

Immune effects of dietary anethole on *Eimeria acervulina* infection

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ABSTRACT The effects of anethole on in vitro and in vivo parameters of chicken immunity during experimental avian coccidiosis were evaluated. Anethole reduced the viability of invasive *Eimeria acervulina* sporozoites after 2 or 4 h of treatment in vitro by 45 and 42%, respectively, and stimulated 6.0-fold greater chicken spleen cell proliferation compared with controls. Broiler chickens continuously fed from hatch with an anethole-supplemented diet and orally challenged with live *E. acervulina* oocysts showed enhanced BW gain, decreased fecal oocyst excretion, and greater *E. acervulina* profilin antibody responses compared with infected chickens given an unsupplemented standard diet. The levels of transcripts encoding the immune mediators IL6, IL8, IL10, and tumor necrosis factor ligand superfamily member 15 (TNFSF15) in intestinal lym-

phocytes were increased in *E. acervulina*-infected chickens fed the anethole-containing diet compared with untreated controls. Global gene expression analysis by microarray hybridization identified 1,810 transcripts (677 upregulated, 1,133 downregulated) whose levels were significantly altered in intestinal lymphocytes of anethole-fed birds compared with unsupplemented controls. From this transcriptome, 576 corresponding genes were identified. The most significant biological function associated with these genes was “Inflammatory Response” in the “Disease and Disorders” category. This new information documents the immunologic and genomic changes that occur in chickens following anethole dietary supplementation that may be relevant to host protective immune response to avian coccidiosis.

Key words: anethole, *Eimeria acervulina*, chicken, immunity, microarray

2013 Poultry Science 92:2625–2634
<http://dx.doi.org/10.3382/ps.2013-03092>

INTRODUCTION

Coccidia protozoa comprise a subclass of single-celled eukaryotic microorganisms belonging to the phylum Apicomplexa. Coccidia of the genus *Eimeria* infect the intestinal epithelia of chickens, turkeys, and some mammalian hosts, causing substantial economic losses to the poultry industry as a result of decreased nutrient absorption, retarded growth rate of broilers, and decreased egg production of layers (Lillehoj and Li, 2004; Lillehoj et al., 2007). Whereas in-feed anticoccidial drugs and antibiotic growth promoters have been used to mitigate some of the negative effects of coccidiosis, the emergence of drug-resistant parasites and increasing legislative restrictions on the use of in-feed antibiotics encourage the development of alternative disease control strategies (Lillehoj and Lillehoj, 2000;

Lillehoj et al., 2011). One promising new avenue to achieve this goal concerns the use of natural dietary immune modulators to improve feed conversion efficiency, increase overall gut health, and promote natural innate immunity (Lee et al., 2010). More specifically, plant-derived phytochemicals have garnered increasing interest as medicinal compounds to ameliorate the ill effects of infectious diseases in mammals and poultry (Duke et al., 2003; Veldhuizen et al., 2006; Burt et al., 2007; Chang et al., 2008; Tsubura et al., 2011).

Anethole, 1-methoxy-4-(1-propenyl)benzene, is an aromatic, unsaturated ether that occurs as a major component of the essential oils of anise (*Pimpinella anisum*), star anise (*Illicium verum*), fennel (*Foeniculum vulgare*), and liquorice (*Glycyrrhiza glabra*), and is widely used as a flavoring agent (Windholz et al., 1983). In mammalian systems, anethole had demonstrable anticarcinogenic, antioxidant, and antiinflammatory activities (al-Harbi et al., 1995; Chainy et al., 2000; Freire et al., 2005), as well as antimicrobial properties against bacteria, fungi, and nematodes (Kubo and Fujita, 2001; Lee et al., 2002). These broad-spectrum pharmacologic

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Received February 4, 2013.

Accepted May 28, 2013.

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activities suggest that anethole may also protect avian hosts against infectious diseases. However, only limited studies have been reported describing the use of anethole in veterinary medicine (Cardozo et al., 2005). Therefore, the current study was undertaken to investigate the effects of anethole on in vitro and in vivo parameters of chicken immunity during experimental avian coccidiosis caused by *E. acervulina*.

MATERIALS AND METHODS

Anethole Preparation

Anethole (99%, Pancosma S.A.) was dissolved in ethanol (10 mg/mL), diluted in PBS (0.1 mg/mL), and sterilized by passage through a 0.45- μ m filter before use.

Parasite Cytotoxicity Assay

Freshly sporulated *E. acervulina* oocysts were prepared and disrupted with 0.5-mm glass beads for 5 to 7 s as described (Kim et al., 2013). Sporocysts were incubated for 45 min at 41°C in PBS containing 0.014 M taurodeoxycholic acid (Sigma-Aldrich, St. Louis, MO) and 0.25% trypsin (Sigma-Aldrich) to release infective sporozoites. Sporozoites were separated from debris by filtration, resuspended in RPMI 1640 medium (Life Technologies, Carlsbad, CA), and isolated by centrifugation at $2,100 \times g$ for 10 min at 4°C. Isolated sporozoites (1.0×10^6 /mL) were incubated with PBS (negative control), 10 μ g/mL of anethole, or 5.0 μ g/mL of chicken purified recombinant NK-lysin (Hong et al., 2008; positive control) for 2 or 4 h at 4°C, and viability was measured by trypan blue exclusion by counting a minimum of 100 sporozoites.

Experimental Birds and Diets

Eighty chickens were randomly housed in Petersime starter brooder units and fed from hatch with a standard diet (control) or a diet supplemented with 15 mg/kg of anethole. The standard grower diet contained 24% CP and 54% carbohydrate. All diets contained 15% vitamin and mineral mixture, 4.7% fat, and 2.4% fiber (USDA/Feed Mill, Beltsville, MD). The composition of the standard diet was prepared as recommended by the NRC (1994). The dose of anethole was chosen based on preliminary dose-response experiments. All experiments were approved by the Agricultural Research Service Institutional Animal Care and Use Committee.

