

Dietary *Curcuma longa* enhances resistance against *Eimeria maxima* and *Eimeria tenella* infections in chickens

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ABSTRACT The effects of dietary supplementation with an organic extract of *Curcuma longa* on systemic and local immune responses to experimental *Eimeria maxima* and *Eimeria tenella* infections were evaluated in commercial broiler chickens. Dietary supplementation with *C. longa* enhanced coccidiosis resistance as demonstrated by increased BW gains, reduced fecal oocyst shedding, and decreased gut lesions compared with infected birds fed a nonsupplemented control diet. The chickens fed *C. longa*-supplemented diet showed enhanced systemic humoral immunity, as assessed by greater levels of serum antibodies to an *Eimeria* merozoite protein, MIC2, and enhanced cellular immunity, as measured by concanavalin A-induced spleen cell pro-

liferation, compared with controls. At the intestinal level, genome-wide gene expression profiling by microarray hybridization identified 601 differentially expressed transcripts (287 upregulated, 314 downregulated) in gut lymphocytes of *C. longa*-fed chickens compared with nonsupplemented controls. Based on the known functions of the corresponding mammalian genes, the *C. longa*-induced intestinal transcriptome was mostly associated with genes mediating anti-inflammatory effects. Taken together, these results suggest that dietary *C. longa* could be used to attenuate *Eimeria*-induced, inflammation-mediated gut damage in commercial poultry production.

Key words: curcumin, avian coccidiosis, intestine, genomics, cytokine

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INTRODUCTION

Turmeric (*Curcuma longa*) is an herbaceous, perennial plant belonging to the ginger family. Its rhizomes are the source of turmeric powder that has historically been used as a spice and food coloring agent (Thomas-Eapen, 2009). *Curcuma longa* possesses several phytochemical compounds exhibiting a wide variety of pharmacological properties, including those against tumor cells, hormonal disorders, inflammation, bacterial infection, oxidative stress, and parasitosis (Araujo and Leon, 2001). The major active ingredient of turmeric is curcumin (diferuloylmethane), a lipophilic polyphenol that is practically insoluble in water, but quite stable in the acidic pH of the stomach (Bengmark et al., 2009; Jurenka, 2009). The pharmacological activities of turmeric are considerably dependent on the bioactivity of curcumin (Sharma, 1976; Mukhopadhyay et al., 1982; Conney et al., 1991; Jayaprakasha et al., 2002).

In particular, several studies have documented the parasitocidal activity of curcumin against *Leishmania*, *Trypanosoma*, *Giardia*, *Schistosoma*, and *Plasmodium* (Rasmussen et al., 2000; Koide et al., 2002; Cui and Miao, 2007; Allam, 2009; Nagajyothi et al., 2012; Said et al., 2012).

Avian coccidiosis is an intestinal disease caused by multiple species of the apicomplexan intracellular parasite, *Eimeria* (McDonald and Shirley, 2009; Shirley and Lillehoj, 2012). Coccidiosis causes significant economic loss to the world poultry industry due to reduced feed conversion in the gut and associated retardation of growth rate (Lillehoj and Li, 2004; Lillehoj et al., 2007). Host immune response to coccidiosis is complex, and *Eimeria*-induced immunopathology and immune protection include many facets of soluble mediators of cytokines and chemokines (Min et al., In press). It is generally accepted that intestinal damage following coccidiosis diminishes nutrient absorption by the gut epithelium, thereby jeopardizing BW growth. What is less clear are the relative roles of parasite invasion and destruction of gut epithelial cells versus bystander tissue damage due to uncontrolled host inflammation. Traditionally, in-feed anticoccidial drugs and antibiotic

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growth promoters have proven highly effective in mitigating the BW loss associated with avian coccidiosis. However, there is an increasing governmental restriction in the use of antibiotics in agricultural animal production in many parts of the world (Lillehoj and Lillehoj, 2000; Lillehoj et al., 2011). Recently, several antibiotic alternative strategies have been explored to enhance innate immunity of poultry and mitigate the use of antibiotics (Lee et al., 2010b; Lillehoj and Lee, 2012). Plant extracts, in particular, are gaining interest as feed additives to ameliorate the ill effects of coccidiosis as convincing scientific evidence demonstrating the beneficial effects of various phytonutrients on infectious diseases and cancers increases (Duke et al., 2003; Veldhuizen et al., 2006; Burt et al., 2007; Chang et al., 2008; Lee et al., 2010a,b; Tsubura et al., 2011). In mammals, it is well known that *C. longa* exerts antimicrobial effects on parasites including apicomplexans (Rasmussen et al., 2000; Koide et al., 2002; Cui and Miao, 2007; Allam, 2009; Nagajyothi et al., 2012; Said et al., 2012). However, there is no information on the effects of *C. longa* on avian coccidiosis. Therefore, this study was undertaken to evaluate the effects of dietary *C. longa* extract on intestinal immunity to avian coccidiosis.

