is unique among the TLR family in that upon activation it recruits several different adaptor molecules including MyD88 and TIR-domain containing adapter protein (MyD88-dependent pathway) and adapter-inducing IFN-β-related adapter molecule and TIR-domain-containing adapter-inducing IFN-β (MyD88-independent pathway) (Keestra and van Putten, 2008).

Epidermal growth factor has been found to upregulate tight junction protein and to tighten epithelial junctions in canine epithelial MDCK (Madin-Darby Canine Kidney Epithelial) cell lines (Singh and Harris, 2004). Further research has shown that the abnormalities of the tight junctional proteins caused by *Giardia lamblia* or *Cryptosporidium andersoni* or *Campylobacter jejun*i were prevented in epithelial cells pretreated with EGF (Buret et al., 2002; Buret et al., 2003; Lamb-Rosteski et al., 2008). In agreement with these previous studies on other tight junctional proteins and other pathogens, we observed that feeding EGF to broilers challenged with *Eimeria* increased the expression of tight junction proteins (occuludin and ZO2). Cell proliferation in the intestine commonly occur during infection and inflammation to replace damaged enterocytes (Buret et al., 2002; Buret et al., 2003; Lamb-Rosteski et al., 2008). Proliferating cell nuclear antigen is a cofactor of DNA polymerase δ that is highly expressed at the S-phase of the cell cycle and thus it has been used as a marker of cell proliferation (Muskhelishvili et al., 2003). The increased crypt depth in *Eimeria* challenged birds correlated well with increased expression of PCNA and higher gut weight indicating the birds prioritized gut development following intestinal insult. Similarly, an increase of cell proliferation was also observed in the crypt base of coccidia-infected

chickens, which exhibited higher length of the crypts and higher PCNA signals in the duodenum epithelium (Sun et al., 2016). The abundance of PCNA signals represents the total mitotic cells present in the mucosa, including the epithelial population and lamina propria cells (Uni et al., 1998; Uni et al., 2000). As in mammals, the intestinal integrity in birds is regulated tightly by many different signaling pathways which balance cell proliferation and differentiation along the crypt-villus axis (Uni et al., 1998; Uni et al., 2000). Jejunal expression of genes for PCNA were numerically higher in EGF fed birds upon challenge with *Eimeria*, these observations agreed with our previous research that revealed that the proliferation of intestinal cells was significantly greater in the EGF fed piglets upon weaning challenge (Kang et al., 2010).

Due to the ubiquitous and fecund nature of the parasite, finding a commercial flock that is not shedding oocysts is rare (Chapman, 2014). To combat coccidiosis in commercial poultry production, biosecurity, house and flock management, and disease prevention methods must be followed (Chapman, 2014). Proper house and flock management may reduce the risk of contamination among birds or from pests (e.g., vermin or flying insects) in a single flock or between flocks due to carryover of used litter or an improperly cleaned poultry house (Chapman, 2014). Prevention of coccidiosis can rely on anti-coccidial drugs or live vaccination or a combination of these approaches in an integrated rotational program (Chapman, 2009; Chapman et al., 2016). However, concerns over the development of resistant *Eimeria* species to existing anti-coccidial drugs and public concern over drugs use in poultry production are limiting chemotherapy options (Chapman and Jeffers, 2014). The principles of *Eimeria* vaccination involves provision of a mild mixed *Eimeria* species challenge within the first d of chick life to build immunity against exposure to field strains later in life (Chapman, 2014). Vaccination at this age increases the risk of enteric disturbances, including coccidiosis, because it requires up to 10 d to develop an adequate acquired immune response (Kogut and Klasing, 2009). Enteric disturbances at this stage of a chick's life may cause an early reduction in growth and increase the chick's susceptibility to secondary infections (Chapman, 2014). The present study highlights the potential therapeutic benefits of EGF and support a role for EGF in protecting the gastrointestinal tract from colonization with enteropathogens and from microbially induced barrier defects.

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