Experimental *E. acervulina* Infection

At 10 d posthatch, chickens given the unsupplemented or anethole-supplemented diets were transferred to large hanging cages (2 birds/cage) and were orally infected with 5.0×10^3 sporulated oocysts of *E. acervulina*. Negative control animals were housed in

neighboring cages, but were uninfected. Body weights (16 birds/group, 8 pens) were measured at 0 and 9 d postinfection. Fecal samples (8 pens) were collected between 6 and 9 d postinfection, and oocyst numbers were determined using a McMaster chamber according to the formula (Lee et al., 2009): total oocysts/bird = oocyst count \times dilution factor \times (fecal sample volume \div counting chamber volume) \div 2.

Antiprofilin Serum Antibody Assay

Peripheral blood (4 birds/group) was collected from uninfected and infected chickens given the unsupplemented or anethole-supplemented diets at 9 d postinfection, and sera were prepared by centrifugation and analyzed for anti-profilin antibody levels by ELISA. Ninety-six-well plates were coated overnight with 10 μ g/well of an *E. acervulina* purified recombinant profilin protein as described (Jang et al., 2011). The plates were washed with PBS containing 0.05% Tween (PBS-T), and blocked with PBS containing 1.0% bovine serum albumin (Sigma-Aldrich). Diluted sera (1:50) were added (100 μ L/well), incubated with agitation for 1 h at room temperature, and washed with PBS-T. Bound antibody was detected with horseradish peroxidase-conjugated rabbit anti-chicken IgG (Sigma-Aldrich) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich) by measuring optical density at 450 nm.

Spleen Lymphocyte Proliferation

Three-week-old Ross/Ross broiler chickens (Longenecker's Hatchery, Elizabethtown, PA) were euthanized by cervical dislocation, and spleens were removed and placed in Petri dishes with 10 mL of Hanks' balanced salt solution (HBSS) supplemented with 100 U/mL of penicillin and 100 μ g/mL of streptomycin (Sigma-Aldrich). Cell suspensions were prepared by gently flushing through a cell strainer and lymphocytes were purified by density gradient centrifugation through Histopaque-1077 (Sigma-Aldrich) as described (Kim et al., 2013). The cells were adjusted to 1.0×10^7 cells/mL in RPMI-1640 medium without phenol red (Sigma-Aldrich) and supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μ g/mL of streptomycin. The cells (100 μ L/well) were added to 96-well flat bottom plates containing an equal volume of serially diluted anethole to achieve a final concentration of 0.01 to 10 μ g/mL, or concanavalin A (Con A, 0.5 mg/mL, Sigma-Aldrich) as a positive control, or medium alone as a negative control. The cells were incubated at 41°C in a humidified incubator (Thermo Scientific, Asheville, NC) with 5% CO₂ for 48 h, and cell numbers were measured using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium; Dojindo Molecular Technologies, Rockville, MD] at 450 nm using a microplate spectrophotometer (Bio-Rad, Hercules, CA).

Microarray Hybridization

Chicken intestinal intraepithelial lymphocytes (IEL) were isolated from uninfected chickens given the un-supplemented or anethole-supplemented diets at 14 d posthatch as described by Ding et al. (2005), and gene expression analysis was performed by microarray hybridization as described by Kim et al. (2013). The intestinal jejunum was removed, cut longitudinally, and washed 3 times with ice-cold HBSS. Tissue samples were incubated in HBSS containing 0.5 mM EDTA and 5% FBS 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% FBS. The IEL were purified by Percoll density gradient centrifugation and washed 3 times with HBSS containing 5% FBS. Total RNA (6 birds/group) was isolated using Trizol (Life Technologies) and equal amounts were pooled into 2 samples from 3 birds each. The RNA were amplified using the Two-Color Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA) with cyanine 3 (Cy3)- or Cy5-labeled CTP. The RNA probes from the control and treatment groups labeled with 2 different colors were hybridized with the Chicken Gene Expression Microarray (Agilent Technologies) containing 43,803 elements. Two biological replicates were conducted with alternation of Cy3- and Cy5-labeled RNA to prevent data distortion from sample labeling (McShane et al., 2003). 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 (Agilent Technologies) was used to qualify and normalize image analysis data and to determine the fold changes in gene expression. Average signal intensities were corrected for background signals and normalized by the block LOW-ESS (locally weighted regression and smoothing scatter plots) methods. Flag information was applied to strain the spots with 100% valid values from each sample, and an asymptotic *t*-test analysis with $P < 0.05$ was performed to analyze the significance differences between the un-supplemented and anethole-supplemented groups. To generate signal ratios, signal channel values

from anethole-fed birds were divided by values from negative controls. All microarray information and data were deposited in the Gene Expression Omnibus database (series record number, GSE41250). Differentially expressed genes between anethole-fed and control chickens were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems). Each identifier was mapped to its corresponding gene in IPA. These identified genes were superimposed onto the global molecular networks contained within IPA. An IPA functional analysis was performed to identify the biological functions associated with the identified genes from the mapped data sets.

Quantitative RT-PCR

Gene expression changes observed by microarray analysis were confirmed by quantitative reverse-transcription (qRT) PCR as described by Hong et al. (2006). Equivalent amounts of the same RNA samples used for microarray hybridizations and the RNA prepared from IEL at 9 d postinfection of *E. acervulina* were reverse-transcribed using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies). Oligonucleotide primers are listed in Table 1. Amplification and detection were carried out using the Mx3000P system and Brilliant SYBR Green qRT-PCR master mix (Qiagen, 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 treatment groups, the cycle threshold (Ct) value of the target gene was normalized to GAPDH and calibrated to the relevant control value. Each analysis was performed in triplicate.