MATERIALS AND METHODS

Experimental Diets

One-day-old commercial broilers (Ross × Ross, Longenecker's Hatchery, Elizabethtown, PA) were randomly housed in Petersime starter brooder units and fed ad libitum with a standard diet (control) or the same diet supplemented with 35 mg/kg of a freeze-dried *C. longa* extract (90%, Pancosma SA, Geneva, Switzerland). Organic phase extraction of *C. longa* rhizomes and lyophilization of the extract were performed as described (Lee et al., 2010b). The dose of *C. longa* extract was based on that used in our previous study (Lee et al., 2010b). The composition of the standard diet was prepared as recommended by the NRC (1994). All experiments were approved by the Beltsville Agricultural Research Service Institutional Animal Care and Use Committee.

Eimeria Infection and Measurement of Disease Parameters

Chickens given the nonsupplemented diet or the *C. longa*-supplemented diet were transferred to hanging cages (2 birds/cage) at 14 d posthatch, where they remained noninfected or were infected by oral gavage with 2.0×10^4 sporulated oocysts of *Eimeria maxima* or *Eimeria tenella* as described (Lee et al., 2010a,b). Two chicken groups (60 chickens/group) were allocated to the trials, one group infected with *E. maxima* and the other group with *E. tenella*. In each group, 40 chickens were infected and 20 birds were left uninfected as controls. Body weights (20 chickens/group) were mea-

sured at 0 and 10 d postinfection, and fecal samples (20 chickens/group, 10 pens) were collected between 5 and 10 d postinfection. The samples from each pen (2 birds/pen) were pooled, and oocyst numbers were determined using a McMaster chamber (HK Inc., Tokyo, Japan) according to the following formula: total oocysts/bird = oocyst count × dilution factor × (fecal sample volume ÷ counting chamber volume) ÷ 2 (Lee et al., 2010b). At 10 d postinfection with *E. maxima*, jejunum lesion scores were determined using 4 randomly selected chickens from each pen in a blinded fashion by 3 independent observers on a numerical scale from 0 (normal) to 4 (severe) as described by Johnson and Reid (1970) and Lee et al. (2010a).

Anti-*Eimeria* Serum Antibody Responses

Serum samples (4 chickens/group) were collected from noninfected and *E. maxima*- and *E. tenella*-infected chickens on the nonsupplemented or *C. longa*-supplemented diets at 10 d postinfection. Ninety-six-well microtiter plates were coated overnight with 100 μL/well of an *E. tenella* purified recombinant microneme 2 (**EtMIC2**) protein as described (Lee et al., 2010a). Our prior study documented that chickens infected with *Eimeria* species other than *E. tenella* produce serum antibodies that cross-react with the *E. tenella*-derived protein (Lee et al., 2010b). The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 1.0% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Diluted sera (1:50) were added (100 μL/well), incubated for 1 h with agitation at room temperature, and washed with PBS-T. Bound antibodies were detected at 450 nm optical density with horseradish peroxidase-conjugated rabbit anti-chicken IgG secondary antibody and 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich). All samples were analyzed in quadruplicate.

Concanavalin A-Stimulated Spleen Cell Proliferation

Spleen cell suspensions (4 chickens/group) from noninfected, and *E. maxima*- and *E. tenella*-infected chickens on the nonsupplemented or *C. longa*-supplemented diets were freshly prepared at 7 d postinfection by gently flushing through a cell strainer. Lymphocytes were purified by density gradient centrifugation through Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). The cells were adjusted to 1.0×10^7 cells/mL in RPMI-1640 medium without phenol red (Sigma) and supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Sigma-Aldrich). The cells (100 μL/well) were added to 96-well flat bottom plates containing 100 μL/well of concanavalin A (**Con A**, 1.0 μg/mL; Sigma-Aldrich, St. Louis, MO) or medium alone as a control. The cells were incubated at 41°C in a humidified incubator (Thermo Fisher Scien-

tific Inc., Waltham, MA) with 5% CO₂ for 48 h and cell proliferation was measured at OD₄₅₀ using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; Dojindo Molecular Technologies, Rockville, MD] and a microplate spectrophotometer (Bio-Rad, Hercules, CA).

Microarray Hybridization

Intestines (6 chickens/group) were removed from noninfected chickens on the nonsupplemented or *C. longa*-supplemented diets at 14 d posthatch and cut longitudinally. Tissues were washed 3 times with ice-cold Hanks' balanced salt solution (HBSS) and incubated in HBSS containing 0.5 mM EDTA and 5% fetal calf serum (FCS) for 20 min at 37°C with constant swirling. Cells released into the supernatant were passed through nylon wool (Robbins Scientific, Sunnyvale, CA), and washed twice with HBSS containing 5% FCS. Intestinal intraepithelial lymphocytes (IEL) were purified by Percoll density gradient centrifugation as described (Kim et al., 2013) and washed 3 times with HBSS containing 5% FCS. Total RNA was isolated using Trizol (Invitrogen, Grand Island, NY) and pooled into 2 equal samples from 3 birds each. The 2 samples from each group were treated as biological replicates and amplified using the Two-Color Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA) with cyanine 3 (Cy3)- or Cy5-labeled CTP. The alternation of the label with Cy3 and Cy5 was conducted between biological replicates to prevent data distortion from sample labeling (McShane et al., 2003). The RNA probes from the control and treatment groups labeled with the 2 different colors were hybridized with a Chicken Gene Expression Microarray (Agilent Technologies) containing 43,803 elements, and 2 arrays were used in this study. Microarray images were scanned, and data extraction and analysis were performed using Feature Extraction software version 10.7.3.1 (Agilent Technologies).

Microarray Data Analysis

GeneSpring GX10 software (Silicon Genetics, Redwood City, CA) was used to normalize image analysis data and to determine the fold changes in gene expression as previously described (Kim et al., 2013). Flag information was applied to strain the spots with 100% valid values from each sample and an asymptotic *t*-test analysis was performed to analyze the significance between the nonsupplemented and *C. longa*-supplemented groups. To generate signal ratios, signal channel values from *C. longa*-fed birds were divided by values from negative controls. Modulated elements were defined as RNA with ≥ 2.0 -fold increased or decreased levels with $P < 0.05$ as determined by Student's *t*-test. All microarray information and data were deposited in the Gene Expression Omnibus database (GSE37813). The differentially expressed genes between the control

and *C. longa*-fed groups were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). Each identifier was mapped to its corresponding gene in IPA. Both 2.0-fold increased and 2.0-fold decreased identifiers were defined as value parameters for the analysis. Functional gene analysis was performed to identify the biological functions and canonical pathways of genes from the mapped data sets. The Fischer's exact test was used to calculate *P*-values to assess the probability of each biological function and pathway assigned to that data set. Pathways of focus genes were algorithmically generated based on their connectivity.

Validation of Gene Expression by qRT-PCR

Gene expression changes observed by microarray analysis were confirmed by quantitative reverse-transcription (qRT) PCR as described (Hong et al., 2006b). Equivalent amounts of the same RNA samples used for microarray hybridizations were reverse-transcribed using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA) and the oligonucleotide primers listed in Table 1. Amplification and detection were carried out using the Mx3000P system and RT² SYBR Green qPCR Mastermix (Qiagen Inc., Valencia, CA). Standard curves were generated using log₁₀ diluted standard RNA and the levels of individual transcripts were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the Q-gene program (Muller et al., 2002). For the calculation of fold changes between the control and *C. longa*-fed groups, the cycle threshold value of the target gene was normalized to GAPDH and calibrated to the relevant control value. Each analysis was performed in triplicate.

Statistical Analysis

Body weight gains, fecal oocyst shedding, intestinal lesion scores, Con A-stimulated spleen cell proliferation, and serum anti-*Eimeria tenella* microneme 2 (Et-MIC2) antibody levels were expressed as the mean and SD values. Comparisons of the mean values were performed by ANOVA and Student's *t*-test using SPSS software (SPSS 15.0 for Windows, Chicago, IL), and differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

Effect of Dietary *C. longa* on BW Gain, Fecal Oocyst Excretion, and Intestinal Lesions Following *E. maxima* or *E. tenella* Infection

Dietary supplementation with the *C. longa* significantly increased BW gains of *E. maxima*- and *E. tenel-*