Statistical Analyses

Data from parasite cytotoxicity assays, BW gains, oocyst shedding, spleen lymphoproliferation, and profilin antibody levels were expressed as the mean \pm SD values. Comparisons of the mean values were performed by ANOVA and the Student's *t*-test using SPSS software (SPSS 15.0 for Windows, Chicago, IL) and were considered significant at $P < 0.05$. An IPA biological

Table 1. Primers used for quantitative reverse-transcription PCR¹

Gene transcript	Forward primer (5' → 3')	Reverse primer (5' → 3')	GenBank accession no.
GAPDH	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCCACA	K01458
IL6	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTCT	AJ309540
IL8	GGCTTGCTAGGGGAAATGA	AGCTGACTCTGACTAGGAAACTGT	AJ009800
IL10	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC	AJ621614
TNFSF15	CCTGAGTATTCCAGCAACGCA	ATCCACCAGCTTGATGTCACTAAC	NM_010245578

¹GAPDH = glyceraldehyde-3-phosphate dehydrogenase. TNFSF15 = tumor necrosis factor ligand superfamily member 15.

function analysis was performed using the Fischer's exact test to calculate the probability of each biological function assigned to that data set and were considered significant at $P < 0.05$.

RESULTS

Effect of Dietary Anethole on In Vitro Sporozoite Viability

Treatment of freshly prepared *E. acervulina* sporozoites with 10 $\mu\text{g}/\text{mL}$ of anethole for 2 or 4 h decreased parasite viability by 45 and 42%, respectively (Figure 1). Anethole-treated parasite viability at both time points was significantly reduced compared with PBS-treated parasites. The reduced sporozoite viability was equivalent to that produced by 5.0 $\mu\text{g}/\text{mL}$ of chicken purified recombinant NK-lysin, previously shown to be cytotoxic for *E. acervulina* parasites (Hong et al., 2008).

Effect of Dietary Anethole on BW Gain and Fecal Oocyst Excretion Following Experimental *E. acervulina* Infection

Dietary supplementation with anethole significantly increased BW gain by 12% in *E. acervulina*-infected

chickens compared with infected birds given the unsupplemented control diet (Figure 2A). There was no difference in weight gains between the uninfected, unsupplemented group and the infected, anethole-supplemented group. In addition, infected chickens fed the anethole-supplemented diet had decreased fecal oocyst excretion compared with the infected, unsupplemented control group (Figure 2B).

Effect of Dietary Anethole on Serum Antiprofilin Antibody Levels and Spleen Cell Proliferation

Eimeria acervulina profilin serum antibody levels and in vitro spleen cell proliferation were measured as parameters of humoral and cellular immunity in the anethole-supplemented and unsupplemented chickens. In *E. acervulina*-infected chickens, serum profilin antibody levels were higher in anethole-supplemented groups compared with the unsupplemented group (Figure 3). At all concentrations (0.01–10 $\mu\text{g}/\text{mL}$), anethole increased the proliferation of spleen lymphocyte compared with the medium-alone control (Figure 4). More importantly, spleen cells treated with 0.04 to 0.30 $\mu\text{g}/\text{mL}$ of anethole showed increased proliferation compared with cells treated with 0.5 $\mu\text{g}/\text{mL}$ of Con A.