Table 1. Primers used for quantitative reverse-transcription PCR

Symbol	Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')	GenBank accession no.
CD28	CD28 molecule	AGACAGTGCAGTCGAAGTCT	GATGGGTGTCTCTCTGACAT	NM_205311.1
CD200	CD200 molecule	ATAGCCACCTGTAGTGCTGT	CCAAGAACCTTAATCCCTCT	NM_001030787.1
FGF2	Fibroblast growth factor 2	TCAAGCAGAAGAAAGAGGAG	GCTTGTACTGTCCAGTCCTT	NM_205433.1
FGG	Fibrinogen γ chain	TTACAGAGGAGATTGGATGG	CTTCTTCAGTTCCCACTTG	NM_204989.1
IL10RB	IL 10 receptor β	ATCTGTACCGATTCCACCAAG	TTTAGAGTCCTGATGGTGCT	NM_204857.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GGTGGTGCTAAGCGTGTAT	ACCTCTGTCATCTCTCCACA	K01458

la-infected chickens between d 0 and 10 postinfection compared with infected birds given a nonsupplemented control diet (Table 2). Chickens fed the *C. longa*-supplemented diet and infected with *E. tenella*, but not with *E. maxima*, showed decreased fecal oocyst excretion compared with the nonsupplemented control group (Table 3). However, intestinal lesion scores were significantly reduced in the chickens fed with *C. longa* extract and infected with *E. maxima* compared with the nonsupplemented and infected controls (Figure 1). Oocyst shedding (unpublished data) and gut lesions (Figure 1) were not detected in noninfected chickens.

Effect of Dietary *C. longa* on Serum Anti-EtMIC2 Antibody Levels and Mitogenic Spleen Response

Dietary supplementation with the *C. longa* extract significantly increased anti-EtMIC2 serum antibody levels in *E. maxima*-infected chickens at d 10 postinfection compared with infected birds given a nonsupplemented control diet (Table 4). By contrast, anti-EtMIC2 antibody levels in *E. tenella*-infected chickens were equal in the nonsupplemented and *C. longa*-fed groups (Table 4). As a measure of cellular immunity, the mitogenic response of splenocytes to Con A was significantly greater in *E. maxima*- and *E. tenella*-infected chickens given the *C. longa* diet compared with the infected/*C. longa*-supplemented controls (Table 5).

Effect of Dietary *C. longa* on Gene Expression in Intestinal Lymphocytes

Microarray hybridization analysis using Agilent Technologies' Chicken Gene Expression Microarray containing 43,803 elements identified 601 mRNA whose levels were altered ≥ 2.0 -fold in IEL from the duodenum of chickens given the *C. longa*-supplemented diet compared with nonsupplemented controls. Of these, 287 were increased and 314 were decreased. This data set was mapped to the corresponding genes of the human, mouse, and rat genomes using Ingenuity Knowledge Base software, and 217 chicken genes were identified and annotated. Biofunctional analysis identified 12 biological functions belonging to the category of "Disease and Disorder" that were significantly associated with these genes (Table 6). Of note, the function "Inflammatory Response" with 21 related genes was the 6th most significant function in this category.

qRT-PCR Validation

The expression patterns observed by genome-wide gene expression analysis were validated by qRT-PCR using 5 selected transcripts whose levels were significantly downregulated in the intestinal IEL of the *C. longa*-fed chickens compared with the nonsupplemented control group. These genes were cluster of differentiation 28 (**CD28**), CD200, fibroblast growth factor 2, fibrinogen γ chain, and IL 10 receptor β , and were cate-

Table 2. Effect of dietary *Curcuma longa* on BW following *Eimeria maxima* or *Eimeria tenella* infection

Treatment ¹	<i>E. maxima</i> ² (g)	<i>E. tenella</i> ² (g)
Control diet + no infection	623.86 \pm 10.66 ^a	680.45 \pm 20.33 ^b
Control diet + <i>Eimeria</i> infection	306.58 \pm 19.17 ^c	643.41 \pm 27.02 ^c
<i>C. longa</i> + <i>Eimeria</i> infection	420.15 \pm 16.38 ^b	719.17 \pm 18.68 ^a

^{a-c}Mean values with different superscripts are significantly different ($P < 0.05$), according to the Duncan's multiple range test ($n = 20$).

¹One-day-old broiler chickens were fed a standard diet alone (control) or a standard diet supplemented with *C. longa*. At 14 d posthatch, the chickens were noninfected or infected with 2.0×10^4 sporulated oocysts of *E. maxima* or *E. tenella*, and BW gains were measured between d 0 and 10 postinfection.

²Mean \pm SD.

Table 3. Effect of dietary *Curcuma longa* on fecal oocyst shedding following *Eimeria maxima* or *Eimeria tenella* infection

Treatment ¹	<i>E. maxima</i> ($\times 10^7$) ²	<i>E. tenella</i> ($\times 10^7$) ²
Control	2.77 \pm 0.12	6.53 \pm 1.55 ^a
<i>C. longa</i>	2.38 \pm 0.18	2.82 \pm 0.60 ^b

^{a,b}Mean values with different superscripts are significantly different ($P < 0.05$), according to Student's *t*-test ($n = 20$).

¹One-day-old broiler chickens were fed a standard diet alone (control) or a standard diet supplemented with *C. longa*. At 14 d posthatch, the chickens were infected with 2.0×10^4 sporulated oocysts of *E. maxima* or *E. tenella*, and fecal oocysts were enumerated between d 5 and 10 postinfection.

²Mean \pm SEM.

gorized within the biological function of "Inflammatory Response" (Table 6). The levels of these 5 transcripts that were downregulated by microarray hybridization were also correspondingly altered when analyzed by qRT-PCR (Table 7).

DISCUSSION

In this report, we document that chickens fed a diet supplemented with an organic extract of *C. longa* showed increased BW gain following infection with *E. maxima* or *E. tenella* compared with chickens given the standard diet alone. In our previous study, feeding broiler chickens with diets containing *C. longa* did not show any toxic effects on the host, but enhanced BW gain and reduced fecal oocyst shedding compared with the noninfected controls following *E. tenella* infection (Lee et al., 2010b). In contrast, oocyst shedding was unaffected in *C. longa*-fed birds infected with *E. maxima*, although this group did exhibit reduced gut lesions compared with nonsupplemented and infected controls. Similar observations have been reported for *E. acervulina*-infected chickens, which showed increased BW gain in the absence of alterations in fecal oocyst excretion following dietary *C. longa* supplementation (Lee et al., 2010b). These results showed that the effect of dietary *C. longa* depends on the species of *Eimeria*, and these effects may reflect differential rates of phytonutrient absorption in the various regions of the gut

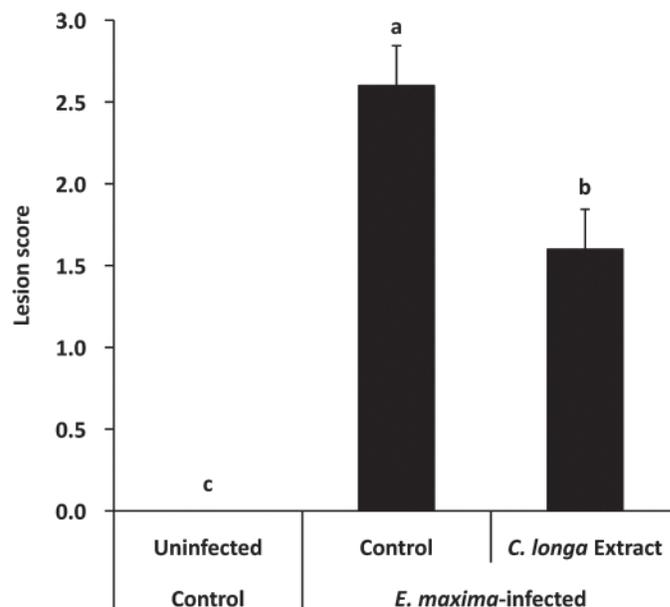


Figure 1. Effect of dietary *Curcuma longa* on intestinal lesions following *Eimeria maxima* infection. One-day-old broiler chickens were fed a standard diet alone (control) or a standard diet supplemented with *C. longa*. At 14 d posthatch, the chickens were noninfected or infected with 2.0×10^4 sporulated oocysts of *E. maxima*. Intestinal jejunum samples were obtained at d 10 postinfection, and lesion scores were determined in a blinded fashion by 3 independent observers from 0 (normal) to 4 (severe). Values are means, with SD represented by vertical bars ($n = 4$). Mean values with different letters (a–c) are significantly different ($P < 0.05$), according to Duncan's multiple range test.

infected as previously suggested by Ravindranath and Chandrasekhara (1980) because *E. maxima* (jejunum), *E. tenella* (cecum), and *E. acervulina* (duodenum) infect different locations in the gut. Alternatively, we cannot rule out the possibility that the *C. longa* may exert differential cytotoxic activity against the denoted *Eimeria* species (Khalafalla et al., 2011).