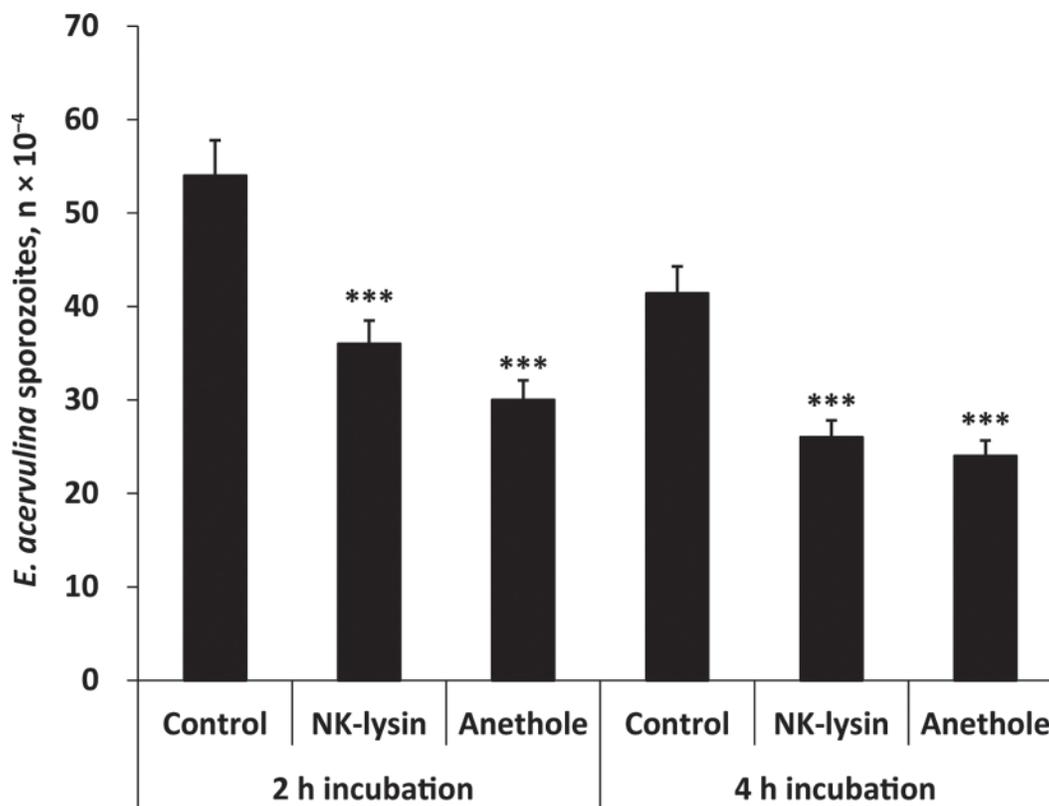


Figure 1. Effect of anethole on in vitro sporozoite viability. *Eimeria acervulina* sporozoites ($1.0 \times 10^6/\text{mL}$) were incubated with PBS (control), 5.0 $\mu\text{g}/\text{mL}$ of chicken recombinant NK-lysin, or 10 $\mu\text{g}/\text{mL}$ of anethole for 2 or 4 h at 4°C, and viability was measured by trypan blue exclusion by counting a minimum of 100 sporozoites. Each bar represents the mean \pm SD value ($n = 3$). *** $P < 0.001$ when comparing the anethole-treated group with the control group according to Student's *t*-test.

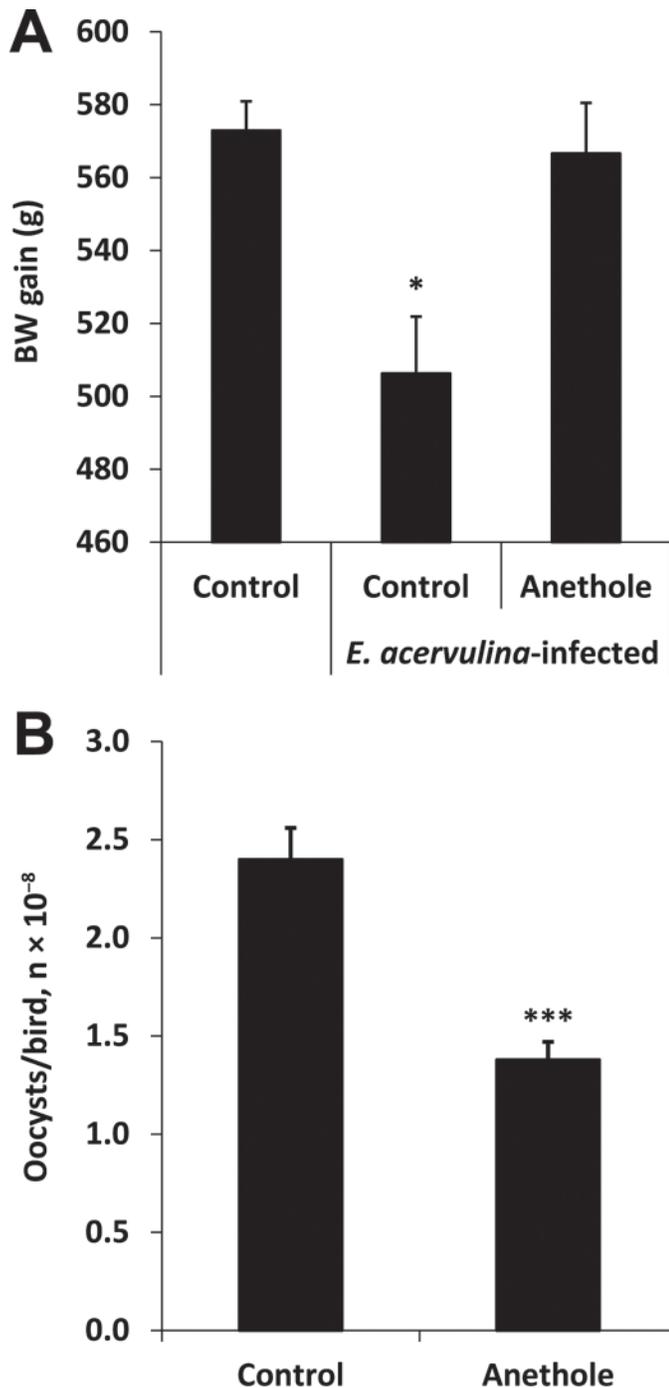


Figure 2. Effect of dietary anethole on BW gain and fecal oocyst excretion following experimental *Eimeria acervulina* infection. Chickens were fed from hatch with unsupplemented (control) or anethole-supplemented diets and either uninfected or orally infected with 5.0×10^3 oocysts of *E. acervulina* at 10 d posthatch. (A) Body weight gains were measured between 0 and 9 d postinfection. Each bar represents the mean \pm SD value ($n = 15$). (B) Fecal samples were collected between 6 and 9 d postinfection and total oocyst numbers were determined using a McMaster chamber. Each bar represents the mean \pm SD value ($n = 20$). * $P < 0.05$ and *** $P < 0.001$ when comparing the anethole-treated and untreated control group followed by *E. acervulina* infection according to Student's *t*-test.