An important conclusion from this study relates to the observed differences in systemic vs. local intestinal immune responses affected by dietary *C. longa*. More specifically, at the systemic level, chickens fed with *C. longa* showed an increased humoral immune response, as measured by higher serum anti-EtMIC2 antibody

Table 4. Effect of dietary *Curcuma longa* on serum anti-EtMIC2 antibody levels following *Eimeria maxima* or *Eimeria tenella* infection

Treatment ¹	<i>E. maxima</i> (mean \pm SEM) ²	<i>E. tenella</i> (mean \pm SEM) ²
Control diet + no infection	0.69 \pm 0.04 ^a	0.62 \pm 0.03 ^b
Control diet + <i>Eimeria</i> infection	0.76 \pm 0.05 ^b	0.71 \pm 0.02 ^{ab}
<i>C. longa</i> + <i>Eimeria</i> infection	1.05 \pm 0.01 ^a	0.77 \pm 0.05 ^a

^{a,b}Mean values with different superscripts are significantly different ($P < 0.05$), according to the Duncan's multiple range test ($n = 16$).

¹One-day-old broiler chickens were fed a standard diet alone (control) or a standard diet supplemented with *C. longa*. At 14 d posthatch, the chickens were noninfected or infected with 2.0×10^4 sporulated oocysts of *E. maxima* or *E. tenella*. Serum samples were obtained at d 10 postinfection, and anti-EtMIC2 antibody levels were measured by ELISA.

²Optical density at 450 nm (mean \pm SEM).

Table 5. Effect of dietary *Curcuma longa* on concanavalin A-induced spleen cell proliferation following *Eimeria maxima* or *Eimeria tenella* infection

Treatment ¹	<i>E. maxima</i> (mean ± SEM) ²	<i>E. tenella</i> (mean ± SEM) ²
Control diet + no infection	0.69 ± 0.04 ^b	0.87 ± 0.01 ^b
Control diet + <i>Eimeria</i> infection	0.76 ± 0.05 ^b	0.91 ± 0.03 ^b
<i>C. longa</i> + <i>Eimeria</i> infection	1.05 ± 0.01 ^a	1.04 ± 0.04 ^a

^{a,b}Mean values with different superscripts are significantly different ($P < 0.05$), according to Duncan's multiple range test ($n = 4$).

¹One-day-old broiler chickens were fed a standard diet alone (control) or a standard diet supplemented with *C. longa*. At 14 d posthatch, the chickens were noninfected or infected with 2.0×10^4 sporulated oocysts of *E. maxima* or *E. tenella*. Splenocytes were isolated at 7 d postinfection and cell proliferation was measured using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium).

²Optical density at 450 nm (mean ± SEM).

levels, compared with the nonsupplemented controls. Because microneme proteins are involved in parasite invasion of host cells (Tomley et al., 1996; Lillehoj et al., 2004), higher antibody response to this protein indicates better protection against coccidiosis. In general, *E. maxima* is more immunogenic than other *Eimeria* species (Shirley and Lillehoj, 2012), and this may explain different anti-EtMIC2 responses between *E. maxima* and *E. tenella*. Birds fed the *C. longa* also showed greater cell-mediated immunity as demonstrated by enhanced spleen cell proliferation response to Con A compared with the nonsupplemented controls. Global gene expression analysis of intestinal IEL from *C. longa*-fed chickens showed differential expression of genes involved in inflammatory response. Because invasion and intracellular development stages of *Eimeria* parasites in the gut are associated with the induction of local inflammatory response (Hong et al., 2006a,c), stimulation of anti-inflammatory response by dietary

C. longa would lead to lesser gut damage. It is tempting to speculate that this dual mode of action of *C. longa* may serve not only to limit *Eimeria* pathogenicity in vivo, but also to minimize bystander inflammatory damage to host tissues.

Among the pharmacological benefits of *C. longa* phytonutrients, particularly curcumin, their activities as inhibitors of inflammation have been extensively documented (Araujo and Leon, 2001). The anti-inflammatory effects of curcumin are mediated through its ability to block nuclear factor- κ B activation in response to various inflammatory stimuli (Singh and Aggarwal, 1995), and to suppress the expression of proinflammatory cytokines, including tumor necrosis factor- α , IL-1 β , IL-6, and IL-8 (Abe et al., 1999; Jagetia and Aggarwal, 2007). In avian coccidiosis, several proinflammatory and T cell helper type 1 cytokines and chemokines (interferon- γ , IL-1 β , lipopolysaccharide-induced TNF- α factor, IL-6, IL-8, and IL-10, and so on) were

Table 6. The significant functions in the "Disease and Disorders" category in chickens given a curcumin-supplemented diet compared with a nonsupplemented diet