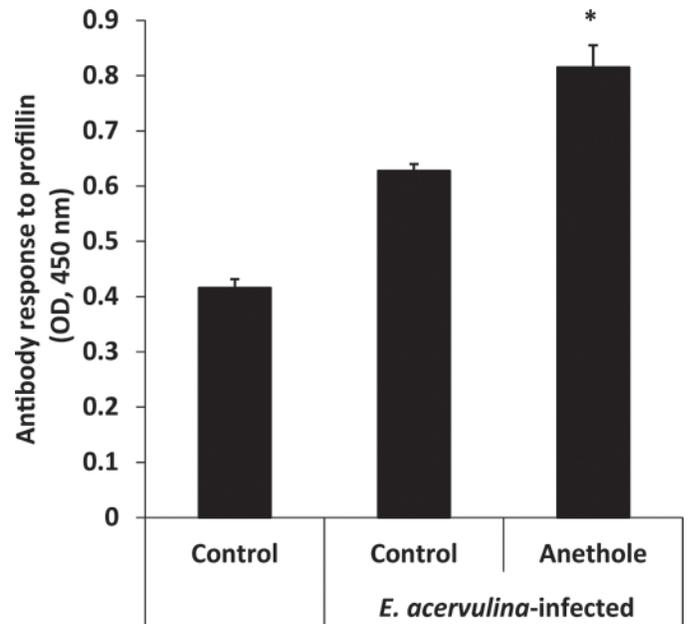


Figure 3. Effect of dietary anethole on serum anti-profilin antibody levels. Chickens were fed the unsupplemented (control) or anethole-supplemented diets, orally infected with 5.0×10^3 oocysts of *Eimeria acervulina* at 10 d posthatch, and serum anti-profilin antibody levels were determined at 9 d postinfection. Each bar represents the mean \pm SD value ($n = 4$). * $P < 0.05$ when comparing the anethole-treated and control groups according to Student's *t*-test. OD = optical density.

Effect of Dietary Anethole on Intestinal Lymphocyte Cytokine Gene Expression

In uninfected chickens, the levels of transcripts encoding IL6, IL8, and tumor necrosis factor ligand superfamily member 15 (TNFSF15) in intestinal IEL were decreased, whereas IL10 transcript levels were increased, in anethole-fed birds compared with unsupplemented controls (Figure 5). Following *E. acervulina* infection, the levels of all 4 mRNA were significantly higher in the anethole-supplemented group compared with the untreated group.

Effect of Dietary Anethole on Global Gene Expression

Microarray hybridization analysis using Agilent Technologies' Chicken Gene Expression Microarray containing 43,803 elements identified 1,810 transcripts whose levels were significantly altered in intestinal IEL of uninfected anethole-treated chickens compared with unsupplemented controls. Of these, 677 mRNA were increased and 1,133 were decreased. This data set was mapped to the human, mouse, rat, and chicken genomes using Ingenuity Knowledge Base software, leading to the identification and annotation of 576 chicken genes.

Biological Functional Analysis

The biological category "Disease and Disorders" was identified as being most significantly associated with

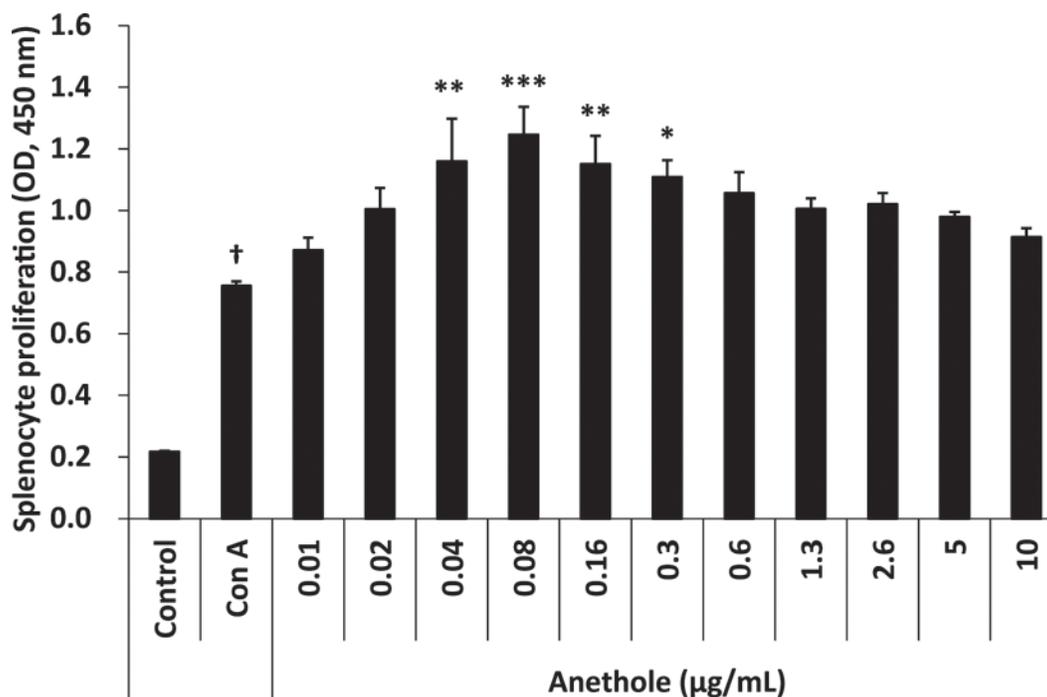


Figure 4. Effect of anethole on spleen lymphocyte proliferation. Spleen cells from uninfected chickens were treated with the indicated concentrations of anethole, concanavalin A (Con A; 5.0 µg/mL), or medium alone (control) for 48 h, and viable cell numbers were measured. Each bar represents the mean \pm SD value ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when comparing the anethole-treated and Con A-treated groups according to Student's t -test. OD = optical density. † $P < 0.05$ when comparing Con A-treated and control groups according to Student's t -test.

the 576 genes corresponding to the mRNA that were differently altered following dietary anethole treatment compared with untreated controls. Biological functional analysis within this category using the IPA database identified 17 biofunctions that were significantly associated with these genes (Table 2). The P -value associated with a particular function in this analysis is a statistical measure of the likelihood that genes from the data set under investigation participate in that function. The most significantly associated biofunction was the “Inflammatory Response” with 47 identified genes (14 up-regulated, 33 downregulated).

DISCUSSION

This study was conducted to determine the effects of dietary supplementation with anethole on experimental *E. acervulina* infection in broiler chickens. In vitro, a direct cytotoxic effect of anethole, which was dose-dependent against the parasite, was observed. Compared with untreated controls, chickens fed an anethole-supplemented diet showed increased BW gain and reduced oocyst shedding following challenge infection with live *E. acervulina*. Dietary anethole treatment increased serum *E. acervulina* proliferin antibody levels in vivo and augmented spleen lymphocyte proliferation in vitro compared with the respective untreated controls. Levels of transcripts encoding IL6, IL8, IL10, and TNFSF15 in intestinal IEL were increased in parasite-infected chickens given the anethole-containing diet compared

with untreated controls. Microarray hybridization identified 1,810 transcripts in gut lymphocytes whose levels were significantly altered in the anethole-fed birds compared with unsupplemented controls, from which 576 corresponding genes were identified. The most significant biological function associated with this altered transcriptome was the “Inflammatory Response” in the “Disease and Disorders” category.

The results from the current study are consistent with those of prior reports. Anethole decreased ethanol-induced gastric lesions without modifying mucus secretion in mice (Freire et al., 2005). The gastroprotective effect of essential oils, such as anethole, seemed to be related to their cytoprotective effects on the gastric mucosa (Hiruma-Lima et al., 2000). Because disruption of the gut epithelium during avian coccidiosis severely restricts feed conversion efficiency, the protective effects of anethole on experimental *E. acervulina* infection may be related, in part, to the protection of the gastrointestinal epithelia against parasitic damage, thereby enhancing nutrient absorption and improving BW gain. These beneficial effects appear to activate humoral and cellular immunities against coccidia and possibly to operate in conjunction with the ability of anethole to directly kill infective parasites. However, other potential mechanisms of action of anethole may also be involved. Ghasemi et al. (2011) suggested that anethole regulates Ca^{2+} channels and Ca^{2+} -activated K^{+} channels in dose-dependent, biphasic manners. Extracellular Ca^{2+} decreased the invasion of *E. tenella*

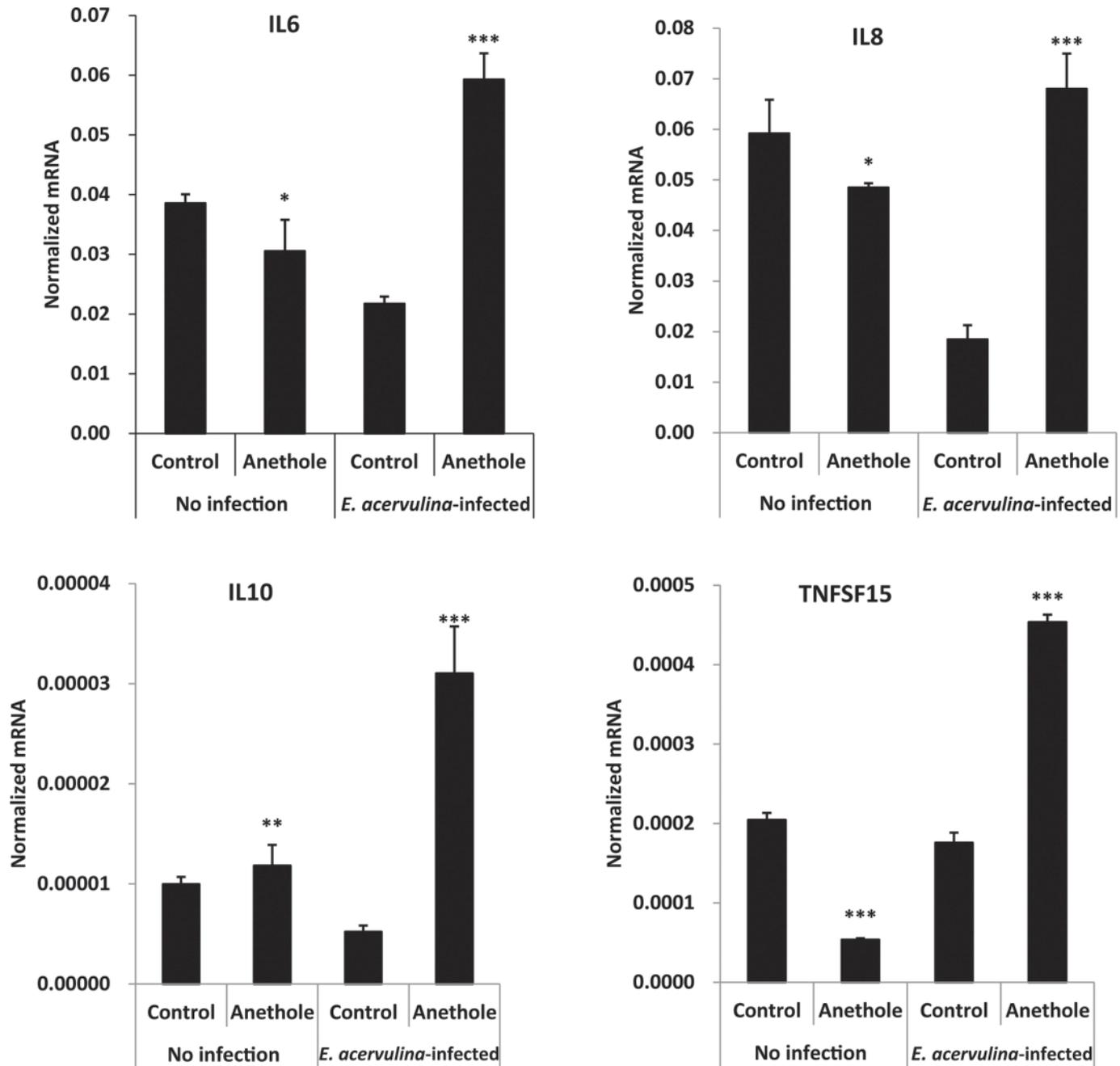


Figure 5. Effect of dietary anethole on intestinal lymphocyte cytokine mRNA levels. Chickens were fed the unsupplemented (control) or anethole-supplemented diets and were uninfected or were orally infected with 5.0×10^3 oocysts of *Eimeria acervulina* at 10 d posthatch. Intestinal intraepithelial lymphocytes were isolated at 14 d posthatch without infection and at 9 d postinfection of *E. acervulina*. The levels of transcripts for IL6, IL8, IL10, and tumor necrosis factor ligand superfamily member 15 (TNFSF15) were quantified by quantitative reverse-transcription PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels. Each bar represents the mean \pm SD value ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when comparing the anethole-treated with the corresponding untreated control groups according to Student's *t*-test.

sporozoites into host cells at concentrations less than 0.9 mM. Ryanodine, a herbal alkaloid that binds to internal Ca^{2+} channels, inhibited *E. tenella* sporozoite invasion (Schubert et al., 2005). Therefore, a Ca^{2+} channel blockade mechanism by anethole may be involved in the reduction of *E. acervulina* infectivity in vivo.