Category ¹	<i>P</i> -value ²	Genes
Nutritional disease	9.5×10^{-4} to 9.5×10^{-4}	<i>HCRT*</i> , <i>INS</i> , <i>POMC*</i> , <i>ADARB1</i> , <i>CCKBR*</i>
Psychological disorders	9.5×10^{-4} to 9.5×10^{-4}	<i>HCRT*</i> , <i>INS</i> , <i>POMC*</i> , <i>ADARB1</i> , <i>CCKBR*</i>
Genetic disorder	3.2×10^{-3} to 4.65×10^{-2}	<i>CTRC</i> , <i>YH10</i> , <i>MYL2*</i> , <i>COL4A6</i> , <i>MRE11A</i> , <i>IKZF1</i> , <i>CRYBA1*</i> , <i>FGG</i> , <i>GSTT1</i> , <i>CEL</i> , <i>LTF</i> , <i>CSF2RA</i> , <i>INS</i> , <i>SLC25A15</i> , <i>PMM2*</i> , <i>DNASE1</i> , <i>UNC13D*</i> , <i>MLLT10*</i> , <i>C1GALT1C1</i> , <i>POLG</i> , <i>NEFH</i> , <i>MPO</i> , <i>CAS</i> , <i>INPP5D*</i> , <i>SLC7A9</i> , <i>TLR5</i> , <i>POMT1</i> , <i>ATCAY*</i> , <i>CTH</i> , <i>PNLIP</i> , <i>PPAT</i> , <i>MYH10</i> , <i>SLC7A9</i> , <i>COL4A6</i> , <i>KL</i> , <i>MDK*</i>
Renal and urological disease	3.20×10^{-3} to 4.65×10^{-2}	<i>LTF</i> , <i>FGF2</i> , <i>INS</i> , <i>MPO</i> , <i>DNASE1</i>
Respiratory disease	3.90×10^{-3} to 4.65×10^{-2}	<i>UNC13D*</i> , <i>CD200</i> , <i>FGF2</i> , <i>COL17A1*</i> , <i>TAC1</i> , <i>MPO</i> , <i>C1QA</i> , <i>POMC*</i> , <i>CDO1</i> , <i>FGG</i> , <i>INPP5D</i> , <i>CD28</i> , <i>GSTT1</i> , <i>TLR5</i> , <i>LTF</i> , <i>CSF2RA</i> , <i>KL</i> , <i>INS</i> , <i>IL10RB</i> , <i>MDK*</i> , <i>DNASE1</i>
Inflammatory response	5.03×10^{-3} to 4.65×10^{-2}	<i>PEBP1*</i> , <i>GSTT1</i> , <i>CD28</i> , <i>MLLT10*</i> , <i>COL4A6</i> , <i>CSF2RA</i> , <i>FGF2</i> , <i>MPO</i> , <i>IKZF1</i> , <i>WDR77</i> , <i>INPP5D*</i> , <i>PPAT</i> , <i>GSTT1</i> , <i>CD28</i> , <i>MYH10</i> , <i>MLLT10*</i> , <i>CSF2RA</i> , <i>C1GALT1C1</i> , <i>MPO</i> , <i>IKZF1</i> , <i>POMC*</i> , <i>INPP5D*</i> , <i>PPAT</i> , <i>FGG</i>
Cancer	7.75×10^{-3} to 4.65×10^{-2}	<i>UNC13D*</i> , <i>CD200</i> , <i>COL17A1*</i> , <i>MPO</i> , <i>IKZF1</i> , <i>C1QA</i> , <i>C1R</i> , <i>CD28</i> , <i>TLR5</i> , <i>LTF</i> , <i>CSF2RA</i> , <i>PPAT</i> , <i>DNASE1</i>
Hematological disease	7.75×10^{-3} to 4.65×10^{-2}	<i>C1R</i> , <i>LTF</i> , <i>TLR5</i> , <i>FGF2</i> , <i>MPO</i> , <i>C1QA</i> , <i>DNASE1</i>
Immunological disease	7.75×10^{-3} to 4.65×10^{-2}	<i>C1R</i> , <i>CTRC</i> , <i>LTF</i> , <i>TLR5</i> , <i>FGF2</i> , <i>MPO</i> , <i>C1QA</i> , <i>DNASE1</i>
Connective tissue disorders	8.23×10^{-3} to 2.35×10^{-2}	<i>C1R</i> , <i>HCRT*</i> , <i>LTF</i> , <i>TLR5</i> , <i>FGF2</i> , <i>POMT1</i> , <i>NEFH</i> , <i>MPO</i> , <i>C1QA</i> , <i>DNASE1</i>
Inflammatory disease	8.23×10^{-3} to 4.65×10^{-2}	
Skeletal and muscular disorders	8.23×10^{-3} to 2.35×10^{-2}	

¹Data sets were analyzed by BioFunction analysis using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA) and are listed in descending order of statistical significance.