Anethole repressed tumor necrosis factor alpha (TNF- α)-dependent nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- κ B) acti-

vation and downstream proinflammatory gene expression (Chainy et al., 2000). Nuclear factor of kappa light polypeptide gene enhancer in B-cells is a major transcription factor that plays a key role in regulating the immune response to infection following binding of pathogenic microorganisms to a variety of cell surface receptors, such as Toll-like receptors (Takeda and Akira, 2005). Our microarray-based biological functional analysis of uninfected anethole-treated chicken intestinal

Table 2. The significant biofunctions and their identified genes in the “Disease and Disorders” category in chickens given an anethole-supplemented diet compared with an unsupplemented diet

Biofunction ¹	P-value ²	Genes ³
Inflammatory response	1.81×10^{-3} to 4.69×10^{-2}	<i>SOCS3, CD3E, IL1RL1, SOCS2*, PIK3R5, TNFSF10, BMPR2*, CCL20, TAC1, PMP2*, BCL11B*, ARAP3, IL6, APP*, HSPA4, GSTT1, CD28, NOTCH2, SCARB1*, INS, PRKAA1, PRKAR1B, MGLL, ERBB2*, IRF2*, FASLG, FUT4*, DNASE1, MARCO, PPARG, IL8, UNC13D*, CD69, DDOST, CRH*, ANXA2, IRF1, MSH2, BCL10*, VAV3, SYK, PELI1, LSP1, GATA3*, JAK3, PTPN22, LGALS1*</i>
Endocrine system disorders	2.75×10^{-3} to 4.23×10^{-2}	<i>PPARG, ZFAT, ACACA*, IL6, WFS1, CCKBR*, FASLG, PTPN22</i>
Gastrointestinal disease	2.75×10^{-3} to 4.5×10^{-2}	<i>ASXL1*, TERT, RAN, TNFSF10, IL6, EP300*, CYP8B1*, EZR, WWOX, ERBB2*, IRF2*, FASLG, CCKBR*, PPARG, IL8, CLPS, FHIT*, TUBG1, IRF1, CHRNG*, MSH2, ITPR3, ACACA*, AMY2A, CTTN*</i>
Hepatic system disease	2.75×10^{-3} to 3.93×10^{-2}	<i>PPARG, IL8, ACACA*, IL6, AMY2A, CCKBR*</i>
Metabolic disease	2.75×10^{-3} to 4.69×10^{-2}	<i>PPARG, CBS*, FXN, MCEE, ACACA, IL6, MMAA, WFS1, APP*, CCKBR*, PTPN22</i>
Hereditary disorder	3.29×10^{-3} to 4.69×10^{-2}	<i>SOCS3, ATXN3, NEFL, ASS1*, TDP1, EIF4E, PRPS1, FXN, ERBB2*, MFN2, PPARG, BEAN1, TBP, POLG, FAM3B, FIG4, IGFBP5*, ANXA2, MRPS16*, IRF1, ARHGDI1, VAV3, MCEE, ATXN10, NFIB*, GATA3*, MMAA, AMY2A</i>
Neurological disease	3.29×10^{-3} to 4.69×10^{-2}	<i>PPARG, RBFOX1*, IL8, APOB*, NEFL, BEAN1, ATXN3, DDC, CA12, POLG, TBP, FIG4, PMP2*, IGFBP5*, TDP1, MRPS16*, APP*, CD28, PRPS1, FXN, ATXN10, NFIB*, AMY2A, MFN2</i>
Skeletal and muscular disorders	3.29×10^{-3} to 2.96×10^{-2}	<i>PPARG, SOCS3, GSC*, PRPS1, NEFL, FIG4, TDP1, IL6, AMY2A, MFN2, TBX22*</i>
Cancer	3.58×10^{-3} to 4.69×10^{-2}	<i>SOCS3, CD3E, ASXL1*, IL1RL1, DDC, ASS1*, TERT, RAN, TNFSF10, BCL11B*, IL6, ETV6, EIF4E, BIRC5, EP300*, MAML1*, GSTT1, NOTCH2, EZR, WWOX, LAMB1*, ERBB2*, FASLG, IRF2*, PPARG, FHIT*, TUBG1, FAM3B, ANXA2, CCK*, IRF1, ARHGDI1, CHRNG*, MSH2, VAV3, JAK3, GATA3*, CTTN*</i>
Infectious disease	3.58×10^{-3} to 3.93×10^{-2}	<i>PPARG, IL8, TNFSF10, ANXA2, IL6, APP*, AMY2A, FASLG, LGALS1*</i>
Organismal injury and abnormalities	3.58×10^{-3} to 3.34×10^{-2}	<i>PPARG, CEL, FHIT*, APOB*, CBS*, SCARB1*, MSH2, IL1RL1, IL6, ASPH*, APP*, FASLG</i>
Cardiovascular disease	5.45×10^{-3} to 4.78×10^{-2}	<i>SOCS3, APOB*, NEFL, PCSK5*, IL1RL1, TNNT2, TAC1, BMPR2*, TNFSF10, IL6, APP*, FGG, EP300*, CD28, CEL, SCARB1*, CBS*, SYK, FASLG, FUT4*</i>
Developmental disorder	5.45×10^{-3} to 4.69×10^{-2}	<i>GSC*, MCEE, NFIB*, MMAA, MRPS16*, TBX22*</i>
Hematological disease	5.45×10^{-3} to 4.69×10^{-2}	<i>MSH2, CD3E, CBS*, DDC, SYK, BMPR2*, BCL11B*, IL6, JAK3, APP*, ETV6</i>
Immunological disease	5.45×10^{-3} to 4.62×10^{-2}	<i>ZFAT, CD3E, DDC, PMP2*, IL6, BCL11B*, APP*, ETV6, CD28, MSH2, WFS1, JAK3, PTPN22, FASLG</i>
Inflammatory disease	5.45×10^{-3} to 3.93×10^{-2}	<i>PPARG, CD28, SOCS3, IL8, IL6, AMY2A, EP300*</i>
Renal and urological disease	5.45×10^{-3} to 4.69×10^{-2}	<i>CD28, IL8, MCEE, IL6, MMAA, FASLG, EP300*</i>

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

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

³Genes (GenBank symbols) corresponding to transcript levels that were altered in anethole-treated compared with untreated chickens.

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

IEL identified 47 transcripts belonging to the biological function of “Inflammatory Response” in the “Disease and Disorders” category. Among these transcripts, IL6 and IL8 were significantly downregulated in uninfected anethole-fed birds compared with unsupplemented controls. In addition, qRT-PCR analysis demonstrated decreased levels of transcripts for the proinflammatory IL6, IL8, and TNFSF15 cytokines/chemokines, and increased levels of transcripts for the anti-inflammatory IL10 cytokine, in uninfected anethole-fed birds compared with controls. In contrast, transcript levels for all 4 immune mediators were increased in *E. acervulina*-infected, anethole-fed birds compared with untreated controls. The latter results is consistent with previous reports demonstrating increased chicken IL6, IL8, IL10, and TNFSF15 production following *Eimeria* infection

(Rothwell et al., 2004; Hong et al., 2006). A similar effect has been noted in the host cytokine response of infected vs. uninfected chickens in the context of phyto-nutrient dietary supplementation (Lillehoj et al., 2011). Prior to *E. tenella* infection, chickens given a diet containing *Capsicum* and turmeric oleoresins had reduced levels of transcripts encoding IL6 and interferon- γ compared with unsupplemented controls, whereas postinfection levels of both cytokines increased with the oleoresin supplementation. It may be possible that in the absence of *Eimeria* infection, anethole upregulates IL10 expression, which, in turn, downregulates IL6, IL8, and TNFSF15 levels, whereas in the presence of *Eimeria* infection, the opposite effects occur as a consequence of the parasite's ability to stimulate proinflammatory cytokine/chemokine expression through its multiple TLR

agonists, including profilin, heat shock protein 70, and glycosylphosphatidylinositol-anchored surface antigens (Yarovinsky et al., 2005; Lillehoj et al., 2007; Chow et al., 2011). Further studies to characterize the activity of anethole and its effects on innate and adaptive immunity will help to resolve these questions.

In mammals, IL6 is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through the IL6 receptor (Kishimoto et al., 1995; Petersen and Pedersen, 2005). Chicken IL6 production was induced by infection with *Staphylococcus aureus* or *Salmonella enterica* serovars Enteritidis and Typhimurium (Kaiser et al., 2000; Zhou et al., 2007). Increased expression of IL6 in chickens may stimulate a population of heterophils, avian equivalents of mammalian neutrophils, with increased capability of responding to and eliminating infectious pathogens (Swaggerty et al., 2004). Interleukin 8 is a CXC chemokine that is produced in the gut of newly hatched chickens following exposure to bacteria (Bar-Shira and Friedman, 2006). Chicken IL8 has both chemotactic and angiogenic functions (Poh et al., 2008). At low concentrations, IL8 is chemotactic for monocyte/macrophages and lymphocytes, whereas at higher concentrations it stimulates the sprouting and growth of new blood vessels (Martins-Green and Feugate, 1998). Interleukin 10 is an anti-inflammatory cytokine that inhibits the function of macrophages and dendritic cells, including their production of proinflammatory cytokines (Moore et al., 2001). Interleukin 10 has been suggested as a master regulator, acting through a negative feedback mechanism to prevent the overexpression of Th1 and Th2 immune responses (Couper et al., 2008). The TNF superfamily is an important regulator of inflammation, immune responses, and tissue homeostasis (Locksley et al., 2001). Chicken TNFSF15 decreased feed intake, increased nitric oxide production, and exhibited in vitro cytotoxic activity against murine and chicken primary fibroblasts (Takimoto et al., 2005).

In conclusion, in vivo feeding of young broiler chickens with anethole improved resistance to experimental *E. acervulina* infection and induced significant alterations of the transcriptome in chicken intestinal lymphocytes involved in an inflammation response-related biological function. These results provide the first immunological evidence that dietary anethole enhances systemic immunity in chickens and increases local protective immunity against experimental avian coccidiosis. Dietary anethole supplementation may represent a possible alternative to reduce pathological effect of coccidiosis and mitigate the use of antibiotics in commercial poultry production.

ACKNOWLEDGMENTS

This project was supported by a Trust agreement between ARS-USDA and Pancosma and the World Class University Program (R33-10013) of the Ministry of Ed-

ucation, Science and Technology of South Korea. We thank Marjorie Nichols, Stacy O'Donnell, and Ashley Cox (Agricultural Research Service, USDA, Beltsville, MD) for technical assistance.

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