²*P*-values were calculated using the right-tailed Fisher's exact test.

*Upregulated genes are denoted by asterisks. Genes without asterisks are downregulated genes.

Table 7. Comparison between microarray hybridization and quantitative reverse-transcription (qRT) PCR for fold changes of mRNA levels in *Curcuma longa*-fed chickens and nonsupplemented controls

Symbol	Gene name	Microarray ¹	qRT-PCR ¹
CD28	CD28 molecule	-2.533 ± 0.005	-1.164 ± 0.182
CD200	CD200 molecule	-4.984 ± 0.061	-23.213 ± 2.336
FGF2	Fibroblast growth factor 2	-2.001 ± 0.018	-9.339 ± 1.626
FGG	Fibrinogen γ chain	-4.922 ± 0.050	-142.224 ± 27.012
IL10RB	IL 10 receptor β	-2.335 ± 0.014	-82.130 ± 7.931

¹Mean ± SD.

elevated in the chicken gut following infections with *E. tenella*, *E. acervulina*, and *E. maxima* (Hong et al., 2006a,c), and these soluble mediators of inflammation have been proposed to be responsible, in part, for intestinal damage during coccidiosis (Hong et al., 2006b). Therefore, dietary curcumin provides beneficial effects to the host by reducing inflammation-induced gut lesions during coccidiosis. Curcumin also downregulates the activities of other proinflammatory mediators, including cyclooxygenase-2, lipoxygenase, and inducible nitric oxide synthase (Jurenka, 2009) and seems to be a highly pleiotropic molecule capable of attenuating the activity of numerous molecular targets involved in inflammation.

For poultry, this is the first report demonstrating the curcumin-induced transcriptional changes in the gut, especially those genes that are mediating the inflammatory response. Among the *C. longa* upregulated genes, proopiomelanocortin is known to block mouse macrophage activation through inhibition of IL-1 β (Getting et al., 2003), and inositol polyphosphate-5-phosphatase suppressed the inflammatory response of macrophages following treatment with *Francisella tularensis* (Santic et al., 2010). Among the *C. longa* downregulated genes were tachykinin precursor 1, myeloperoxidase, colony-stimulating factor 2 receptor α , lactotransferrin, and CD28. Tachykinin precursor 1 was shown to regulate *Clostridium difficile* toxin A-mediated murine intestinal inflammation (Anton et al., 2004) and to induce activation of human neutrophils in vitro (Iwamoto et al., 1990). Neutrophil-derived myeloperoxidase was reported to stimulate rat alveolar macrophages, resulting in increased inflammatory and cytotoxic states and contributing to the overall lung inflammatory response (Grattendick et al., 2002). Colony-stimulating factor 2 receptor α enhanced chemotactic activity and chemokinesis of neutrophils (Gomez-Cambronero et al., 2003), and lactotransferrin increased the activation of neutrophils and macrophages (Sorimachi et al., 1997; Ueta et al., 2001). Cluster of differentiation 28 increased the migratory activity of human peripheral blood neutrophils in response to IL-8 (Venuprasad et al., 2001). Downregulation of 5 gene transcripts that are related to the “inflammatory response” function (Table 6) in the gut IEL of *C. longa*-fed chickens was confirmed using qRT-PCR, although in some cases differences were observed between the microarray hybridization and qRT-PCR results. These variations maybe due to differences in

the normalization methods used, the different fluorescent dyes employed, or both, in the 2 techniques (Lee et al., 2002).

In conclusion, in vivo feeding of young broiler chickens with an organic extract of *C. longa* rhizomes, known to contain the natural polyphenol phytonutrient, curcumin, improved resistance to experimental *E. maxima* and *E. tenella* infections, as demonstrated by increased BW gain in both *Eimeria* spp. challenge infections, reduced fecal oocyst shedding in *E. tenella*, but not in *E. maxima* infection, and decreased gut lesions with *E. maxima* infection compared with birds given a non-supplemented diet. Chickens given the *C. longa* diet also showed increased systemic humoral (serum anti-EtMIC2 antibody levels) and cellular (Con A-induced spleen cell proliferation) immune responses compared with controls. At the local tissue level, *C. longa* induced significant alterations in the transcriptome of chicken intestinal lymphocytes, through up- or downregulation of inflammatory- and immune-related genes in a manner consistent with an overall anti-inflammatory effect of curcumin. Collectively, these results suggest that dietary immunomodulation by *C. longa*-derived phytochemicals may represent an alternative way to reduce negative effects of avian coccidiosis in commercial poultry production.

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